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Anti-CCP antibodies, a highly specific marker for (early) rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic, destructive autoimmune disease affecting the joints. With more sophisticated and effective therapies becoming available and with the understanding that early intervention is crucial in preventing irreversible joint damage, it is more and more important to diagnose RA at a very early stage in the disease. To facilitate diagnosis during the early stages of the disease, when often not all clinical symptoms are manifest, a good serological marker is needed. Antibodies directed to citrullinated proteins provide this ability. The most sensitive assay to detect these antibodies is the so-called anti-cyclic citrullinated peptide (CCP) enzyme-linked immunosorbent assay (ELISA) assay. In this review, the diagnostic and prognostic potential and the general utility in clinical practice of anti-CCP antibodies are discussed. Furthermore, we elaborate on the mechanisms involved in the generation of citrullinated autoantigens and the possible role of the anti-CCP antibodies and their antigens in the disease.

Keywords: anti-CCP antibodies; Rheumatoid arthritis; Citrullinated proteins; Serological marker diagnosis

1. Introduction

Rheumatoid arthritis (RA) is a common, systemic autoimmune disease affecting 0.5–1% of the population [1,2]. It is characterized by chronic inflammation of the synovial joints, which

Abbreviations: ACR, American College of Rheumatology; AFA, anti-filaggrin antibodies; AKA, anti-keratin antibodies; APC, antigen-presenting cells; APF, antiperinuclear; BiP, binding protein; CCP, cyclic citrullinated peptide; CCP1, first generation CCP; CCP2, second generation CCP; ELISA, enzyme-linked immunosorbent assay; HCV, hepatitis C virus; HLA, human leukocyte antigen; JRA, juvenile RA; MC, mixed cryoglobulinemia; MCTD, mixed connective tissue disease; MTX, methotrexate; PAD, peptidylarginine deiminase; PR, palindromic rheumatism; RA, rheumatoid arthritis; RF, rheumatoid factor; ROC/AUC, receiver operator characteristic/area under the curve; SE, shared epitope; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphisms; UA, undifferentiated arthritis.

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commonly leads to progressive joint destruction and consequent disability and reduction of quality of life [3]. Disease outcome may vary from mild symptoms to severe systemic disease when joint destruction is accompanied by extraarticular manifestations (i.e., rheumatoid nodules, vasculitis). Mortality in the latter group is significantly increased compared to the former group and the general population [4,5].

With more sophisticated and effective therapies becoming available (reviewed in [6]) and with the understanding that early intervention is crucial in preventing irreversible joint damage [7–9], it is more and more important to diagnose RA at a very early stage in the disease. Although the 1987 American College of Rheumatology (ACR; formerly, American Rheumatism Association) classification criteria for RA [10] are often used in clinical practice as diagnostic tool for RA, they are not very well suited for the diagnosis of early RA [11–13]. The ACR criteria rely heavily on the expression of clinical symptoms of RA, but in early RA these clinical parameters are often not (yet) manifest. Therefore, a specific and sensitive (serological) marker, which is present very early in the disease, is needed. Rheumatologists need to be able to target the use of potentially toxic and expensive drugs to those patients where the benefits clearly outweigh the risks [13,14]. Therefore a good marker should ideally be able to predict the erosive or nonerosive progression of the disease. Fortunately, the anti-CCP (cyclic citrullinated peptide) antibodies meet the demands for a good and useful marker for early RA and this will be the focus of this review.

2. Autoantibodies in RA

2.1. RA-associated antibodies

The serum of RA patients contains a large repertoire of antibodies directed against self-proteins. Most of these autoantibodies, however, can also be detected in patients with other (autoimmune) conditions, and are therefore not specific for RA. Even the well known rheumatoid factor (RF) antibodies, directed against the Fc domain of IgG molecules (reviewed in [15]), which are part of the ACR criteria [10] have only moderate specificity for RA. They can be found in patients with other autoimmune diseases, in patients with infectious diseases and in 3–5% of the healthy population (10–30% for elderly individuals) [16,17].

Other RA-associated antibodies (reviewed in [18]) include anti-RA33 (anti-hnRNP-A2) [19–21], anti-calpastatin [22,23], ANCA (anti-neutrophil cytoplasmic antibodies) [24,25], ANA (antibodies to nuclear antigens) [26,27], anti-collagen type II [28–30], anti-fibronectin [31], and anti-GPI (glucose-6-phosphate isomerase) [32,33]. Most of these autoantibodies are also present in a variety of other autoimmune diseases like systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) and to some extent also in healthy individuals. Fortunately, some autoantibody systems with a better specificity for RA also exist: anti-BiP (p68), anti-Sa and anti-citrullinated protein antibodies (APF, AKA, anti-filaggrin, anti-CCP).

2.2. RA-specific antibodies

Auto-antibodies directed to BiP (heavy chain binding protein), formerly known as anti-p68, occur in about 64% of RA patients and have been reported to be highly specific for the disease

[34,35]. BiP is a Hsp70 family stress protein that normally is ubiquitously expressed in the endoplasmic reticulum. During conditions of cellular stress, like in the rheumatoid synovium, BiP is overexpressed and partly relocates to the cell surface [35]. The major epitopes for anti-BiP are N-acetylglucosamine carbohydrate groups that are normally not present on BiP, and these modifications could be important for the relocation [36]. The BiP antigen appears to be a target of RA-specific T-cell immune responses as well [35,37,38]. Anti-BiP antibodies can also be found in mice with established collagen or pristane induced arthritis [39]. No data exist on the predictive value or the presence of anti-BiP in early RA. Furthermore, the reported specificity of these antibodies awaits further confirmation by independent clinical studies.

The Sa system is highly specific (92–99%), although only moderately sensitive (30–40%) for RA [40–43]. Originally, the Sa antigen was described as a set of two bands of 50 and 55 kDa that are recognized by RA sera on immunoblots containing extracts from normal human spleen or placenta [40]. Then, an Sa-like antigen was found in human endothelial cells [44] and more recently, several protein bands of various molecular weights have been mentioned as being Sa-related antigens [45]. The Sa-antigen can also be detected in the rheumatoid pannus tissue [40]. Although the true nature of the Sa antigen remains to be revealed, it has been suggested that it is citrullinated vimentin in which the citrulline moiety acts as the autoantigenic hapten and vimentin as the hapten carrier [44]. Indeed, citrullination of vimentin has been described in mouse and human macrophages, cells that are abundantly present in the RA synovium [46,47]. If the Sa antigen(s) are indeed citrullinated, the anti-Sa antibodies should be classified within the family of anti-citrullinated protein antibodies.

