



Review

Closing the serological gap: promising novel biomarkers for the early diagnosis of rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation and damage of the joints affecting about 0.5% of the general population. Early treatment in RA is important as it can prevent disease progression and irreversible damage of the joints. Despite the high diagnostic value of anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF), there is a strong demand for novel serological biomarkers to further improve the diagnosis of this abundant disease. During the last decades, several autoantigens have been described in RA including Ra33 (hnRNP A2), fibrinogen, fibronectin, alpha-enolase, type II collagen, immunoglobulin binding protein (BiP), annexins and viral citrullinated peptide (VCP) derived from Epstein Barr Virus-encoded protein (EBNA-2). More recent discoveries include antibodies to carbamylated antigens (anti-CarP), to peptidyl arginine deiminase type 4 (PAD4), to BRAF (v raf murine sarcoma viral oncogene homologue B1) and to 14 autoantigens identified by phage display technology. This review provides a current overview of novel biomarkers for RA and discusses their future potential to improve the diagnosis of the disease.

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1. Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation and damage of the joints affecting about 0.5% of the general population [1]. Early treatment in RA is important as it can prevent irreversible damage of the joints. Despite the strong diagnostic value of anti-citrullinated protein antibodies (ACPA) and

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rheumatoid factor (RF), there is a strong demand for novel serological biomarkers to further improve the early diagnosis of this abundant disease. During the last decades, several autoantigens have been described, which are discussed in this review.

2. Anti-citrullinated protein antibodies (ACPA)

ACPA are an important serological marker in the diagnosis of RA [2,3]. ACPA can be detected up to 10 years before RA patients first present to a clinician, predicting the future development of RA [4]. In addition, the presence of ACPA is associated with a specific disease course [5]. Historically, a combination of several serologic markers, including RF, anti-perinuclear factor (APF) and anti-keratin antibody (AKA), has been used to aid in the diagnosis of RA [2]. With the discovery in 1998 that the underlying antigen in the APF/AKA tests contained citrulline [6], the development of novel assays to detect ACPA was facilitated [2]. In 2010, ACPA were added as one of the American College of Rheumatology (ACR)/The European League Against Rheumatism (EULAR) disease classification criteria for RA [7,8]. Whereas the first generation of the cyclic citrullinated peptide (CCP) test relied on a peptide derived from the flaggrin protein [6], the second- and third-generation CCP (CCP2, CCP3) tests are based on artificial optimized peptides to detect ACPA [9,10], thereby enhancing the presentation efficacy of the citrulline-containing epitope(s). The CCP2 peptide sequence has been identified by screening peptide libraries of extremely high complexity with sera of RA patients which resulted in a highly immunogenic antigen [10]. In contrast, CCP3 was designed by combinatorial peptide engineering and contains multiple citrullinated epitopes displayed in a conformational structure to increase epitope exposure and thus immunoreactivity, especially for early RA (unpublished data). Later on, CCP3.1 has been developed that detects ACPA IgG/IgA and that is until today the only assay that has been approved by the FDA for the early detection of RA (510 k number: K072944).

Over the past few years, many studies have evaluated the diagnostic performance of ACPA assays on a variety of diagnostic platforms [11–16]. A meta-analysis showed that 71.7% of 18,061 RA patients analyzed in these combined studies were positive in the CCP2 test compared to only 1% of 4937 healthy controls and 6% of 15,971 non-RA disease controls [2]. In early RA patients, 61.6% proved to be positive for CCP2 ($n = 4589$) [17].

In conclusion, both sensitivity and specificity of the ACPA tests are significantly higher than those of the RF test [3]. Because of the relatively low pre-test probability of patients routinely tested for RF and ACPA (about 15%) for having RA, the increased specificity of ACPA compared to RF gives the ACPA tests a much greater positive predictive value (PPV) [18,19].

