Clinica Chimica Acta 414 (2012) 266–272

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Third generation anti-citrullinated peptide antibody assay is a sensitive marker in rheumatoid factor negative rheumatoid arthritis

Andreas Swart [a], Rufus W. Burlingame [b], Irmgard Gürtler [a], Michael Mahler [b,*]

[a] Center for Rheumatic Diseases, Neuss, Germany
[b] INOVA Diagnostics, Inc., San Diego, CA, USA

ARTICLE INFO

Article history:
Received 26 July 2012
Received in revised form 27 August 2012
Accepted 14 September 2012
Available online 25 September 2012

Keywords:
CCP
Citrullinated cyclic peptide
Rheumatoid arthritis
RA
Autoantibodies

ABSTRACT

Introduction: We compared 2 anti-citrullinated protein antibody (ACPA) assays using a routine patient cohort.
Methods: Two-hundred ninety-five sera were collected from patients for whom ACPA was ordered and tested for ACPA by QUANTA Lite® CCP 3 (INOVA Diagnostics, Inc., San Diego) and EliA® CCP (CCP, Phadia, Germany). Rheumatoid factor (RF) was determined using Quantex RF(II) (Biokit, Spain).
Results: Acceptable qualitative (96.6%, kappa = 0.93) and quantitative agreements (Spearman rho = 0.77; $p < 0.0001$) were observed between the two ACPA assays. Nine samples were CCP3 +/CCP2 − and one sample was CCP2 +/CCP3 −. Of the 9 CCP3 +/CCP2 − patients, 6 (66.7%) had RA, one patient had ankylosing spondylitis, one osteoarthritis and one psoriatic arthritis. The CCP3 −/CCP2 + patient had juvenile RA. At the manufacturer's cut-offs, the sensitivities and specificities were 77.3%/98.1% (CCP2), 81.6%/96.8% (CCP3) and 65.2%/89.6% (RF), respectively. At 98.7% specificity level, the sensitivities in the total cohort were 59.6% (CCP2) and 69.5% (CCP3) while the sensitivities in the RF-negative group were 49.0% (CCP2) and 57.1% (CCP3).
In the RF-negative group, sensitivities for patients with a disease duration of ≤5 years were 38.7% (CCP2) and 51.6% (CCP3).
Conclusion: Discrimination between RA and non-RA patients was better using CCP3, most pronounced in RF-negative RA.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Anti-citrullinated protein antibodies (ACPAs) are an important serological marker in the diagnosis of rheumatoid arthritis (RA) [1–3]. Historically, a combination of several serologic markers, including rheumatoid factor (RF, a test for anti-IgG autoantibodies), anti-perinuclear factor (APF) and anti-keratin antibody (AKA), has been used in the diagnosis of RA [1]. RF is considered a moderately specific test for RA, whereas the APF/AKA-indirect immunofluorescence (IIF) based assays were reported to be highly specific [1]. APF and AKA assays, however, were laborious, time consuming and difficult to standardize because they required human buccal cells or rat tissue sections and IIF microscopy [1]. With the discovery in 1998 that the underlying antigen in the APF/AKA tests contained citrulline [4], the development of novel assays to detect ACPA was facilitated [1]. The following studies confirmed that ACPA are indeed highly specific for RA and were recently added as one of the American College of Rheumatology (ACR)/The European League Against Rheumatism (EULAR) disease classification criteria for RA [5,6]. Whereas the first generation of the cyclic citrullinated peptide (CCP) test relied on a peptide derived from the filaggrin protein, the second- and third generation CCP (CCP2, CCP3) tests are no longer based on filaggrin but on peptides specifically designed and optimized (mimotypes) [7,8] to detect ACPA, thereby enhancing the presentation efficacy of the citrulline-containing epitope.

Over the past few years, many studies have evaluated the diagnostic performance of ACPA assays on a variety of diagnostic platforms [9–14]. 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 [1]. In early RA patients, 61.6% proved to be positive for CCP2 (n = 4589) [15].

In conclusion, both sensitivity and specificity of the CCP2/CCP3 tests are significantly higher than those of the RF test [2]. The lower specificity of the RF-IgM is due to its occurrence in other systemic autoimmune rheumatic diseases (SARDs), in a variety of infectious diseases, and even in a significant percentage of the healthy population [1]. 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

Abbreviations: ACPA, anti-citrullinated protein antibody; BiP, Immunoglobulin binding protein; CCP, cyclic citrullinated peptide; LR, likelihood ratio; MCV, mutated citrullinated vimentin; RF, Rheumatoid factor; VCP, viral citrullinated peptide; RU, relative units; SARD, systemic autoimmune rheumatic disease; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

* Corresponding author at: INOVA Diagnostics, 9900 Old Grove Rd., San Diego, CA 32131-1638, USA. Tel.: +1 858 586 9900; fax: +1 858 586 9911.
E-mail addresses: mmahler@inovadx.com, m.mahler.job@web.de (M. Mahler).

0009-8981/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2012.09.015

Table 1
Prevalence of anti-CCP2 and anti-CCP3 antibodies in different disease cohorts.