3. History of anti-citrullinated protein antibodies

3.1. The original systems: APF and AKA

In 1964 the antiperinuclear factor (APF) was described for the first time [48]. These autoantibodies are specific for RA (reviewed in [49]) and can be detected by indirect immunofluorescence using human buccal mucosa cells as the antigenic substrate. Light and immunoelectron microscopy revealed that the perinuclear factor is localized in the keratohyalin granules [50,51]. These granules are 0.5–4 μm amorphous structures of densely packed proteins located in the cytoplasm of epithelial cells. Detailed examination revealed precise colocalization of the perinuclear factor with (pro)filaggrin [51].

In 1979, RA specific antibodies directed against keratinized epithelium of the stratum corneum were described [52]. These antibodies can be detected by indirect immunofluorescence using rat or human esophagus cryostat sections as the antigenic substrate. Based on their immunofluorescence pattern, these antibodies were tentatively named anti-keratin antibodies (AKA) [52]. This name turned out to be a rather unfortunate choice, because AKA are reactive with filaggrin instead of keratin [53,54]. So it turned out that, although discovered independently, AKA and APF antibodies are directed to the same antigen [55]. Many reports had beforehand shown, that APF and AKA share many features. Although sensitivity of AKA is lower than that for APF, presence and titers of both antibodies are correlated to each other, to the presence of RF and to the severity of the disease (reviewed in [49]).

Filaggrin (filament aggregating protein), the common antigen of APF and AKA, cross-links keratin filaments in order to form very rigid cytoskeletal structures. It is synthesized as a large, heavily phosphorylated precursor protein, consisting of 10–12 filaggrin subunits [56]. During the final stages of differentiation, profilaggrin maturates via a multistep process, which includes dephosphorylation, citrullination, and cleavage into separate filaggrin subunits. Although the exact mechanisms involved are not completely understood, it is believed that citrullination of profilaggrin opens up the structure of the protein, rendering it susceptible to cleavage into its separate subunits [57,58]. Finally, the mature filaggrin subunits bind to keratin filaments to form dense macrofibrils that protect the keratins from proteolytic degradation [59,60].

3.2. Citrulline-specific reactivity

Based on the knowledge that mature filaggrin and not profilaggrin is the target of the APF and AKA antibodies [51,61,62], synthetic citrulline-containing peptides were developed and tested for their reactivity with RA sera [63]. Citrulline is a nonstandard amino acid, as it is not incorporated into proteins during protein translation. It can, however, be generated by post-translational modification of arginine residues by peptidylarginine deiminase (PAD; EC 3.5.3.15) enzymes (Fig. 1) (reviewed in [64]). Using a citrulline-containing peptide which was derived from filaggrin sequences in an enzyme-linked immunosorbent assay (ELISA), antibodies could be detected in up to 48% of RA sera with 98% specificity. Only peptides containing citrulline were reactive. Peptides in which citrulline was substituted by another amino acid were not reactive at all, indicating that the citrulline moiety is the antigenic determinant recognized by the APF and AKA antibodies [63]. These data were confirmed by the observation that filaggrin purified from rat esophagus or human epidermis is reactive both with RA sera and with specific anti-citrullinated protein antibodies [65].

RA sera show a remarkable variety in the reactivity pattern toward different citrulline-containing peptides, indicating that the amino acids flanking the citrulline residue are important for the antigenicity of the epitope and that anti-citrullinated protein activities such as APF and AKA are strongly polyclonal responses [63]. It has been established that these antibodies are produced locally in the synovium of RA patients [66–68]. This suggests the presence of citrullinated protein(s) in the RA synovium causing an antigen-driven maturation of anti-citrullinated

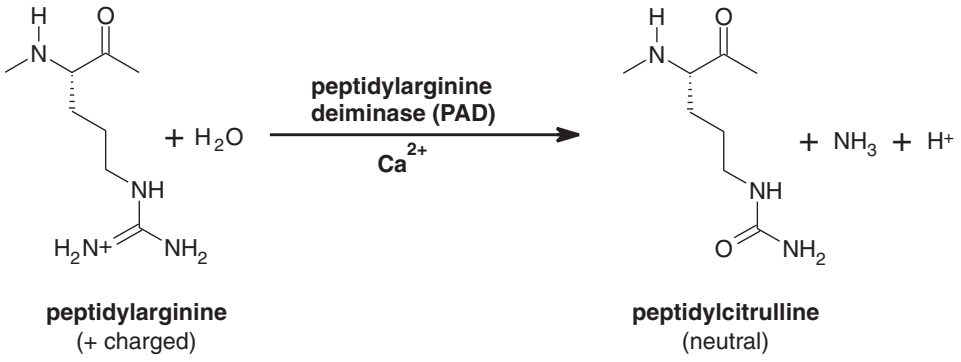

Fig. 1. Citrullination (deimination) of peptidylarginine by PAD.
The guanido group of arginine is hydrolyzed yielding a ureido group and ammonia.

protein specific B-cells at the site of inflammation in RA. Filaggrin, however, is expressed in differentiating epithelial cells, but not in the synovium [69]. It thus appears that anti-filaggrin antibodies (AFA) originate from a response against one or more synovial citrullinated proteins and that their reaction with citrullinated filaggrin is caused by cross-reactivity. Filaggrin is a highly heterogeneous protein, displaying considerable sequence variation (~40%) between subunits [56]. Furthermore, variable phosphorylation (on average 22 potential phosphorylation sites per filaggrin subunit) [56] and variable citrullination [70] contribute to an extensive charge heterogeneity. Due to these properties, filaggrin can be regarded as a natural library of citrullinated epitopes, which makes it a mimic of synovial citrullinated antigens.