Recent studies comparing different types of ACPA assays [18] showed that, in general, the peptide-based assays have a somewhat better sensitivity and specificity than the protein-based assays. Among the studies comparing CCP2- and CCP3-based assays, a few reported a higher sensitivity of the anti-CCP3 peptide assay compared to anti-CCP2 tests [13,14] while other investigations did not support these conclusions [15]. It has been speculated that the reported higher sensitivity of CCP3 may only be found in cohorts with early RA, whereas the sensitivity may be similar in groups with established disease. Jaskowski et al. [16] found that in RF-negative RA patients, anti-CCP3 antibodies were more prevalent than anti-CCP2 antibodies. In agreement with this observation, a recent study performed by Swart et al. (unpublished data) showed that in early and RF-negative RA patients, the sensitivity of CCP3 is significant higher than that of CCP2.

Despite intense efforts that have gone into standardizing ACPA detection, significant differences persist between ACPA assays [12], even between different assay using the same peptide antigen (CCP2) [11,12]. Recently, it has been shown that the anti-CCP2 titer in early RA is correlated with the epitope diversity (epitope spreading) [20].

These data indicate that patients with early RA and especially in the prediagnostic phase have antibodies to only one or very few epitopes. Therefore, an antigenic construct combining different epitopes as on CCP3 results in a significant number of early-RA patients having antibodies to the combination epitope on CCP3. This may be different for other ACPA assays [10]. Identifying patients at risk at a very early stage is highly desirable in view of the irreversible joint damage and permanent disability that can follow delayed diagnosis and treatment of RA [1].

3. Additional autoantigens recognized by autoantibodies in sera from RA patients

Following the success of the CCP test, several alternative methods for detecting ACPA have been developed, including assays based on citrullinated proteins instead of peptides, such as mutated citrullinated vimentin (MCV; Orgentec, Mainz, Germany) [21], flaggrin (CPA; Genesis, London, UK). In addition, a viral citrullinated peptide has been discovered (VCP; VCP1 and VCP2) [9,21,22]. The limited data and contradictory results from comparative studies on anti-MCV autoantibodies [23–25] compared to anti-CCP assays are inconclusive with respect to the sensitivity and specificity of this assay.

In addition, several other autoantigens have been suggested as target of autoantibodies in RA including Ra33 (hnRNP A2) [2,26], fibrinogen [2,27], fibronectin [27], alpha-enolase [27], type II collagen, immunoglobulin binding protein (BiP) [28] and annexins [29]. Anti-Ra33 antibodies have been reported to identify about 25%–30% of RA patients which are negative for ACPA and which are associated with a mild form of RA [26]. The specificity of anti-Ra33 antibodies is much lower than that of ACPA and more comparable with RF (~90%) [26]. Epitope mapping studies have identified several linear epitopes on Ra33. Some of the epitopes are also recognized by autoantibodies in sera from patients with other pathologies [30]. None of these markers are currently widely used in routine diagnosis of RA.

4. Recent discoveries of novel markers for early RA diagnosis

Although, ACPA have significantly improved the diagnosis of RA, it is unquestionable that novel biomarkers are required for a better diagnosis of early and seronegative RA. Recently, such autoantigens have been described mainly by three research groups [31–36]. Despite all of these biomarkers are very promising, none has yet been transferred into commercial/clinical use.

4.1. Anti-CarP antibodies

In 2010 it has been shown that homocitrulline (hCit)-containing proteins can trigger the formation of citrulline reactive antibodies in rabbits [37]. Although hCit and citrulline (Cit) are both post-translationally modified amino acids and quite similar in structure, there are significant differences. hCit is one methylene group longer and is generated chemically from lysine by cyanate [32]. In contrast, Cit is formed enzymatically from arginine by peptidyl arginine deiminase type 4 (PAD4). Shi et al. [32] identified anti-CarP antibodies that recognize hCit containing proteins in ACPA positive but importantly also in ACPA-negative RA patients using carbamylated foetal calf serum (FCS) as the antigen and non-modified FCS as control which was tested with sera from RA patients ($n = 557$) and healthy controls ($n = 305$). In their cohort, 16% of anti-CCP2 antibody-negative RA patients were positive for anti-CarP IgG and 30% for anti-CarP IgA [32]. Additionally, it was demonstrated that besides the carbamylated proteins of bovine origin, also carbamylated human fibrinogen is specifically recognized by autoantibodies in patients with RA. In this cohort, the presence of anti-CarP antibodies at baseline was associated with a severe disease course characterized by rapid radiological progression [32].