Disease cohort	N=	CCP2, no. pos (% pos, 95% CI)	CCP3, no. pos (% pos, 95% CI)	RF, no. pos (% pos, 95% CI)
RA	141	109 (77.3, 69.5–83.9)	115 (81.6, 74.2–87.6)	92 (65.2, 56.8–73.1)
RA (RF negative)	49	24 (49.0, 34.4–63.7)	28 (57.1, 42.2–71.2)	N/A
RA (disease duration ≤2 years)	34	26 (76.5, 58.8-89.3)	27 (79.4, 62.1-91.3)	20 (58.8, 40.7–75.4)
RA (disease duration ≤5 years)	77	54 (70.1, 58.6–80.0)	59 (76.6, 65.6–85.5)	46 (59.7, 47.9–70.8)
RA (RF negative, disease duration ≤5 years)	31	12 (38.7, 21.8–57.8)	16 (51.6, 33.1–69.8)	N/A
Ankylosing spondylitis	13	0 (0.0)	1 (7.7, 0.2–36.0)	0 (0.0)
Degenerative spine disease	6	0 (0.0)	0 (0.0)	0 (0.0)
Fibromyalgia	6	0 (0.0)	0 (0.0)	1 (16.7, 0.4–64.1)
Osteoarthrosis	47	1 (2.1, 0.1–11.3)	1 (2.1, 0.1–11.3)	7 (14.9, 6.2–28.3)
Polymyalgia rheumatica	20	0 (0.0)	0 (0.0)	0 (0.0)
Psoriasis arthritis	14	0 (0.0)	1 (7.1, 0.2–33.9)	0 (0.0)
CTD	18	1 (5.6, 0.1–27.3)	1 (5.6, 0.1–27.3)	4 (22.2, 6.4–47.6)
Others	30	1 (3.3, 0.1–17.2)	1 (3.3, 0.1–17.2)	4 (13.3, 3.8–30.7)

RA = rheumatoid arthritis; RF = rheumatoid factor; CTD = connective tissue disease (SLE, SSc, PM, UCTD, MCTD); SLE = systemic lupus erythematosus; SSc = systemic sclerosis; PM = polymyositis; UCTD = undifferentiated connective tissue disease; MCTD = mixed connective tissue disease.

value (PPV) and likelihood ratios [16,17]. Bossuyt at al. showed that the likelihood ratio for RA was 27.7 for ACPA and only 4.8 for RF [17].

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), filaggrin (CPA; Genesis, London, UK) or a viral citrullinated peptide (VCP; VCP1 and VCP2) [7,18,19]. The limited data and contradictory results from comparative studies on anti-MCV autoantibodies [20–22] 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) [1,23], fibrinogen [1,24], fibronectin [24], alpha-enolase [24], type II collagen, immunoglobulin binding protein (BiP) [25] and viral citrullinated peptide (VCP) derived from Epstein Barr Virus encoded protein (EBNA-2) [19]. None of these markers are widely used in routine diagnosis of RA.

Recent studies comparing different types of ACPA assays [16] showed that, in general, the peptide-based assays have a somewhat better sensitivity and specificity than the protein-based assays. Although based on the same antigenic peptide, not all CCP2 assays show the same performance characteristics [9,10]. 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 [11,12] while other investigations did not support these conclusions [13]. 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. found that in RF-negative RA patients anti-CCP3 antibodies were more prevalent than anti-CCP2 antibodies [14]. In an effort to clarify these contradictory results we decided to stratify our RA patient cohort, both according to disease, as well as to duration and the presence of RF in the serum, evaluating the two ACPA assays in a setting of the routine patient population.

2. Materials and methods

2.1. Patients and sera

Sera were collected from 295 patients for whom a CCP test was ordered. All patients were derived from a single clinical center (Center for Rheumatic Diseases, Neuss, Germany) and were collected between January 13th and March 29th 2011. Since the majority of patients were diagnosed before the new (2010) criteria were published [5], the diagnosis of RA was homogeneously based on the revised 1987 ACR diagnostic criteria [26]. In our cohort 98/141 (69.5%) of RA patients are female and 43/141 (30.5%) are male. The mean age and the average disease duration were 62.3 years (SD 12.0) and 8.5 years (SD 8.8), respectively. Patient identity was not disclosed and the data was anonymously used in accordance with the latest version of the Helsinki Declaration of human research ethics.

2.2. Immunoassays

ACPA were determined by QUANTA Lite® CCP3 (INOVA Diagnostics, Inc., San Diego, CA) and EliA® CCP (Phadia, Freiburg, Germany). Rheumatoid factor (RF) was determined using turbidimetry and Quantex RF(II) (Biokit, Barcelona Spain). Antibodies to CCP3 and RF were determined in the laboratory of the Clinic for Rheumatic Disease, Neuss, Germany. Automated QUANTA Lite® CCP3 ELISA was carried out on an

Table 2
Sensitivity and specificity of CCP2 and CCP3 at different cut-off values and in relation to rheumatoid factor and disease duration.

Group	Assay (cut-off)	Sensitivity (95% CI)	Specificity (95% CI)	LR+/LR−	AUC
At manufacturer's c/o	CCP2 (10.0)	77.3% (69.5–83.9%)	98.1% (94.4–99.6%)	39.7/0.23	0.891 (0.850–0.932)
	CCP3 (20.0)	81.6% (74.2–87.6%)	96.8% (92.6–98.9%)	25.1/0.19	0.893 (0.850–0.937)
	RF (15.0)	65.2% (56.8–73.1%)	89.6% (83.7–93.9%)	6.28/0.39	0.800 (0.742–0.848)
At same specificity 98.7%	CCP2 (62)	59.6% (51.0–67.7%)	98.7% (95.4–99.8%)	45.9/0.41	0.891 (0.850–0.932)
	CCP3 (103.99)	69.5% (61.2–77.0%)	98.7% (95.4–99.8%)	53.5/0.31	0.893 (0.850–0.937)
	RF (180.2)	15.6% (10.0–22.7%)	98.7% (95.4–99.8%)	12.1/0.86	0.795 (0.742–0.848)
In RF negative RA (n = 49)	CCP2 (10.0)	49.0% (34.4–63.7%)	98.1% (94.4–99.6%)	25.1/0.52	0.739 (0.646–0.833)
	CCP3 (20.0)	57.1% (42.2%–71.2%)	96.8% (92.6–98.9%)	17.6/0.44	0.723 (0.620–0.827)
In RF negative RA (n = 49) and at same specificity 98.7%	CCP2 (62)	24.5% (13.3–38.9%)	98.7% (95.4–99.8%)	18.9/0.77	0.739 (0.646–0.833)
	CCP3 (103.99)	40.8% (27.0–55.8%)	98.7% (95.4–99.8%)	31.4/0.60	0.723 (0.620–0.827)
In RF negative RA with disease duration ≤5 years (n = 31)	CCP2 (10.0)	38.7% (21.8–57.8%)	98.1% (94.4–99.6%)	19.9/0.63	0.699 (0.577–0.821)
	CCP3 (20.0)	51.6% (33.1–69.8%)	96.8% (92.6–98.9%)	15.9/0.50	0.680 (0.543–0.818)
In RF negative RA with disease duration ≤5 years (n = 31) and at same specificity 98.7%	CCP2 (62)	19.4% (7.5–37.5%)	98.7% (95.4–99.8%)	14.9/0.82	0.699 (0.577–0.821)
	CCP3 (103.99)	32.2% (16.7–51.4%)	98.7% (95.4–99.8%)	24.8/0.69	0.680 (0.543–0.818)