3.3. Diagnostic potential

The APF and AKA assays never became mainstream diagnostic tests, because of their inconvenient and laborious immunofluorescence test format, which hampers interlaboratory standardization. To circumvent this problem several attempts have been made to set up assays using filaggrin as the antigenic substrate. Immunoblotting and ELISA assays using filaggrin purified from human skin as an antigen performed very well with respect to specificity [71,72]. Sensitivities, however, were quite disappointing (41% and 47% respectively, at 99% specificity) [71,72] and appeared to be dependent on the method of purification of the filaggrin. Slack et al. [73] calculated sensitivities of 12% and 16% for two different filaggrin preparations, while only one out of five positive sera reacted with both preparations. This reflects a major disadvantage of these assays; it is difficult to obtain an antigen preparation that is pure and contains a reproducible citrulline content. Nogueira et al. [74] compared the diagnostic sensitivities of four different AFA assays which were 40%, 37%, 22%, and 31% (at 99% specificity) for classic AKA immunofluorescence, filaggrin immunoblot, ELISA with filaggrin purified from human skin and an ELISA using recombinant human filaggrin citrullinated by PAD in vitro, respectively. Concordance between the assays was moderate (correlation coefficients between 0.50–0.86) [74]. Recently a more sophisticated ELISA assay using recombinant rat filaggrin citrullinated in vitro has been described, which showed a sensitivity of 62% at a specificity of 99% [75]. So it seems that for AFA assays a qualitative good substrate can be obtained by in vitro citrullination of recombinant filaggrin. Nevertheless, preparation of sufficient amounts of purified recombinant filaggrin is a laborious and specialized task and even with in vitro citrullinated proteins, it is difficult to obtain an antigen with a reproducible citrulline content. This will most likely hamper interlaboratory standardization. Such problems do not occur when synthetic citrullinated peptides are used, because in such cases the precise epitopes are known [63,76].

4. The CCP1 and CCP2 systems

To increase the sensitivity of the citrulline-containing peptide ELISA [63], peptides were modified to adopt a structure in which the citrulline moiety is optimally exposed for antibody binding ([76] and our unpublished 3D-NMR observations). With a single cyclic citrullinated peptide (CCP), antibodies could be detected in 68% of RA sera with a very high specificity

(98%) [76]. This filaggrin-derived cyclic peptide was used as the antigenic substrate in the CCP1 test (results described below).

Although the sensitivity of the CCP1 assay was higher than that of the classical APF and AKA assays and most AFA assays, it was not as high as the sensitivity of the IgM-RF [18]. As explained above, the CCP1 peptide is derived from filaggrin sequences, and since filaggrin is not expressed in the synovium, it is most likely not the natural antigen for the anti-citrullinated protein antibodies. Other peptides, not related to filaggrin, could therefore potentially provide better epitopes for detection of the antibodies. To obtain such novel peptides, dedicated libraries of citrulline-containing peptides were screened with RA sera (as described in [77–79]). From these libraries, novel citrullinated peptides were obtained and incorporated into a second generation CCP test (CCP2). The cyclic peptide(s) in the CCP2 test have no homology with filaggrin or other known proteins. This test has a sensitivity comparable to the IgM-RF test (82% in a cohort of mainly chronic RA patients at 98.5% specificity, vs. 80% sensitivity for RF) [13]. The CCP2 ELISA is commercially available (Euro-Diagnostica, Arnhem, The Netherlands; Axis-Shield, Dundee, Scotland; INOVA, San Diego, USA) and since all these companies use the same type CCP2 peptides, standardization is achieved quite easily. The CCP2 test has now been used in several studies that will be discussed below.

5. CCP for the clinician

A good serological marker should be:

1. Sensitive and highly specific for the disease
2. Detectable very early in the disease
3. Able to prognose disease outcome

5.1. Anti-CCP is extremely specific for RA

A good serological marker for RA should be highly specific for the disease and be able to distinguish RA from other arthritides that mimic RA. In the original studies of Schellekens et al. [76], in which anti-CCP was measured in cohorts of RA patients, in patients with other rheumatic diseases, in patients with infectious diseases and in healthy controls, it could already be concluded that anti-CCP antibodies (anti-CCP1 in this case) are very specific for RA. These results were subsequently confirmed by Bizzaro et al. [80], Bas et al. [81], and many others. These earlier studies have been reviewed by van Boekel et al. [18]. More recent studies confirmed this specificity also for the second generation CCP2 test. In a large multicenter trial 78% of RA patients, 15% of healthy controls and 50% of disease controls tested positive for IgM-RF, while 79% of RA patients, none of the healthy controls and only 5% of disease controls were positive for anti-CCP2 [82]. In another large cohort study anti-CCP2 was positive in 82% of (chronic) RA patients, 1% of healthy controls, and 2% of disease controls (80%, 1%, and 12%, respectively for IgM-RF) [13]. A recent study by Pinheiro et al. with 150 RA patients independently confirms these results (80% sensitivity at 98% specificity) [83]. CCP2 thus has a comparable sensitivity but a much higher specificity for RA than RF. A summary of the results from these cohorts, supplemented with data from other cohort studies is given in Table 1.

In a recent study by Lee and Schur, a somewhat lower specificity of only 90% for CCP2 is reported (66% sensitivity; RF 72% sensitivity and 80% specificity) [84]. Most of the false positives in this cohort were juvenile RA (JRA) patients, 29% of which were positive for anti-CCP. Previous reports [85,86] have shown that anti-CCP (CCP1 at that time) antibodies can be present in a subset of JRA patients, mostly in polyarticular, IgM-RF positive (which is rare in JRA [87]), erosive JRA patients. The JRA patients in the Lee and Schur cohort comprised adults (average age 31, range 21–50) with longstanding disease (average disease duration 21 years) and high prevalence of erosions (87%) [84]. Some of these JRA patients might actually have adult RA with childhood-onset, which could explain the high frequency of anti-CCP antibodies in the JRA group of this cohort.

When the level of the anti-CCP titer is taken into account, specificity appears to be almost absolute. In a study by Bizzaro et al. [80] 7 out of 232 controls (3%) tested positive for anti-CCP1, three of them with high titers (>1000 units; cut-off for CCP1 is 50 units). After reexamination, two of the three patients with high anti-CCP titer were diagnosed with RA. The third patient had died. None of the four patients that were weakly positive for anti-CCP had developed signs of RA [80]. Higher titers of anti-CCP are thus almost exclusively observed in RA patients.

