

# New biomarkers in rheumatoid arthritis

A. Willemze,\* R.E.M. Toes, T.W.J. Huizinga, L.A. Trouw

Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands,  
\*corresponding author: tel: +31 (0)71-5263265, fax: + 31 (0)71-5266752, e-mail: a.willemze@lumc.nl

## ABSTRACT

Rheumatoid arthritis (RA) is a common autoimmune disease affecting around 1% of the population. Although major advances have been made in the treatment of RA, still relatively little is known on disease pathogenesis and aetiology. From treatment studies it has become clear that treating patients early in their disease course will provide the best results. However, especially in the early phase of arthritis, in particular when the patients do not yet fulfil the criteria for RA, it is difficult to decide which patients would benefit most from an early and aggressive intervention. Good biomarkers are important to guide decisions in the clinical management of RA. Next to the well-known rheumatoid factor (RF) and the anti-citrullinated protein antibodies (ACPA), several new markers are now likely to become available with interesting potential. Besides antibody responses directed against citrullinated proteins, also antibodies against carbamylated proteins (anti-CarP) have recently been shown to be present in RA. 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. Apart from the antibodies that help in establishing the diagnosis and prognosis, also novel biomarkers that reflect clinical disease activity scores are being discovered. The development of biomarker-based disease activity scores might allow easy and frequent monitoring of patients to rapidly adjust treatment.

## KEYWORDS

Rheumatoid arthritis, ACPA, anti-CarP, biomarkers, MBDA

## INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases, affecting up to 1% of the Dutch population. RA is characterised by persistent synovitis,

systemic inflammation and the presence of several autoantibodies.<sup>1</sup> RA is a highly heterogeneous disease and therefore classification criteria were developed. These criteria were developed to arrive at relatively homogenous patient groups to be able to better compare studies performed across the world. In the development of the American College of Rheumatology (ACR) 1987 criteria, clinical cases of classic RA patients were included, which resulted in the inclusion of patients with conceivably different disease mechanisms.<sup>2</sup> Therefore, using this classification system there was still considerable variation in disease course and treatment responses among the RA patients. Given the fact that the sensitivity of the 1987 ACR criteria for RA is relatively low for recent-onset RA, the ACR/European League against Rheumatism 2010 criteria were developed, which display a higher sensitivity but a somewhat lower specificity for RA. Recent advances in biomarker discovery have been included in the new 2010 criteria for RA by inclusion of a set of autoantibodies, directed against citrullinated proteins (ACPA) (*figure 1*).<sup>3,4</sup> ACPA have a very high specificity for RA.<sup>3</sup>

Both genetic and environmental risk factors influence the development of RA. An estimated 50% of the risk to develop RA is mediated via genetic risk factors.<sup>1</sup> Of the genetic risk factors, the strongest predisposing variants are found in the human leucocyte antigen (HLA) alleles. These HLA molecules are essential for antigen presentation in the immune response. Although the association between HLA and RA is still incompletely understood, the association suggests that antigen presentation and the type of immune activation that leads to (auto)antibody formation are important in RA. Of the environmental risk factors, smoking is the most prominent with its largest effect in autoantibody positive patients.

Recent data have clearly shown that treatment (very) early in the disease course will provide the best results and will largely prevent irreversible damage to the joints. With these new approaches the classical picture of massive erosions,