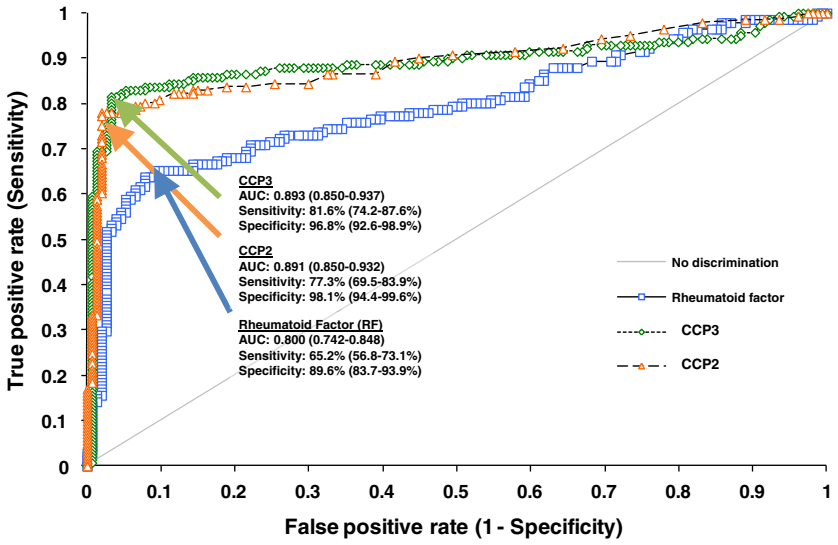

Fig. 1. Receiver operating characteristic analysis. A receiver operating characteristic analysis comparing CCP2, CCP3 and rheumatoid factor (RF) was performed. Sensitivities, specificities and area under the curve (AUC) values are presented in the figure. Both ACPA assays showed similar performance characteristics and outperformed RF.