Various diseases can be confused with RA in their early stages. Patients with early SLE for instance, can be misdiagnosed as having RA, because they can be RF positive and can present erosive polyarthritis [88]. Mediwake et al. [89] showed that anti-CCP status can be

Table 1
Overview of CCP2 sensitivity and specificity

	CCP2			IgM-RF		
	n	*pos*	%	*n*	*pos*	%
Rheumatoid Arthritis	1117	865	77%	1119	827	74%
Healthy individuals	431	1	0%	335	38	11%
Osteoarthritis	81	7	9%	79	14	18%
Juvenile Rheumatoid Arthritis[a]	21	6	29%		*not tested*	
Various connective tissue diseases[b]	814	44	5%	573	217	38%
Various inflammatory diseases[c]	225	2	1%	160	14	9%
Various arthropathies[d]	297	13	4%	72	5	7%
Various viral infections[e]	188	2	1%	171	43	25%
Various bacterial infections[f]	147	2	1%	179	21	12%
Various parasitic infections[g]	93	2	2%	99	22	22%
Total non-RA	2297	79	3%	1668	374	22%
Specificity			97%			78%

Combination of CCP2 cohort studies described in [13,82,84]. Other CCP2 studies described in the text are [83,94,97,99,157]

[a] = includes adult JRA patients (see text for details) [84].

[b] = including systemic lupus erythematosus, mixed connective tissue disease, scleroderma, Sjögren's syndrome, CREST syndrome, polymyositis/dermatomyositis.

[c] = including Crohn's disease, colitis ulcerosa, polymyalgia rheumatica, vasculitis, Wegener's Granulomatosis, sarcoidosis, autoimmune thyroditis.

[d] = reactive arthritis, gout, psoriatic arthritis, fibromyalgia, Reiter's syndrome, inflammatory polyarthritis, ankylosing spondylitis.

[e] = including Epstein-Barr, Hepatitis C, Parvovirus B19, Rubella, Viraemia.

[f] = including inflammatory endocarditis, *Legionella, Lyme borrelia, Yersinia, Salmonella*, tuberculosis, *Streptococcus pyogenis.*

[g] = including malaria, *Mycoplasma, Toxoplasma*, Chaga's disease, *Schistosoma, Leishmania*.

used to distinguish RA from erosive SLE. In a large cohort of SLE patients, 10 patients had erosive disease, six of whom were RF positive (60%), six were anti-RA33 positive (60%), whereas only two were anti-CCP1 positive (20%). Of the nonerosive patients, 18% was RF positive, 6% RA-33 positive, and 0.5% was CCP1 positive [89]. Also, patients with chronic hepatitis C virus (HCV) infection often display extrahepatic manifestations among which arthropathy is common, affecting up to 20% of HCV-infected individuals. HCV-related arthritis is commonly presented as rheumatoid-like, symmetrical inflammatory polyarthritis involving mainly small joints, or, less commonly, as monoarthritis or oligoarthritis, usually of the large joints [90,91]. Because many HCV infected patients are RF positive, especially those that display mixed cryoglobulinemia (MC), RF can not be used to discriminate HCV-related arthritis from RA [90,92,93]. In a group of 50 randomly selected HCV patients, none were positive for anti-CCP2 (44% were positive for RF) and of 29 HCV patients with MC only 2 tested positive for anti-CCP2 (76% were RF-positive) [94].

An interesting study on palindromic rheumatism (PR) showed that these patients may also contain anti-CCP and AKA antibodies [95]. PR is characterized by short-lasting attacks of acute arthritis that start abruptly and usually affect single joints, with no remnant clinical or radiographic changes. Some of these patients, and especially those with a positive RF, develop a connective tissue disease, mainly RA. Anti-CCP1 was found in 56% of patients with pure PR (AKA in 36%), about the same proportion of early RA patients was positive for anti-CCP (55%). In contrast, only 2.5% of patients with seronegative spondylarthropathy was anti-CCP positive. These data suggest that PR might be an incomplete expression of RA, and that the CCP positive PR patients may be in the process of developing RA [95].

In conclusion we can say that due to its high specificity, anti-CCP makes a reliable diagnosis for RA possible.

5.2. Anti-CCP is present early in disease

When a random population of a rheumatology clinic is tested for anti-CCP, about 2–5% of the patients test positive but do not suffer from RA (our unpublished observations). A similar phenomenon can be observed when the population of an early arthritis clinic is tested (F. van Gaalen, unpublished data). Two recent studies have provided clear evidence that such supposedly false positives might be in the early phase of developing RA. Nielen and coworkers measured anti-CCP1 and IgM-RF levels in serial blood samples of 79 former blood donors who later developed RA [96]. Of these patients, 41% became anti-CCP1 positive at a median of 4.8 years before the onset of the complaints; 28% at a median of 2.0 years for IgM-RF. Anti-CCP antibodies could be detected in predisease serum samples up to 14 years before onset of the first symptoms of RA; IgM-RF up to 10 years. Of the 2138 control samples, 1.1% was positive for IgM-RF and 0.6% for anti-CCP1. Thus, anti-CCP detected more positive subjects, longer before the start of the complaints and with a higher specificity compared to IgM-RF [96]. In a similar type of study, Rantapää-Dahlqvist and collaborators analyzed early blood samples from 83 blood donors who subsequently developed RA [97]. Anti-CCP2 antibody was detectable in 25% of the subjects 1.5 to 9 years before onset of the first symptoms of the disease. In the last 1.5 years before presentation of the first symptoms, the sensitivity of anti-CCP2 increased to 52%. More than 70% of the patients were anti-CCP2 positive at their

first visit at the clinic. RF could also be detected in the predisease sera, albeit at a lower sensitivity (IgM-RF 15% and 30%; IgG-RF 12% and 27%; IgA-RF 29% and 39%, more than 1.5 years and less than 1.5 years before disease onset, respectively). They conclude that anti-CCP antibody has the highest ability to predict the future development of RA [97].

Other recent studies point to the same conclusion. Jansen and coworkers [98] tried to discriminate RA from undifferentiated polyarthritis, and found that the combined presence of IgM-RF and anti-CCP1 is able to predict which patients with early arthritis ultimately develop RA with a sensitivity of 55.4% and a specificity of close to 97%. Similar observations were made by van Gaalen et al. [99]. In a large cohort of 936 patients with recent onset arthritis, 318 patients could not be properly classified and were categorized as undifferentiated arthritis (UA). After 3 years of follow-up, 40% of these UA patients had developed RA. Of the UA patients that were negative for anti-CCP2 at baseline, 25% developed RA. In contrast, of the UA patients with a positive anti-CCP2 test at baseline 93% progressed to RA (odds ratio 37.8).