Table 1
Overview of novel autoantigens (biomarkers) in RA.

Antigen	(aa) Sequence, or antigen information	Sensitivity, no RA (%), pos	Specificity, no (%) controls, pos	% of CCP-negative patients, pos	Reference
BRAF p10	(20) RKTRHVNILLFMGYSTKPQL	n.p./180 (35)	n.p./60 (93.0) ^a	40	[33,36]
BRAF p25	(20) YSNINNRDQIIIFMVGRGYLS	n.p./180 (19)	n.p./60 (100.0) ^a	21	[33,36]
PAD4-P22	(20) VRVFQATRGLSSKCSVVLG	15/29 (51.7)	7/66 (89.4)	n.p.	[33,34]
PAD4-P28	(20) LLDTSNLELPEAVVFQDSVV	24/29 (82.8)	30/66 (54.5)	n.p.	[33,34]
PAD4-P61	(20) PFGPVINGRCCLEEKVCSLL	8/29 (27.8)	0/66 (100.0)	n.p.	[33,34]
PAD4-P63	(20) EPLGLQCTFINDFFTYHIRH	n.p. (55)	n.p. (90.0)	n.p.	[33,34]
CarP IgA	Carbamylated FCS	n.p./557 (43.0)	n.p./305 (94.7)	30	[32]
CarP IgG	Carbamylated FCS	n.p./557 (44.9)	n.p./305 (97.0)	16	[32]
UH-RA.1	(9) EKRQEITTE	9/92 (9.8)	4/121 (96.5)	n.p.	[31]
UH-RA.2	(5) SISTS	2/92 (2.2)	0/121 (100.0)	n.p.	[31]
UH-RA.7	(5) SSQDV	3/92 (3.3)	0/121 (100.0)	n.p.	[31]
UH-RA.9	(23) RSCHHGCTFTEDQHWECEGDDAV	4/92 (4.4)	0/121 (100.0)	n.p.	[31]
UH-RA.10	(34) (see Somers et al.)	6/92 (6.5)	0/121 (100.0)	n.p.	[31]
UH-RA.11	(65) (see Somers et al.)	12/92 (13.0)	0/121 (100.0)	n.p.	[31]
UH-RA.13	(7) QDSCQEN	2/92 (2.2)	2/121 (98.3)	n.p.	[31]
UH-RA.14	(7) KEELWRQ	11/92 (12)	0/121 (100.0)	n.p.	[31]
UH-RA.15	(49) (see Somers et al.)	5/92 (5.4)	0/121 (100.0)	n.p.	[31]
UH-RA.16	(176) (see Somers et al.)	5/92 (5.4)	0/121 (100.0)	n.p.	[31]
UH-RA.17	(76) (see Somers et al.)	3/92 (3.3)	0/121 (100.0)	n.p.	[31]
UH-RA.20	(22) RGLHLPSGAPKDEPSHSGMESTV	2/92 (2.2)	0/121 (100.0)	n.p.	[31]
UH-RA.21	(28) PGGFRGEFMLGKPDPKPEKGLGSPYIE	27/92 (29.4)	6/121 (95.0)	n.p.	[31]
UH-RA.22	(32) (see Somers et al.)	2/92 (2.2)	0/121 (100.0)	n.p.	[31]
UH 11 plex	Combination of 11 peptide results	34/92 (37.0)	0/121 (100.0)	44	[31]
UH 14 plex	Combination of 14 peptide results	50/92 (54.4)	12/121 (90.1)	67	[31]

NOTE: For some markers, the number of positive patients (n.p.) was not provided in the reference; therefore, only percent values are presented. CarP = carbamylated protein; PAD4 = peptidyl arginine deiminase type 4; UH = University of Hasselt; RA = rheumatoid arthritis; pos, positive.