**Figure 1.** ACR 1987 criteria and ACR/EULAR 2010 criteria: Classification criteria for rheumatoid arthritis

ACR 1987 criteria	ACR/EULAR 2010 criteria																																
<ul style="list-style-type: none"> <li>• Morning stiffness (at least 1h)</li> <li>• Arthritis of three or more joint areas</li> <li>• Arthritis of hand joints (&gt;1 swollen joints)</li> <li>• Symmetric arthritis</li> <li>• Rheumatoid nodules</li> <li>• Serum rheumatoid factor</li> <li>• Radiographic changes (erosions)</li> </ul>	<table border="0"> <tr> <td>• Joint involvement</td> <td>Score</td> </tr> <tr> <td>1 large joint</td> <td>0</td> </tr> <tr> <td>2-10 large joints</td> <td>1</td> </tr> <tr> <td>1-3 small joints (large joints not counted)</td> <td>2</td> </tr> <tr> <td>4-10 small joints (large joints not counted)</td> <td>3</td> </tr> <tr> <td>&gt; 10 joints (at least one small joint)</td> <td>5</td> </tr> <tr> <td>• Serology</td> <td></td> </tr> <tr> <td>Negative RF and negative ACPA</td> <td>0</td> </tr> <tr> <td>Low-positive RF or low-positive ACPA</td> <td>2</td> </tr> <tr> <td>High-positive RF or high-positive ACPA</td> <td>3</td> </tr> <tr> <td>• Acute-phase reactants</td> <td></td> </tr> <tr> <td>Normal CRP and normal ESR</td> <td>0</td> </tr> <tr> <td>Abnormal CRP or abnormal ESR</td> <td>1</td> </tr> <tr> <td>• Duration of symptoms</td> <td></td> </tr> <tr> <td>&lt;6 weeks</td> <td>0</td> </tr> <tr> <td>≥6 weeks</td> <td>1</td> </tr> </table>	• Joint involvement	Score	1 large joint	0	2-10 large joints	1	1-3 small joints (large joints not counted)	2	4-10 small joints (large joints not counted)	3	> 10 joints (at least one small joint)	5	• Serology		Negative RF and negative ACPA	0	Low-positive RF or low-positive ACPA	2	High-positive RF or high-positive ACPA	3	• Acute-phase reactants		Normal CRP and normal ESR	0	Abnormal CRP or abnormal ESR	1	• Duration of symptoms		<6 weeks	0	≥6 weeks	1
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<p>Four of seven criteria must be present. Criteria 1-4 must have been present for at least 6 weeks.</p>	<p>A score of ≥6 is the cutpoint for rheumatoid arthritis. Patients can also be classified as having rheumatoid arthritis if they have: 1) erosive disease typical for rheumatoid arthritis, 2) long-standing disease previously satisfying the classification criteria.</p>																																
<p>ACR = American College of Rheumatology; EULAR = European League Against Rheumatism; RF = rheumatoid factor; ACPA = anti-citrullinated peptide antibodies; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate. Adjusted from Scott <i>et al.</i><sup>1</sup></p>																																	

deformities and disability can be prevented in a large proportion of patients. However, having the opportunity to treat patients at a (very) early phase of their disease course also poses a problem. Many patients with early RA and especially those suffering from arthralgia or undifferentiated arthritis may undergo spontaneous remission, whereas patients may also progress to full-blown RA. A recent study showed that about a third of the undifferentiated arthritis patients go into remission, pointing to the relevance of biomarkers for diagnosis.<sup>4</sup> Thus, the aim is to identify those people who will require early (and aggressive) intervention and at the same time prevent over-treatment in those who do not need such an intervention. Next to clinical parameters, biomarkers are also helpful in making such decisions. In this review we will describe several new biomarkers which are now available or are currently being developed to guide such decisions.

### ANTI-CITRULLINATED PROTEIN ANTIBODIES (ACPA)

The first RA-associated antibody, rheumatoid factor (RF), was discovered 1940,<sup>5</sup> and was later found to be directed to the Fc region of IgG. In the past two decades, it has been shown that the autoantibodies with the highest specificity for RA are antibodies directed against proteins containing citrulline epitopes. These antibodies are called ACPA and were first described as anti-perinuclear factor antibodies (APF).<sup>6</sup> ACPA can recognise the non-classical