The conclusion from all these studies is that anti-CCP antibodies are present early in disease, and that their presence is able to accurately predict the development of RA.

5.3. Anti-CCP predicts erosive disease

It is known for some time that APF/AKA/AFA are able to predict erosive disease progression in (early) RA patients. APF/AKA/AFA-positive patients clearly exhibit higher radiological damage scores than negative patients [100–105]. Because the APF/AKA/AFA belong to the same family of anti-citrullinated protein antibodies as anti-CCP, it may be expected that also anti-CCP antibodies are able to predict erosive disease.

Kroot and coworkers [106] were the first to study the predictive value of anti-CCP1 in patients with recent-onset rheumatoid arthritis. Their results with this relatively unsensitive first generation CCP test clearly showed the enormous potential of these antibodies. Patients with anti-CCP1 had developed significantly more severe radiological damage after 6 years of follow-up than patients without anti-CCP1. However, in multiple regression analysis the additional predictive value of this antibody in this cohort of patients was only moderate [106].

Visser et al. [107] used CCP1 serology as an additional help in predicting disease outcome. A standardized diagnostic evaluation was performed on 524 newly referred patients with early arthritis. Potentially diagnostic determinants, among them IgM-RF and CCP1, were entered in a logistic regression analysis. Arthritis outcome was recorded at 2 years' follow-up. The discriminative ability of the model and various parameters was expressed as a receiver operating characteristic (ROC) area under the curve (AUC). The ROC/AUC of the model was 0.84 for discrimination between self-limiting and persistent arthritis, and 0.91 for discrimination between persistent nonerosive and persistent erosive arthritis. The discriminative abilities of the revised 1987 ACR criteria for rheumatoid arthritis were significantly lower with ROC/AUC values of 0.78 and 0.79. The developed prediction model consisted of seven variables, in which anti-CCP1 had the highest weight factor [107].

A recent study from Vencovský and coworkers [108] confirmed that anti-CCP antibodies are able to predict erosive and progressive disease. In a prospective study they measured baseline levels of several autoantibodies, and found that significant differences in erosions and disease progression were detected by anti-CCP1, AKA and APF (as described above, all

three antibody specificities are directed to citrullinated antigens) and by RF (IgM, IgA, and IgG). Combined analysis of anti-CCP1 and IgM-RF provided the most accurate prediction [108]. Meyer and coworkers [109] followed 191 patients with recent onset RA prospectively for 5 years. The serum samples of these patients were examined for AKA, APF, and CCP1; erosions were evaluated using Sharp scores modified as described by van der Heijde [110]. Their results showed that the likelihood of a total Sharp score increase after 5 years was significantly greater among patients with anti-CCP antibodies (odds ratio 2.5) than patients with RF (odds ratio 0.7). They concluded that antibodies to citrullinated proteins/peptides are good predictors of radiographic joint damage [109].

Jansen et al. [111] investigated radiographic progression of RA in a group of 282 early arthritis patients, which included patients with undifferentiated oligoarthritis and polyarthritis. Variables most predictive for radiographic progression of the disease after 2 years of follow-up were anti-CCP positivity (32% anti-CCP1 positive at baseline) and joint damage at entry. In a study by Manetti and coworkers [112], early arthritis patients that presented bone erosions were significantly more often anti-CCP2 positive and on average with higher titer than patients without bone lesions. Their data indicate that anti-CCP antibodies can be a useful marker in the early phase of the disease to differentiate subjects likely to have progressive bone erosions from those with self-limiting or nonerosive disease, especially when high antibody titers are presented.

In a group of patients with Felty's syndrome (very severe RA complicated with leukopenia and splenomegaly), very high anti-CCP titers were observed in all patients ($>$1000 CCP1 units, n $=$12, our unpublished observations).

All these studies indicate that presence of anti-CCP antibodies is correlated with erosive disease progression. The antibodies can therefore be used in clinical practice to help in planning a therapeutic strategy.

5.4. Take home message

The data discussed above, show that anti-CCP indeed is a good serological marker for RA. CCP is well suited as a front line diagnostic test for RA and especially early RA, because it can often be detected before complete clinical manifestation of the disease. CCP can also help to identify candidates for aggressive treatment because of its prognostic potential. Due to its high specificity for RA, anti-CCP can be used to differentiate RA from other forms of arthritis that may be confused with RA because they can be erosive and RF positive. In RF seronegative patients, CCP can be helpful in confirming the diagnosis of RA, because approximately 40% of RF negative RA patients is positive for anti-CCP2.

Addition of anti-CCP to the ACR criteria would improve the accuracy of the criteria. Because, in most studies, a combination of IgM-RF and anti-CCP has a higher diagnostic and prognostic potential than either of these serological markers alone, it seems logical to add anti-CCP as an eighth criterion.

6. For the researcher: citrullinated autoantigens

6.1. Local presence of citrullinated autoantigens at the site of inflammation in RA

The anti-CCP antibodies are probably produced locally in the synovium as they constitute a higher proportion of IgG in synovial fluid and synovial tissue compared to serum (1.4 and

7.5-fold, respectively) [66,67]. Furthermore anti-CCP producing plasma cells have been isolated from the synovium of RA patients [66,68]. This local antibody production requires the existence of citrullinated antigens in the inflamed synovium and consequently also the presence of PAD enzymes. Five isotypes of PAD exist in mammals (reviewed in [64]). PAD1 and PAD3 are mainly found in epidermis and hair follicles [113–116]. PAD2 is expressed in a variety of tissues, including muscle, brain and hemopoietic cells [117–119], while PAD4 (this human enzyme was formerly known as PAD5) is predominantly found in hemopoietic cells [120–122]. The recently described PAD6 (originally annotated as ePAD) is only expressed in egg cells and embryonic tissue [47,123].