^a Specificity based on healthy controls (other controls tested).

Despite the promising data presented in this study, further studies using disease controls are needed to verify the specificity of anti-CarP antibodies. In two other studies published as congress abstracts, it was reported that carbamylated vimentin [38] and fibrinogen [39] can be used to detect anti-CarP antibodies in RA patients. Although the sensitivity of the assays based on carbamylated proteins was significantly lower than ACPA, the results are of high interest for two reasons; (a) the results confirm the observation that RA patients contain antibodies reacting with hCit containing proteins and (b) they show that the modification of different antigens can enhance the reactivity with RA patient sera. In the study by Bang et al. [38], vimentin was significantly more reactive than enolase after carbamylation which indicates that hCit might represent the key target for autoantibody binding, but that surrounding amino acids or even the whole molecule is contributing to the immunogenicity. Unfortunately, only one of these studies stratified the RA cohort in terms of ACPA reactivity and found that 5% of ACPA-negative patients exhibit reactivity to carbamylated fibrinogen [39].

4.2. Antigens identified by phage display

Somers et al. [31] identified several novel autoantigens using phage display technology using pooled sera from early and seronegative RA patients. In total, 14 antigens have been identified which significantly vary in size (from 5 to 176 amino acids), cellular function and immune reactivity. The sensitivities of the novel marker antigens varied between 2% and 29% with specificities between 95% and 100% (see Table 1). In ACPA-negative RA patients, autoantibodies to at least 1 of 11 or 1 of 14 were found in 44% or 67% of the patients. Although these data are very promising, the combined approach would require multiplex testing in RA which is not a common approach in the diagnostic market yet.

4.3. Antigens identified by proteomic approaches (PAD4 and BRAF)

Using a proteomic approach, Auger et al. [33,35] identified PAD4 and BRAF (v raf murine sarcoma viral oncogene homologue B1) as novel autoantigens in RA. Additionally, the same group also performed epitope

mapping studies and described epitopes on both antigens [34,36]. Autoantibodies to PAD4 recognize peptides located both in the N-terminal domain (211–290) and the C-terminal domain (601–650) [34]. Four epitopes were described: PAD4-P22 (VRVFQATRGLSSKCSVVLG), PAD4-P28 (LLDTSNLELPEAVVFQDSVV), PAD4-P61 (PFGPVINGRCCLEEKVCSLL) and PAD4-P63 (EPLGLQCTFINDFFTYHIRH). Autoantibodies to BRAF recognize two major epitopes: BRAF p10 (RKTRHVNILLFMGYSTKPQL) and BRAF p25 (YSNINNRDQIIFMVGRGYLS) (see Table 1).

5. Comparison of different markers

Since none of the novel markers have the clinical utility of ACPA, it is unlikely that they will replace ACPA. Therefore, the novel markers or combination of different markers (UH.RA 11 plex and UH.RA 14 plex) were compared in the CCP-negative RA patient cohort (see Fig. 1). Sensitivities in this important group of patients ranged from 16% to 40% (BRAF p10) for a single marker and up to 67% using the UH.RA 14 plex [31]. Following the CCP3.1 approach [40], an IgA/IgG screening test for anti-CarP antibodies might provide the most sensitive assay. The specificity was not considered in this analysis, mostly because the control cohort included different pathologies and different numbers of samples. Since the likelihood ratios are depended on both, sensitivity and specificity [19], further studies are mandatory to define the clinical utility of these novel biomarkers. Besides the diagnostic value, other aspects should be considered, which include the prognostic value of the individual autoantibody assays.