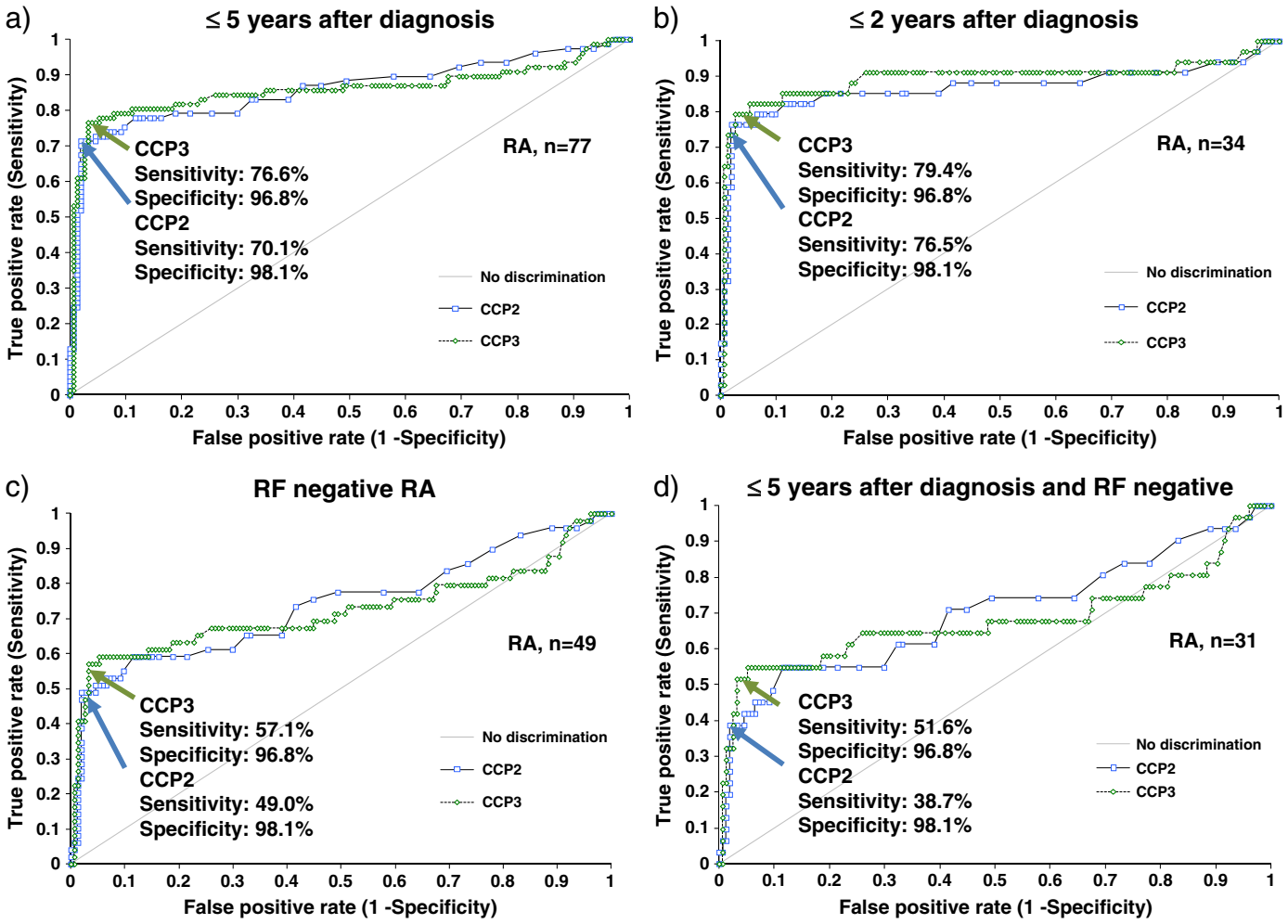

Fig. 2. Receiver operating characteristic (ROC) analysis in different subpopulations of rheumatoid arthritis (RA) patients. A ROC analysis comparing CCP2 and CCP3 was performed using different RA subpopulations. a.) with a disease duration of ≤5 years (n=77), b.) with a disease duration of ≤2 years (n=34), c.) rheumatoid factor negative RA patients (n=49) and d.) rheumatoid factor negative RA patients with a disease duration of ≤5 years (n=31). Sensitivities, specificities and area under the curve (AUC) values are presented in Table 2. RF negative RA and a disease duration of ≤2 years was not analyzed due to a small sample size. For further information and statistical analysis, see Table 2.

AP22 Speedy instrument (DAS, Rome Italy) as part of the routine diagnostic procedure. In addition, samples were also tested in a single run to verify the results and to confirm integrity of samples. Results showed 100% qualitative agreement and Spearman rho was 1.0. EliA® CCP was performed on a Phadia® 250 instrument at an academic hospital laboratory in the U.S. All tests were done according to manufacturers' instructions. Unless stated, cut-off values recommended in the instruction for use were applied (EliA® CCP = 10 U/ml, QUANTA Lite® CCP3 = 20 units). In the following text, EliA® CCP is referred to as CCP2 and QUANTA Lite® CCP 3 as CCP3 (based on the antigen used).

2.3. Statistical evaluation

Data were statistically evaluated using Analyse-it software (ver 2.03; Analyse-it Software, Ltd., Leeds, UK). Receiver-operating characteristic (ROC) analysis was carried out to analyze the discrimination between RA patients and controls. Differences between likelihood ratios were calculated using BDTcomparator as described previously [27,28]. For all statistical methods, a $p<0.05$ was considered as significant.

3. Results

3.1. Anti-CCP2 and anti-CCP3 antibodies in rheumatoid arthritis

At the manufacturers' cut-offs, 109/141 (77.3%) RA patients were positive for anti-CCP2, 115/141 (81.6%) for anti-CCP3 antibodies and 92/141 (65.2%) for RF. In the control groups 3/154 (1.9%) were positive for anti-CCP2 [1 osteoarthritis (OA), 1 connective tissue disease (CTD) and 1 non-rheumatic disease], 5/154 (3.2%) for anti-CCP3 antibodies [1 ankylosing spondylitis (AS), 1 OA, 1 CTD, 1 psoriatic arthritis (PA) and 1 non-rheumatic disease] and 16/154 (10.4%) for RF (see Table 1). Thus, the sensitivities and specificities were 77.3% and 98.1% (CCP2), 81.6% and 96.8% (CCP3), and 65.2% and 89.6% (RF), respectively (see Table 2). At a cut-off which yields 98.7% specificity, the sensitivities were 59.6% (CCP2), 69.5% (CCP3) and 14.2% (RF). The area under the curve (AUC) values obtained from ROC analysis were 0.891 for CCP2, 0.893 for CCP3 and 0.800 for RF (Fig. 1). The difference in the AUC between the CCP2 and CCP3 tests was not statistically significant ($p=0.9002$) whereas the difference of both CCP tests to RF was significant ($p<0.01$).

3.2. Anti-CCP2 and anti-CCP3 antibodies in RF negative RA and disease duration of ≤5 years

In the RF-negative RA group (n = 49), 49.0% of the patient samples were positive for anti-CCP2 and 57.1% for anti-CCP3 antibodies (Table 2, Fig. 2). Thus in the RF-negative RA group, the sensitivities were 49.0% for anti-CCP2 and 57.1% for anti-CCP3 antibodies (Fig. 2c). In patients with a disease duration of less than or equal to 5 years (n = 77) 54 or 70.1% were positive for anti-CCP2 while 59 or 76.6% were positive for anti-CCP3 antibodies (Fig. 2a). In RF-negative RA patients with a disease duration of less or equal to 5 years (n = 31), 12