Recently, the existence of 17 single nucleotide polymorphisms (SNPs) in the PAD4 gene was reported by Suzuki et al. [124]. Because the SNPs are located closely together on the chromosome, they segregate together in distinct haplotypes. Two haplotypes account for 85% of all individuals investigated. One of these two major haplotypes was more frequently present in RA patients than in controls. RA patients that are homozygous for the RA-susceptible PAD4 haplotype were significantly more often positive (87%) for anti-citrullinated protein antibodies (AFA) than other RA patients (67%; $P < 0.05$). Four of 17 SNPs are located in exons of PAD4, two of which are strongly correlated with RA ($P < 0.001$). Three of the four exonic SNPs result in amino acid substitutions. Possible consequences for protein function were not investigated, but the authors did show that the RA-susceptible haplotype increases PAD4 mRNA stability. This could result in more PAD4 enzyme being produced, and subsequently lead to increased citrullination of proteins and an increased chance of developing anti-CCP antibodies [124].

6.2. *Proteins are citrullinated during cell death*

An example of a citrullinated protein present in inflamed RA joints is extravascular citrullinated fibrin [125]. Citrullinated fibrin(ogen) is readily recognized by RA sera [125,126]. In inflamed RA synovia the oxygen metabolism is in disequilibrium, which leads to sites with oxygen excess (and subsequent generation of reactive oxygen species) and on the other hand to sites of hypoxia, which can cause synovial tissue microinfarctions [127,128]. At these sites, plaques of extravascular fibrin can be found. The inflamed synovium also contains many PAD2-expressing macrophages and sometimes PAD4-containing granulocytes [47,122,129]. Normally, PAD enzymes are present intracellularly (either in the cytosol or, in the case of PAD4, in the nucleoplasm [122]) but they are not active [47]. Calcium ions are required for activity of the PAD enzymes, but the cytosolic calcium concentration in normal cells (10^{-7} M) is much lower than the threshold Ca^{2+} concentration for PAD activity (~10^{-5} M, our unpublished observations and [130]). When cells are dying, for example as a result of extensive oxidative stress in the inflamed synovium, PAD enzymes might leak out of the cells, become activated (the extracellular Ca^{2+} concentration is ~10^{-3} M, sufficient for PAD activity [130]) and induce the citrullination of extracellular proteins, like fibrin. Furthermore, the integrity of the plasma membrane is lost during cell death [131,132], causing influx of Ca^{2+} from the extracellular space and subsequent activation of intracellular PAD [46,47,122,133,134]. Citrullination of intracellular proteins after calcium influx has been described in granulocytes (citrullination of histones) [122,134] and macrophages (citrullination of vimentin) (Fig. 2) [46,47].

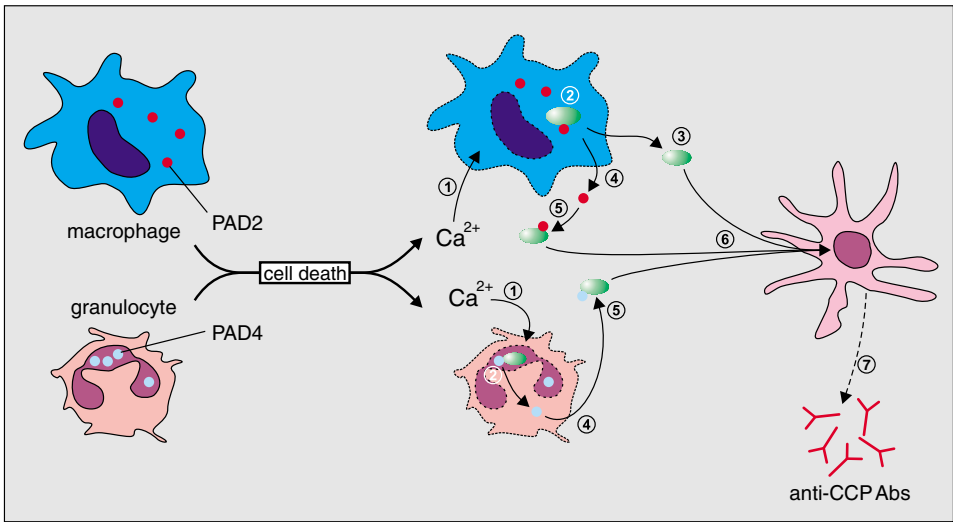

Fig. 2. Citrullination of proteins following cell death.
Macrophages and granulocytes express PAD enzymes (PAD2 and PAD4, respectively). These enzymes are inactive under physiological conditions, because cytosolic Ca^{2+} concentrations (required for PAD activity) are normally too low. During death of these cells, Ca^{2+} homeostasis is lost and Ca^{2+} can enter the cell (1), thereby activating the PAD enzymes. Intracellular proteins (like vimentin in macrophages or histones in granulocytes) are citrullinated (2) and can potentially leak out of the dead cells (3). Furthermore, PAD enzymes can leak out of the dead cells (4) and citrullinate extracellular proteins (like fibrin) (5). The citrullinated proteins can be exposed to the immune system (6), which could –given the right conditions (genetic factors and danger signals) are present– lead to an immune response (7).

Although it has not unequivocally been shown that citrullinated vimentin is present in the inflamed synovium of RA patients, much circumstantial evidence suggests it is. The cells of the synovial lining are of mesenchymal origin and thus contain vimentin [135]. The majority of these cells are infiltrated macrophages that show signs of activation [129] (in RA 80–100% of synovial lining cells are macrophage-like cells, compared to 20–30% in normal synovium [136]). Short-term activated macrophages are protected from apoptosis, whereas long-term (>24 hours) activated macrophages are very prone to cell death [137]. Calcium influx in the dying macrophage triggers activation of PAD and subsequent citrullination of vimentin (and possibly other cellular proteins) [47]. Furthermore, it has been suggested that the Sa-antigen, which can be detected in the RA synovial tissue [40], is identical to citrullinated vimentin [44].

As explained above, RA sera showed a remarkable variety in their reactivity pattern toward different citrulline-containing peptides, which is indicative of a polyclonal immune response, possibly caused by the existence of multiple citrullinated antigens [63]. Therefore, it is not unlikely that next to citrullinated fibrin(ogen) and citrullinated vimentin, also other citrullinated proteins are autoantigenic in RA. Indeed, the presence of various unidentified citrullinated proteins has been described in the RA synovium [125].