6. Future perspectives

Once more diagnostically relevant biomarkers will be established, modern multiplexing techniques for the simultaneous detection of a wide spectrum of markers may provide additional benefit in diagnosis as much as in classification of RA subtypes [41]. The novel biomarkers presented and discussed here have the potential to become part of the diagnostic algorithm and multiplex approaches for the diagnosis of RA in the near future.

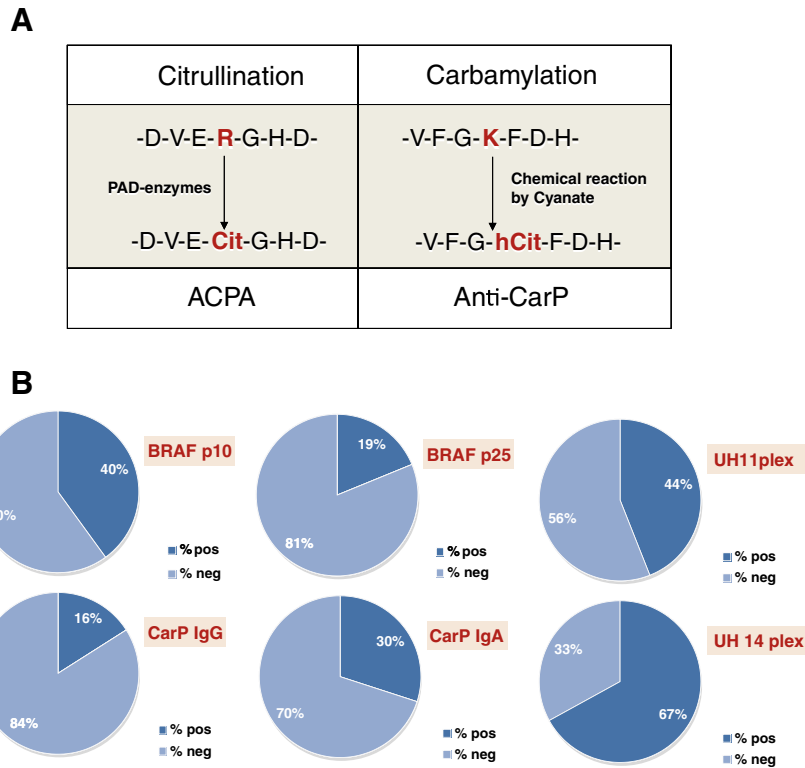


Fig. 1. Characteristics of different novel biomarkers for ACPA-negative rheumatoid arthritis. Schematic overview of the post-translational modifications citrullination and carbamylation is shown. (a) Citrullination modifies an arginine (R) present in the amino acid sequence of a protein into a citrulline, whereas carbamylation modifies a lysine (K), present at another position, into a homocitrulline. Citrullinated proteins are recognized by ACPA whereas carbamylated proteins are recognized by anti-CarP antibodies. (b) Sensitivities of six different biomarkers (or combination of biomarkers), BRAF p10, BRAF p25, CarP IgG, CarP IgA, UH-RA 11 plex, and UH-RA 14 plex, are presented.

Competing interest

Leendert A. Trouw has no competing interest to declare. Michael Mahler is employed at INOVA Diagnostics, Inc. selling autoimmune diagnostic assays.

Abbreviations

ACPA	anti-citrullinated protein antibodies
AUC	area under the curve
BiP	immunoglobulin binding protein
CCP	cyclic citrullinated peptide
LR	likelihood ratio
MCV	mutated citrullinated vimentin
RF	rheumatoid factor
VCP	viral citrullinated peptide
SARD	systemic autoimmune rheumatic disease

Take-home messages

- Several novel biomarkers have been described showing promising results.
- Individual biomarkers might detect up to 40% of ACPA-negative patients. Combinations of different novel biomarkers might have the potential to detect even up to 70% of ACPA-negative RA patients.
- Further studies are necessary to clearly define the clinical utility of novel biomarkers for the early diagnosis of RA.

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