amino acid citrulline, embedded on a protein backbone.<sup>7,8</sup> Citrulline is a non-encoded amino acid, generated by a post-translational modification of arginine mediated by protein-arginine deiminase (PAD) enzymes.<sup>9</sup> This modification takes place during a variety of biological processes, including inflammation. In 1998, the first ELISA using citrullinated peptides was developed<sup>8</sup> and was followed, in 2000, by the first ELISA based on artificial cyclic citrullinated peptides (CCP).<sup>10</sup> The first commercial version of this test, the CCP2 assay, became available in 2002 and enabled routine testing for antibodies directed against citrullinated epitopes as a biomarker for RA.<sup>10-14</sup> As well as the CCP2 assay, a few other assays for ACPA, such as CCP3 and MCV (mutated citrullinated vimentin), have made their way into the clinic. These assays differ slightly in terms of specificity and sensitivity.<sup>15</sup> As anti-CCP2 antibodies recognise a variety of citrullinated peptides and proteins, these antibodies are now called ACPA.<sup>16</sup>

ACPA can recognise a variety of citrullinated antigens, including citrullinated fibrinogen,<sup>17,18</sup> citrullinated vimentin (which is also known as the Sa antigen),<sup>19</sup> citrullinated type II collagen,<sup>20</sup> citrullinated  $\alpha$ -enolase<sup>21</sup> and many more citrullinated proteins. An increase or shift in the antigen recognition profile (a phenomenon known as epitope spreading) can have important pathophysiological consequences, as has been described in, for example, systemic lupus erythematosus<sup>22</sup> and pemphigus. Autoantibodies such as anti-desmoglein antibodies present in patients with pemphigus vulgaris have been convincingly shown to mediate a pathogenic

effect, through transfer into experimental animals.<sup>23</sup> Furthermore, in pemphigus, reactivity against different desmoglein epitopes is associated with different outcomes.<sup>24</sup> Nonetheless, current evidence indicates that epitope spreading to defined antigens does not correlate with clinical phenotype.<sup>25-27</sup> In contrast, an overall broadening of the ACPA-recognition profile is noted before disease precipitation after which a stabilisation occurs.<sup>28,29</sup> Isotype switching is another event involved in enhancing the efficacy of (auto)antibodies, and leads to an increase in the diversity of antibody response that enables the activation of more immune effector mechanisms. ACPA can be present in different forms, including IgG, IgA, IgM and IgE.<sup>30-33</sup> Healthy family members of patients with RA display fewer ACPA isotypes than the patients.<sup>30</sup> Nonetheless, multiple ACPA isotypes are present before the onset of RA.<sup>31</sup> Likewise, the ACPA isotype distribution does not seem to significantly expand anymore during disease progression from undifferentiated arthritis to RA, indicating that most of the expansion of isotype usage by ACPA, like the epitope recognition profile, takes place before the onset of arthritis.

ACPA are strongly associated with RA, which suggests they have a prominent role in disease pathogenesis.<sup>34-36</sup> Selective B-cell depletion has been shown to be effective in the treatment of RA, providing evidence for the involvement of B cells and possibly autoantibodies in its pathogenesis.<sup>37,38</sup> Furthermore, most ACPA-positive patients with RA seem to be ACPA positive years before the onset of disease,<sup>39</sup> although the extent of the ACPA repertoire seems to be limited at this preclinical stage. Likewise, in patients with arthralgia, the development of arthritis is predicted not only by the presence of ACPA, but also by their levels.<sup>40,41</sup> High titres of ACPA are associated with the recognition of more citrullinated epitopes. Indeed, patients with arthralgia who have an extended ACPA repertoire are at higher risk of developing arthritis.<sup>41</sup> Similarly, ACPA-positive patients with early arthritis who do not fulfil the American College of Rheumatology (ACR) classification criteria for RA are more likely to develop RA if their ACPA response is reactive to more citrullinated epitopes.<sup>29</sup> These findings are consistent with the notion that a 'broader' ACPA recognition profile is associated with the transition towards (persistent) disease, and resemble the observations made in pemphigus with the exception that, thus far, no specific anti-citrullinated epitope or protein reactivity has been identified that would predict disease course in RA.