7. For the researcher: possible pathoimmunological role in RA

Many animal models are being used to study the mechanisms of inflammation in RA (for an overview see [138]). We recently showed that synovial proteins are citrullinated during

inflammation in both an acute (streptococcal cell wall arthritis) and a chronic destructive (collagen-induced arthritis) mouse model for arthritis [139]. One of the citrullinated proteins in the inflamed mouse synovium could be identified as fibrin. However, in contrast with the human disease, the mice did not produce antibodies against the citrullinated proteins [139]. In fact none of many animal models of arthritis tested (including rodent, canine and primate models) appears to be positive for anti-CCP (our unpublished observations).

It thus appears that the citrullination of synovial proteins is an inflammation-associated process, not a process specific for RA. But why is the humoral response so specific for RA and does it contribute to the disease process?

7.1. The HLA connection

The anti-CCP antibodies are mainly of the IgG class, although IgM and IgA anti-CCP can also be detected, albeit at a much lower prevalence. IgG1 is the prevailing subclass; roughly a quarter of the anti-CCP positive sera also contain IgG4, while IgG2 and IgG3 are rarely found (our unpublished observations and [140]). This subclass distribution is characteristic of a T-cell dependent antibody production and thus suggests HLA involvement [141]. The genetic predisposition for RA by certain HLA haplotypes, for example, HLA-DR4 (HLA-DRB1*0401 and *0404) has been known for over 25 years [2].

Recent molecular modeling data indicate that peptides containing citrulline, but not arginine, can be bound by *0401 MHC molecules [142]. This citrulline-specific interaction might be the basis of a citrulline-specific immune response. T-cell proliferation assays with HLA-DRB1*0401 transgenic mice showed that stimulation with citrullinated peptides, not with arginine peptides, induced proliferation and activation of T-cells [142]. These data suggest that the specific structure of HLA-DR4 molecules plays an important role in the induction of autoimmunity to citrullinated proteins.

Although there is not an absolute requirement for HLA-DR4 in order to develop anti-CCP antibodies, there is a strong correlation between HLA-DR4 status and anti-CCP positivity in RA patients [143]. As described above, presence of anti-CCP antibodies often precedes onset of clinical symptoms of RA and can therefore predict development of RA [97]. In an expansion of this study, Berglin and coworkers [144] observed that anti-CCP2 antibodies combined with the presence of HLA-DR4 genes had a higher accuracy in predicting RA (OR 66.8), compared to anti-CCP2 (OR 25.1) or HLA-DR4 (1.9) alone.

7.2. The modified self protein

Even if the correct genetic background is present, additional factors are needed to induce an immune response against self proteins. Many known autoantigens become modified during cell death and in particular during apoptosis (for an overview [145]). These modifications include proteolytic cleavage by caspases or granzyme B [146], (de)phosphorylation [147,148], transglutamination [149] but also citrullination [46,47]. When these modified self proteins are inefficiently cleared, they will be presented to the immune system and might not be recognized as self [150]. If sufficient danger signals are present (like in an inflammatory environment), this can lead to an immune response against the modified proteins [150,151]. In this manner, an initial small inflammation (caused by an external factor) may cause the

death of inflammatory cells and consequently induce the citrullination of synovial proteins (Fig. 2) [139]. In an appropriate genetic environment, this may lead to presentation of citrullinated peptides by antigen-presenting cells (APCs) and consequently to an activation of T-cells. Given the right conditions are present, this initial immune response can snowball into a systemic disease that becomes manifest many years later.

7.3. Importance of anti-CCP autoantibodies in the disease

Although no direct evidence exists for a role of anti-CCP antibodies in the pathophysiology of RA, several observations suggest they might. First, high anti-CCP antibody titers are correlated with severe and erosive disease progression as discussed above. Second, the predominance of IgG1 anti-CCP is compatible with effector mechanisms involving complement activation and/or the engagement of Fc gamma receptors (our unpublished observations and [140,141]). Furthermore, treatment responses after B-cell depletion by anti-CD20 antibody therapy (rituximab) suggest an important function for B-cells in the pathology of RA [152–154]. In a group of RA patients treated with rituximab, levels of circulating anti-CCP and RF (only IgA-RF and IgG-RF) antibodies decreased significantly more than their corresponding total serum immunoglobulin classes (IgM-RF decrease was in parallel with total IgM decrease) [155]. In contrast, antibacterial antibodies did not significantly decrease during the treatment. The majority of the patients (70%) showed a marked clinical improvement, which lasted on average 15 months. Relapse was closely correlated with a rise in levels of autoantibodies and preceded by return of B cells [155]. A drop of anti-CCP and RF titers was also observed by Alessandri and colleagues in RA patients that were treated with anti-TNFα (infliximab, reviewed in [156]) therapy in combination with low-dose (MTX) [157]. Serum titers of anti-CCP and IgM-RF decreased significantly after 6 months of therapy ($P = 0.0003$ and $P = 0.02$ respectively), while no significant change of anti-CCP levels was observed in patients treated with MTX alone. Clinical response and changes in anti-CCP titers were correlated; patients with best clinical improvement during the therapy had lowest anti-CCP titers at baseline and showed strongest decrease in titer during treatment [157]. All these data suggest that anti-CCP antibodies might be involved in the disease process in RA.

Interesting in this respect is a recent study by Lard et al. [158] showing that a specific IL-10 promoter polymorphism is associated with progression of joint damage. IL-10 is a pleiotropic cytokine (reviewed in [159]). It has many antiinflammatory functions (i.e., inhibition of production of proinflammatory cytokines, inhibition of APC maturation, etc.), but can also stimulate inflammation by enhancing B-cell proliferation, differentiation and antibody production [160,161]. The specific single nucleotide polymorphism (−2849 (AG/GG)) that is described in the Lard study, is associated with high IL-10 production and consequently with high antibody production [158]. Anti-CCP positive RA patients with the high IL-10 haplotype have significantly higher anti-CCP titers and more severe erosions than anti-CCP positive patients with a low IL-10 haplotype [158].