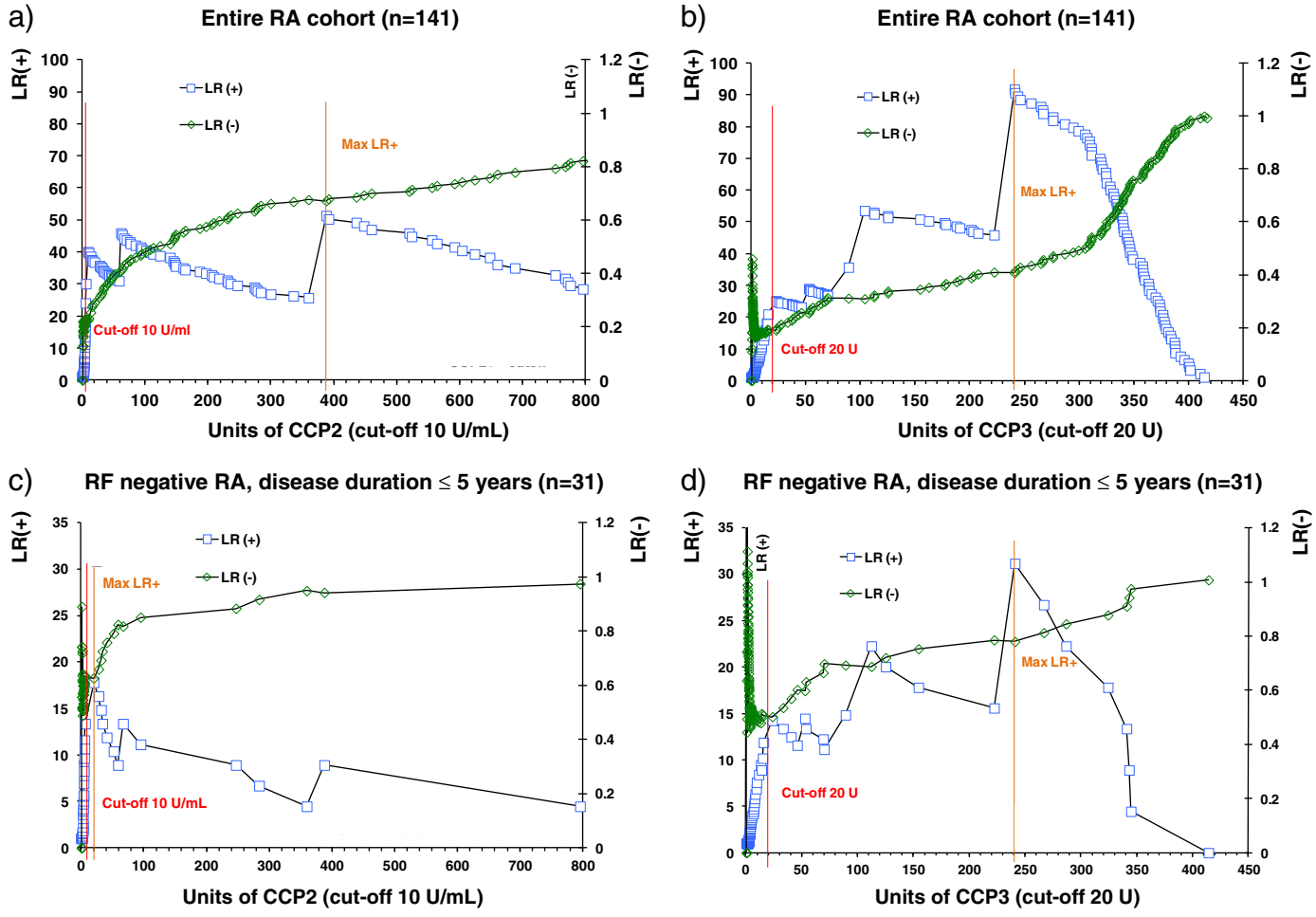

Fig. 3. Likelihood ratios for rheumatoid arthritis (RA). The results of a.) CCP2 and b.) CCP3 were used to calculate the likelihood ratio (LR) for RA at different cut-off values. In both assays, the LR(+) increased with increasing titers of anti-citrullinated protein antibodies (ACPA). For CCP2 the LR(+) reached 51.3 at a cut-off of 388 U/mL and 91.7 for CCP3 (cut-off 240.9 U). In c.) and d.) the LR for CCP2 c.) and CCP3 d.) in rheumatoid factor negative RA with a disease duration of ≤5 years is shown. In this cohort the LR(+) for CCP2 reached 17.8 at a cut-off of 20 U/ml and 31.2 for CCP3 (cut-off 240.9 U).

or 38.7% of patients were anti-CCP2 and 16 or 51.6% were ACPA positive by the CCP3 test (Fig. 2d). Confidence intervals are shown in Table 2.

3.3. Likelihood ratios of anti-CCP2 and anti-CCP3 antibodies in different cohorts in relation to antibody titers

The likelihood ratios for anti-CCP2 and anti-CCP3 antibodies were calculated at different cut-off values (see Fig. 3). At the cut-off values recommended by the manufacturers the LR+ for CCP2 was higher than for CCP3 (39.7 vs. 25.1, $p=0.3764$). At this cut-off the LR− was 0.23 (CCP2) and 0.19 (CCP3, $p<0.025$). However, at higher thresholds CCP3 was significantly more predictive for RA. The highest LR+ for CCP2 was found at a cut-off of 388 U/mL (LR+ = 51.3) and for CCP3 at a cut-off of 240.9 U (LR+ = 91.7, $p=0.1655$). At this cut-off the LR− was 0.67 (CCP2) and 0.47 (CCP3, $p<0.0001$). At the same specificity (98.7%), the LR+/LR− were 45.9/0.41 for CCP2 and 53.5/0.31 for CCP3 ($p=0.4761$ for LR+, $p=0.0018$ for LR−; see Table 2). In the RF negative group (at the recommended cut-off), the LR+/LR− were 25.1/0.52 for CCP2 and 17.6/0.44 for CCP3 ($p=0.4946$ for LR+, $p=0.0709$; see Table 2, Fig. 3). At the same specificity (98.7%) in the RF negative group, the LR+/LR− were 18.9/0.77 for CCP2 and 31.4/0.60 for CCP3 ($p=0.0688$ for LR+, $p=0.0050$ for LR−; see Table 2).