During a B-cell response against recall antigens, isotype switching and affinity maturation typically occur in germinal centres. Following somatic hypermutation, different B-cell clones will compete for antigens

presented on follicular dendritic cells. B cells that express immunoglobulins of sufficiently high avidity will acquire the signals necessary for survival and proliferation. As a result, the total avidity of the immune response—defined as the overall binding strength of polyclonal antibodies to a multivalent antigen—increases, because low avidity B cells will not be stimulated and will eventually disappear from the population. The avidity maturation of antibody responses against recall antigens, mostly following vaccination, has been studied extensively, but autoantibody responses seem to behave differently.<sup>42-44</sup> For example, the avidity of ACPA is significantly lower than the avidity of antibodies to the recall antigens tetanus toxoid and diphtheria toxoid, pointing to a different regulation of autoantibody responses as compared with recall antigens. In individual patients with RA, ACPA do not show avidity maturation during longitudinal follow-up and even in patients who displayed extensive isotype switching, ACPA avidity was relatively low,<sup>45,46</sup> indicating that these two maturation processes are uncoupled in the ACPA response.

#### ACPA AND TREATMENT OUTCOMES

Diseases with distinct pathogeneses might benefit from different treatment strategies; therefore, ACPA-positive and ACPA-negative disease are treated differently. Methotrexate is the most prominent disease-modifying antirheumatic drug (DMARD). A few years ago, we performed a double-blind placebo-controlled randomised trial comparing two treatment strategies in patients with undifferentiated arthritis. Interestingly, the outcome of this study indicated that ACPA-positive patients with undifferentiated arthritis treated with methotrexate are less likely to progress to RA, and do so at a later time point as compared with a placebo-control group. Unexpectedly, no effect of methotrexate therapy on progression to RA in the ACPA-negative group was observed.<sup>47</sup> Interestingly, among patients with undifferentiated arthritis, those with low or intermediate ACPA levels respond better to methotrexate than patients with high ACPA levels.<sup>48</sup> The data from this randomised trial not only indicate that the two ACPA subgroups respond differently to methotrexate treatment, but also that in patients with high ACPA levels methotrexate monotherapy might be insufficient. Indeed, the presence of ACPA and IgM RF together with elevated levels of C-reactive protein (CRP) is predictive of more rapid radiographic progression in patients with RA. Patients with ACPA and IgM RF are also more likely to respond insufficiently to methotrexate monotherapy for recent-onset RA.<sup>49</sup>

It is not only in regard to response to DMARDs that ACPA status seems to matter. In a trial published in 2011,<sup>50</sup> 208

patients with RA refractory to therapy with TNF blockers were treated with rituximab. Rituximab is a monoclonal antibody directed towards the B-cell marker CD20, and has been shown to be an effective treatment in RA. In these patients, the presence of ACPA predicted a better EULAR response to rituximab at 24 weeks. Thus, rituximab might have a greater role in ACPA-positive RA patients than in ACPA-negative individuals.<sup>50</sup> The mechanisms of rituximab efficacy, and of B-cell involvement in RA, are incompletely known; the basis for these differing outcomes remains to be elucidated.

The absence of ACPA and IgM RF are independent predictors of drug-free remission.<sup>51</sup> The course of ACPA-positive disease seems to be characterised by more persistent inflammation than its ACPA-negative counterpart. Together, these data indicate that treatment decisions in RA can be guided by ACPA status. Seroconversion is uncommon among ACPA-positive and ACPA-negative patients; therefore, it does not seem to be useful to repeat ACPA measurements in daily practice.<sup>52,53</sup> Thus, these data support the hypothesis that RA can be classified into two different disease subsets, and suggest that developing different classification criteria for ACPA-positive and ACPA-negative RA might help to further optimise treatment strategies.