A possible mechanism by which anti-CCP antibodies could contribute to the progression of the disease is depicted in Fig. 3. An initial small inflammation can lead to the death of PAD-containing cells and the generation of synovial citrullinated proteins as described above and shown in detail in Fig. 2. In anti-CCP positive patients, the locally produced anti-CCP antibodies will form immune complexes with these citrullinated proteins. The immune

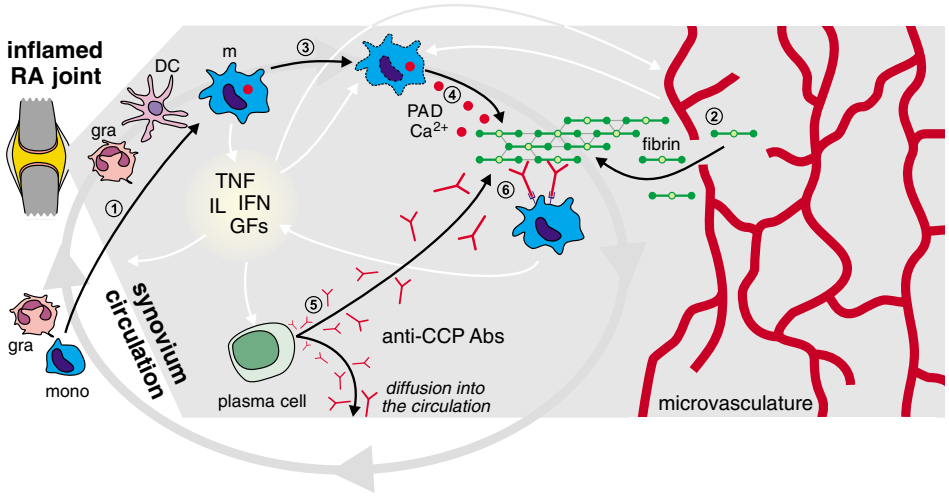

Fig. 3. Possible role of anti-CCP antibodies in the pathofysiology of RA.
Inflammatory cells infiltrate the inflamed RA synovium and become activated [(1)]. The activated cells produce various inflammatory mediators (indicated by the yellowish circle). The microvasculature in the inflamed synovium is in disequilibrium, partly due to the action of the inflammatory mediators. This leads to sites of hypoxia, which can cause synovial tissue micro-infarctions [(2)]. Fibrinogen can leak into the synovium and form plaques of extravascular fibrin (indicated as a green mesh). On the other hand, the microvasculature imbalance can lead to sites of oxygen excess and subsequent generation of reactive oxygen species. These reactive oxygen species can cause death of the infiltrated cells [(3)]. As described in Figure 2, PAD enzymes may leak out of the dying cells and become activated by the high concentration of extracellular Ca^{2+} [(4)]. Subsequently, extracellular proteins (including the extravascular fibrin) will be citrullinated. Anti-CCP antibodies, that are actively produced in the synovium [(5)], can bind to the citrullinated proteins. The immune complexes that are formed can be bound by inflammatory cells via their Fcγ receptors [(6)] which causes an activation of the cells. The activated cells produce novel cytokines to maintain the pool of inflammatory mediators in the synovium. This completes the circle and the inflammation process will continue.
Abbreviations used: IL, interleukins; TNF, tumour necrosis factors; IFN, interferrons; GFs, growth factors; mono, monocytes; gra, granulocytes; DC, dendritic cells; mφ, macrophages. White arrows indicate molecular signals.

complexes will subsequently cause activation of inflammatory cells and production of proinflammatory cytokines. The cytokines promote infiltration of more inflammatory cells into the synovium, that will eventually die and give rise to the production of more citrullinated proteins. In this manner, anti-CCP antibodies might contribute in the perpetuation of the inflammations and the chronicity of the disease.

8. The future of CCP

The current second generation CCP system meets many of the requirements of a useful serological marker for RA. It is highly specific for the disease, can be detected early in the disease and because of its prognostic potential, it may provide an indication for aggressive treatment. With regard to the sensitivity of anti-CCP, there still is some room for improvement. The ideal marker would have a sensitivity of 100%, but because RA is such a complex and

diverse condition, absolute sensitivity is impossible. As described above, the antibody response to citrullinated epitopes is strongly polyclonal. By increasing the number of citrullinated epitopes that are presented in the CCP test, it should be possible to increase the sensitivity of the assay. Knowledge on the identity of the citrullinated candidate autoantigens could be helpful in designing such additional epitopes. In principle, it is also possible to use the candidate autoantigens described above, citrullinated fibrin(ogen) and citrullinated vimentin, as antigens in diagnostic assays. The use of entire proteins does, however, pose some major problems. First it is difficult to prepare sufficient amounts of high quality antigen and to control the degree of citrullination of these antigens. A bigger problem is the existence of circulating antibodies directed against the noncitrullinated protein. Because these antibodies are not directed against the citrulline moiety, they are not specific for RA and can therefore lead to false positives. Anti-vimentin autoantibodies for instance, have been described in autoimmune diseases [162], infectious diseases [163,164] and even in healthy individuals [165]. The use of synthetic citrullinated peptides gives absolute control over the exact epitopes presented. Peptides that give false positive results (some peptides are reactive with sera even when citrulline is replaced by arginine, our unpublished observations) can be selectively left out. That is why an approach using synthetic peptides as antigenic substrates should be preferred. Recent developments in microarray technology have opened up the possibility to measure reactivity against a large number of individual peptides at the same time [166]. If the availability of this technology improves, peptide microarrays could become a platform for a third generation CCP test.

A big pro of antigen-array technology is the ability to obtain multiplex autoantibody profiles with the ease of use and speed of a single ELISA [166–168]. Sophisticated computer programs are needed to assess the presence of hundreds of autoantibody markers and to link each autoantibody profile to a certain diagnosis. This allows clinicians to obtain serological information to aid diagnosis of a wide variety of autoimmune diseases. Although this array technology looks very promising, it is still in its childhood. Future studies need to confirm proof of concept. Until that time, the CCP2 ELISA is the best diagnostic marker for RA.

9. Conclusion

Anti-CCP antibodies meet all the requirements of a good serological marker for RA. Their high specificity and prognostic value make them a valuable diagnostic tool. Furthermore, all the available experimental data is compatible with a possible role in the disease process.

Currently, the revised 1987 ACR criteria are considered the gold standard for the classification of RA. Incorporation of anti-CCP positivity as an extra criterion would improve the accuracy of the ACR criteria [99,107]. Therefore, we suggest to add anti-CCP as an eighth criterion.

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