3.4. Agreement between anti-CCP2 and anti-CCP3 antibodies

Acceptable qualitative (96.6%, 95% CI 93.9–98.4%; K = 0.93, 95% CI 0.89–0.97) and quantitative agreements (Spearman rho = 0.77; $p<0.0001$) were observed between the CCP2 and CCP3 assays (see Table 3). Nine samples were CCP3+/CCP2− and 1 sample was CCP2+/CCP3−. Of the nine CCP3+/CCP2− patients, 6 or 66.7% had RA, 1 patient had AS, 1 OA and 1 PA. The CCP3−/CCP2+ patient had juvenile RA.

Table 3
Agreement between CCP2 and CCP3 in different cohorts.

		CCP2			Percent agreement (95% confidence)
		Positive	Negative	Total	
All patients (n = 295)					
CCP3	Positive	111	9[a]	120	Pos. agree = 99.1% (95.1–100.0%)
	Negative	1[b]	174	175	Neg. agree = 95.1% (90.0–97.7%)
	Total	112	183	295	Total agree = 96.6% (93.9–98.4%)
RA patients (n = 141)					
CCP3	Positive	109	6	115	Pos. agree = 100.0% (96.7–100.0%)
	Negative	0	26	26	Neg. agree = 81.3% (63.6–92.8%)
	Total	109	32	141	Total agree = 95.7% (91.0–98.4%)
RF negative RA patients (n = 49)					
CCP3	Positive	24	4	28	Pos. agree = 100.0% (85.8–100.0%)
	Negative	0	21	21	Neg. agree = 84.0% (63.9–95.5%)
	Total	24	25	49	Total agree = 91.8% (80.4–97.7%)
RF negative RA patients, disease duration ≤ 5 years (n = 31)					
CCP3	Positive	12	4	16	Pos. agree = 100.0% (73.5–100.0%)
	Negative	0	15	15	Neg. agree = 78.9% (54.4–93.9%)
	Total	12	19	31	Total agree = 87.1% (70.2–96.4%)
RA patients, disease duration ≤ 2 years (n = 34)					
CCP3	Positive	26	1	27	Pos. agree = 100.0% (86.8–100.0%)
	Negative	0	7	7	Neg. agree = 87.5% (47.3–99.7%)
	Total	26	8	34	Total agree = 97.1% (84.7–99.9%)
RA patients, disease duration ≤ 5 years (n = 77)					
CCP3	Positive	54	5	59	Pos. agree = 100.0% (93.4–100.0%)
	Negative	0	18	18	Neg. agree = 78.3% (56.3–92.5%)
	Total	54	23	77	Total agree = 93.5% (85.5–97.9%)

[a] Six patients had RA, 1 OA, 1 CTD and 1 non-rheumatic disease.
[b] Patient had juvenile RA.

4. Discussion

Anti-citrullinated protein antibodies (ACPA) are an important serological marker in the diagnosis of RA [1,2,17,29]. Although several studies were designed to compare the performance of various assays, it hitherto remains uncertain as to which test provides the best clinical utility.

Despite intense efforts that have gone into standardizing ACPA detection [30], significant differences persist between ACPA assays [10]. 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 [8]. 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). Therefore both peptides may not contain bona fide autoantigens, but have been proven to measure RA specific antibodies (mimotypes) [8]. Recently it has been shown that the anti-CCP2 titer in early RA is correlated with the epitope diversity (epitope spreading) [31,32]. These data indicate that patients with early RA and especially in the prediagnostic phase have antibodies to only one or very few epitopes. It has been demonstrated that the source of antigen is the most important variable in determining the performance characteristics of an ACPA assay [8]. Unfortunately, the sequences and immunological characteristics of CCP2 and CCP3 have not been published, since they are proprietary sequences owned by two companies [29]. Therefore it remains speculative why patients with RF negative RA and disease duration of less or equal to 5 years preferentially recognize CCP3 over CCP2. However, the high sensitivity of the CCP3 antigen is highly desirable in view of the irreversible joint damage and permanent disability that can follow a delayed diagnosis and treatment of RA [1].

The conclusion from one of the more comprehensive evaluations of the analytical performance and diagnostic utility of ACPAs demonstrated that the CCP2 and CCP3 assays evaluated in the present study are among the best commercial ACPA assays [10].

In a recent study [33], these 2 ACPA assays were compared and it was concluded that the specificity of CCP2 was superior to that of CCP3. As their cohort was limited (only 52 patients were positive in all CCP tests) their discrepant result may be due to low cohort size, cohort composition and/or the manual performance of the CCP3 ELISA. In our study setting samples were tested for CCP3 using a controlled automated procedure and confirmed in a second (batched) testing. In this setting, the performance difference between CCP3 and CCP2 in the entire cohort was non-significant. Both studies are based on a retrospective cross-sectional study design, however, in our study the clinical status of patients with discrepant test results was verified by chart review after an average of 12 months. Even though no change of diagnosis had taken place during clinical follow-up in these patients, based on the high PPV of ACPA it is important to follow up these patients. It has been found that ACPA can be measured in the serum of many RA patients up to 10 years before their first presentation to a clinician, predicting the future development of RA [34].

As Bossuyt et al. [17] stated, it is important for the comparison of diagnostic assays, such as ACPA, to take into account the likelihood ratio of the test under evaluation. Therefore we also analyzed the likelihood ratios of CCP3 and CCP2 in the present study: in our cohort, the LR+ was highly dependent on the cut-off used. Although at the manufacturers' cut-off values the LR+ of CCP2 was higher than for CCP3, at higher thresholds CCP3 was significantly more predictive for RA than CCP2. This observation is in line with the 2010 disease classification criteria of RA which make high levels of ACPA a classification criterion [5]. However, the different assay performance in disease prediction underlines the importance that every clinical center evaluates their post-test probability of disease using the type of ACPA assay used and their own patient cohort. This has become even more

Table 4
Comparison of results of the present study with findings by Jaskowski et al. [14].