#### ACPA AND DISEASE OUTCOME

The emerging relevance of ACPA status to treatment decisions is not only based on differential treatment efficacies, but is also supported by differences in disease outcome. Typically, 50 to 70% of the patients with RA are ACPA positive.<sup>54</sup> Although ACPA-positive and ACPA-negative patients with RA show a very similar clinical presentation in the early phase of the disease,<sup>55,56</sup> their subsequent disease course is different: extra-articular manifestations are clearly associated with ACPA status. For example, ACPA positivity is associated with an increased risk of developing ischaemic heart disease<sup>57</sup> or lung pathology.<sup>58</sup> Likewise, ACPA-positive patients have more destructive joint disease than ACPA-negative patients.<sup>56,59-61</sup> ACPA-positive patients develop erosions earlier and more abundantly than patients without ACPA.<sup>62</sup> Owing to their more severe disease course, ACPA-positive patients require a more aggressive treatment regimen than ACPA-negative patients.<sup>63</sup> Indeed, in the BeSt study, ACPA-positive patients initially treated with DMARD monotherapy displayed greater radiographic joint destruction after two years than ACPA-negative patients.<sup>63</sup> In patients initially treated with combination therapy, by contrast, no difference with respect to joint destruction was observed between ACPA-positive and ACPA-negative patients.

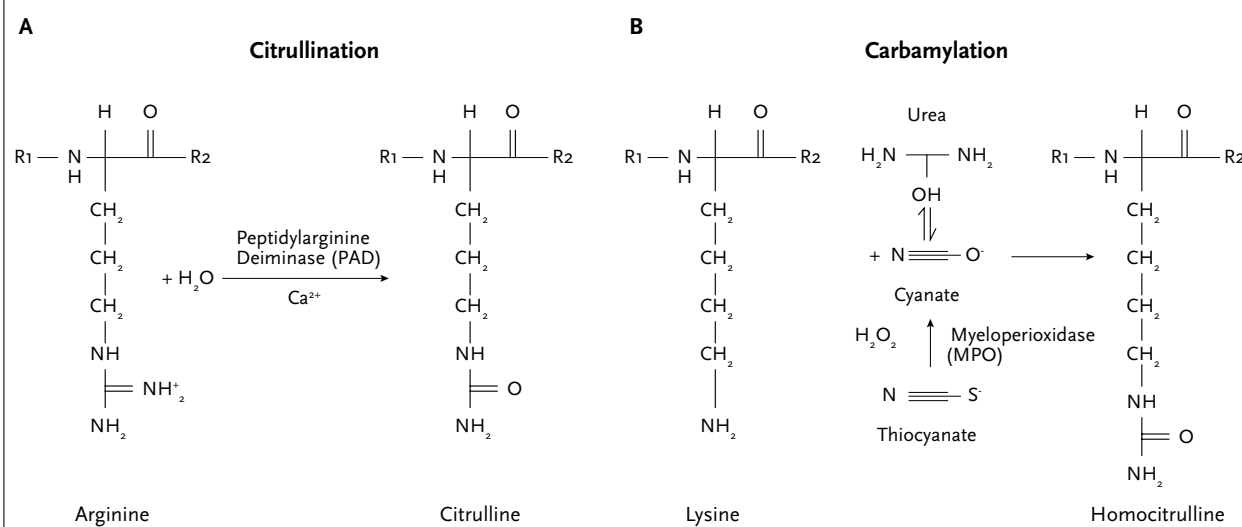
These observations suggest that effective treatment with combination therapy, together with steering treatment according to disease activity, can prevent radiographic progression, even in patients with risk factors for severe damage, such as ACPA-positive patients.

Most ACPA characteristics, such as the fine-specificity profile, are not associated with the rate of joint destruction once RA is established.<sup>25,26</sup> The ACPA isotype profile seems, however, to be an exception—in two cohorts ACPA isotype diversity has been associated with a higher risk of radiographic progression, equating to a 1.4-fold increase in risk per isotype used in the ACPA response, illustrating that an extended isotype usage is associated with a worse outcome.<sup>64</sup> Altogether, evidence is emerging that ACPA-positive and ACPA-negative RA represent two different disease entities with different outcomes, and, possibly, different responses to medication. The latter notion is especially important as it indicates that treatment regimes can be further optimised by guiding their development by ACPA status.