Disease cohort	Current study			Jaskowski et al. [14]		
	N=	CCP2	CCP3	N=	CCP2	CCP3
RA	141	77.3 (69.5–83.9)	81.6 (74.2–87.6)	137	65 (56–73)	76 (68–83)
RA (RF negative)	49	49.0 (34.4–63.7)	57.1 (42.2–71.2)	28	19 (7–39)	35 (17–56)
RA (RF positive)	98	86.7 (78.4–92.7)	88.8 (80.8–94.3)	109	76 (67–84)	85 (77–91)

important with the new set of classification criteria as they identify more patients in a given population introducing a certain risk of over-diagnosis and unnecessary treatment with disease-modifying anti-rheumatic drugs (DMARD) [35]. Our patients were classified according to the 1987 ACR classification criteria for RA, but also fulfilled the 2010 criteria [5]. Despite the high sensitivity, the 2010 ACR criteria still miss some RA patients, especially symmetrical seronegative arthritis and limited joint involvement [36]. In agreement with Jaskowski et al. [14] we found a higher prevalence of anti-CCP3 (57.1%) than anti-CCP2 antibodies (49.0%) in RF-negative RA patients. Although both studies used a similar number of RA patients, the number of RF negative patients was significantly higher in our cohort (49 vs. 28) resulting in large 95% confidence intervals (see Table 4). The most pronounced difference between CCP3 and CCP2, however, was found in RF-negative RA patients with disease duration less or equal to 5 years. In this cohort (n = 31) the sensitivity of CCP3 was 51.6% compared to 38.7% for CCP2. Interestingly, we found that all 3 non-RA patients with a CCP3-positive/CCP2-negative result had a recent diagnosis and included 1 patient with AS, 1 with OA and 1 with PA. Although the diagnosis of these three patients did not change during the observation period, based on the strong predictive value of ACPA [34], a future development of RA cannot be ruled out. We clearly acknowledge that our cohort is small and such numbers are not significant, however they do show that much broader attempts are needed to demonstrate test performance differences in this subgroup in a statistically significant way, preferentially in a uniform multi-center and prospective design encompassing different cohort compositions. Studies focusing on patients with symptoms of ≤3 months [37] are mandatory to analyze the performance of ACPA assays in early RA.

Even though 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 [3]. Recently, such autoantigens have been described [38,39] which have not yet been transferred into commercial use. Shi et al. identified homocitrullin as a key determinant for the binding of autoantibodies in ACPA-negative RA patients [39]. Somers et al. identified several novel autoantigens using phage display technology [38]. The sensitivities of the novel marker antigens varied between 2% and 29% with specificities between 95% and 100% and are expected to further contribute to closing the diagnostic gap in ACPA/RF negative-RA. Once more diagnostically relevant biomarkers have been 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.

5. Conclusion

Discrimination between RA and non-RA patients was better using CCP3, most pronounced in RF-negative RA with a disease duration of ≤5 years.

Acknowledgments

We thank Brian McEvilly (INOVA Diagnostics) for proof reading and valuable suggestions.

References

[1] van Venrooij WJ, van Beers JJ, Pruijn GJ. Anti-CCP antibodies: the past, the present and the future. Nat Rev Rheumatol 2011;7:391-8.
[2] Wiik AS, van Venrooij WJ, Pruijn GJ. All you wanted to know about anti-CCP but were afraid to ask. Autoimmun Rev 2010;10:90-3.
[3] Trouw LA, Mahler M. Closing the serological gap: promising novel biomarkers for the early diagnosis of rheumatoid arthritis. Autoimmun Rev in press, http://dx.doi.org/10.1016/j.autrev.2012.05.007.
[4] Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. J Clin Invest 1998;101:273-81.
[5] Aletaha D, Neogi T, Silman AJ, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 2010;62:2569-81.
[6] Aletaha D, Neogi T, Silman AJ, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis 2010;69:1580-8.
[7] Kessenbrock K, Raijmakers R, Fritzler MJ, Mahler M. Synthetic peptides: the future of patient management in systemic rheumatic diseases? Curr Med Chem 2007;14:2831-8.
[8] Levesque MC, Zhou Z, Moreland LW. Anti-cyclic citrullinated peptide testing for the diagnosis of rheumatoid arthritis and the quest for improved sensitivity and predictive value. Arthritis Rheum 2009;60:2211-5.
[9] Vander CB, Nogueira L, Van PJ, et al. Do all anti-citrullinated protein/peptide antibody tests measure the same? Evaluation of discrepancy between anti-citrullinated protein/peptide antibody tests in patients with and without rheumatoid arthritis. Ann Rheum Dis 2008;67:542-6.
[10] Bizzaro N, Tonutti E, Tozzoli R, Villalta D. Analytical and diagnostic characteristics of 11 2nd- and 3rd-generation immunoenzymatic methods for the detection of antibodies to citrullinated proteins. Clin Chem 2007;53:1527-33.
[11] Santiago M, Baron M, Miyachi K, et al. A comparison of the frequency of antibodies to cyclic citrullinated peptides using a third generation anti-CCP assay (CCP3) in systemic sclerosis, primary biliary cirrhosis and rheumatoid arthritis. Clin Rheumatol 2008;27:77-83.
[12] Correia ML, Carvalho S, Fortuna J, Pereira MH. Comparison of three anti-CCP antibody tests and rheumatoid factor in RA and control patients. Clin Rev Allergy Immunol 2008;34:21-5.
[13] van der Linden MP, van der Woude D, Ioan-Facsinay A, et al. Value of anti-modified citrullinated vimentin and third-generation anti-cyclic citrullinated peptide compared with second-generation anti-cyclic citrullinated peptide and rheumatoid factor in predicting disease outcome in undifferentiated arthritis and rheumatoid arthritis. Arthritis Rheum 2009;60:2232-41.
[14] Jaskowski TD, Hill HR, Russo KL, Lakos G, Szekanecz Z, Teodorescu M. Relationship between rheumatoid factor isotypes and IgG anti-cyclic citrullinated peptide antibodies. J Rheumatol 2010;37:1582-8.
[15] van Venrooij WJ, Zendman AJ, Pruijn GJ. Autoantibodies to citrullinated antigens in (early) rheumatoid arthritis. Autoimmun Rev 2006;6:37-41.
[16] Pruijn GJ, Wiik A, van Venrooij WJ. The use of citrullinated peptides and proteins for the diagnosis of rheumatoid arthritis. Arthritis Res Ther 2010;12:203.
[17] Bossuyt X, Coenen D, Fieuws S, Verschueren P, Westhovens R, Blanckaert N. Likelihood ratios as a function of antibody concentration for anti-cyclic citrullinated peptide antibodies and rheumatoid factor. Ann Rheum Dis 2009;68:287-9.
[18] Pratesi F, Tommasi C, Anzilotti C, et al. Antibodies to a new viral citrullinated peptide, VCP2: fine specificity and correlation with anti-cyclic citrullinated peptide (CCP) and anti-VCP1 antibodies. Clin Exp Immunol 2011;164:337-45.
[19] Bartoloni E, Alunno A, Bistoni O, et al. Diagnostic value of anti-mutated citrullinated vimentin in comparison to anti-cyclic citrullinated peptide and anti-viral citrullinated peptide 2 antibodies in rheumatoid arthritis: an Italian multicentric study and review of the literature. Autoimmun Rev 2012;11:815-20.
[20] Dejaco C, Klotz W, Larcher H, Duftner C, Schirmer M, Herold M. Diagnostic value of antibodies against a modified citrullinated vimentin in rheumatoid arthritis. Arthritis Res Ther 2006;8:R119.
[21] Soos L, Szekanecz Z, Szabo Z, et al. Clinical evaluation of anti-mutated citrullinated vimentin by ELISA in rheumatoid arthritis. J Rheumatol 2007;34:1658-63.
[22] Damjanovska L, Thabet MM, Levarth EW, et al. Diagnostic value of anti-MCV antibodies in differentiating early inflammatory arthritis. Ann Rheum Dis 2010;69:730-2.
[23] Nell VP, Machold KP, Stamm TA, et al. Autoantibody profiling as early diagnostic and prognostic tool for rheumatoid arthritis. Ann Rheum Dis 2005;64:1731-6.
[24] Chandra PE, Sokolove J, Hipp BG, et al. Novel multiplex technology for diagnostic characterization of rheumatoid arthritis. Arthritis Res Ther 2011;13:R102.

[25] Shoda H, Fujio K, Shibuya M, et al. Detection of autoantibodies to citrullinated BiP in rheumatoid arthritis patients and pro-inflammatory role of citrullinated BiP in collagen-induced arthritis. Arthritis Res Ther 2011;13:R191.
[26] Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
[27] Fijorek K, Fijorek D, Wisniowska B, Polak S. BDTcomparator: a program for comparing binary classifiers. Bioinformatics 2011;27:3439-40.
[28] Nofuentes JA, Del Castillo JD. Comparison of the likelihood ratios of two binary diagnostic tests in paired designs. Stat Med 2007;26:4179-201.
[29] Silveira IG, Burlingame RW, von Muhlen CA, Bender AL, Staub HL. Anti-CCP antibodies have more diagnostic impact than rheumatoid factor (RF) in a population tested for RF. Clin Rheumatol 2007;26:1883-9.
[30] Rottmann M. Anti-CCP: challenges in quantifying auto-antibodies and creating international reference materials. Scand J Clin Lab Invest Suppl 2010;242:44-5.
[31] Willemze A, Bohringer S, Knevel R, et al. The ACPA recognition profile and subgrouping of ACPA-positive RA patients. Ann Rheum Dis 2012;71:268-74.
[32] Sokolove J, Bromberg R, Deane KD, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. PLoS One 2012;7: e35296.
[33] Elrefaei M, Boose K, McGee M, et al. Second Generation Automated Anti-CCP Test Better Predicts the Clinical Diagnosis of Rheumatoid Arthritis. J Clin Immunol 2012;32:131-7.
[34] Kokkonen H, Mullazehi M, Berglin E, et al. Antibodies of IgG, IgA and IgM isotypes against cyclic citrullinated peptide precede the development of rheumatoid arthritis. Arthritis Res Ther 2011;13:R13.
[35] Cader MZ, Filer A, Hazlehurst J, de Pablo P, Buckley CD, Raza K. Performance of the 2010 ACR/EULAR criteria for rheumatoid arthritis: comparison with 1987 ACR criteria in a very early synovitis cohort. Ann Rheum Dis 2011;70:949-55.
[36] Fautrel B, Combe B, Rincheval N, Dougados M. Level of agreement of the 1987 ACR and 2010 ACR/EULAR rheumatoid arthritis classification criteria: an analysis based on ESPOIR cohort data. Ann Rheum Dis 2012;71:386-9.
[37] Aletaha D, Eberl G, Nell VP, Machold KP, Smolen JS. Attitudes to early rheumatoid arthritis: changing patterns. Results of a survey. Ann Rheum Dis 2004;63:1269-75.
[38] Somers K, Geusens P, Elewaut D, et al. Novel autoantibody markers for early and seronegative rheumatoid arthritis. J Autoimmun 2011;36:33-46.
[39] Shi J, Knevel R, Suwannalai P, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A 2011;108:17372-7.