#### ANTI-CARBAMYLATED PROTEIN (ANTI-CARP) ANTIBODIES

ACPA recognise 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 different type of post-translational modification are also recognised 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.<sup>65</sup> 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. Renal failure, a condition with increased urea concentrations, is known to be associated with enhanced protein carbamylation.<sup>66</sup> Also smoking, the most prominent environmental risk factor for RA, enhances carbamylation by increasing the cyanate concentration.<sup>67</sup> 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.<sup>67</sup> 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 2*). In

**Figure 2.** Illustration of citrullination and carbamylation. Citrullination (A) and carbamylation (B) occur on different amino acids via different mechanisms, but yield similar end-products



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<sub>2</sub> group longer. The resemblance between the two modifications and the likely presence of carbamylated proteins in the joint prompted us to test for the presence of antibodies directed against carbamylated proteins.

Therefore, we developed a novel assay that specifically detects the presence of antibodies directed against carbamylated proteins (anti-CarP).<sup>68</sup> Using this assay we could show that RA patients indeed also harbour antibodies directed against carbamylated proteins. Importantly we observed these anti-CarP antibodies not only in ACPA-positive but also in ACPA-negative RA patients. This suggests that antibodies recognising one modification do not necessarily cross-react with the other modification. This notion was further supported experimentally by inhibition studies and Western blotting.<sup>69</sup> When analysing the clinical status of anti-CarP antibody positive RA patients, we observed that the presence of anti-CarP antibodies was associated with a higher rate of joint damage. This phenomenon was especially observed in the ACPA-negative subgroup. Especially for these ACPA-negative patients no prognostic markers were available and the identification of anti-CarP antibodies in this group may be useful clinically. These observations suggest that the population of RA patients is more heterogeneous than initially thought as, perhaps, besides ACPA-positive disease also anti-CarP-positive RA might represent an additional disease entity with

its own genetic/environmental contributions. However, conformational studies are required to support this notion. Several major questions are still open regarding anti-CarP such as: why do some people make these antibodies? Do they contribute to the disease process and if so, how? What are the possibilities to interfere with these putative processes? Does the presence of anti-CarP antibodies predict progression towards disease in subjects at risk to develop arthritis, and if so, will it have added value as a biomarker next to ACPA testing? As indicated above international replication studies are needed to confirm and expand the presented observations. Interestingly, the anti-CarP immune response is not only restricted to humans, but can also be induced in mice and rabbits by vaccination with carbamylated proteins.<sup>70,71</sup> This might allow to study the driving mechanisms underlying the anti-CarP response and if and how it contributes to arthritis.

#### BIOMARKER-BASED DISEASE ACTIVITY INDICES

Accurate and frequent assessment of RA disease activity is critical for optimal treatment planning. In numerous studies it has been shown that treatment guided by disease activity improves outcome in RA.<sup>72,73</sup> Moreover, several studies have suggested that frequent measurement of disease activity and subsequent tight control of disease activity is associated with good clinical outcome. In a study

from Utrecht, monthly assessment of disease activity was proposed.<sup>74</sup> However, in daily clinical practice it is often not feasible to schedule monthly patient visits to assess disease activity. As such, a 'simple' biomarker that replaces the clinical assessment of disease activity is relevant. To this end a novel algorithm has been developed to determine a multi-biomarker disease activity (MBDA) score based upon measurement of the concentrations of 12 serum biomarkers (SAA, IL-6, TNF-RI, VEGF-A, MMP-1, YKL-40, MMP-3, EGF, VCAM-1, leptin, resistin and CRP).<sup>75</sup> This MBDA score was significantly associated with conventional disease activity measured, the so-called disease activity score, which is a composite index of the number of swollen and painful joints, the assessment of the patient on disease activity and an acute phase response. Importantly, this association has been observed in several Dutch and American cohorts from RA patients.<sup>76,77</sup> Moreover changes in MBDA scores were able to discriminate clinically relevant reductions in disease activity scores, suggesting that this test has the potential to be used to evaluate disease activity measures.

## CONCLUSION

Both with regard to the prognosis as well as follow-up of patients, biomarkers have been developed that can help the clinical management of RA patients.

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