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Autoantibodies to Disease-Related Proteins in Joints as Novel Biomarkers for the Diagnosis of Rheumatoid Arthritis

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Objective. This study was undertaken to develop and characterize a multiplex immunoassay for detection of autoantibodies against peptides derived from proteins known to play a role in development of arthritis and that are also expressed in joints.

Methods. We selected peptides from the human counterpart of proteins expressed in the joints, based on mouse models that showed these to be targeted by pathogenic or regulatory antibodies in vivo. Using bead-based flow immunoassays measuring IgG antibodies, we selected triple helical or cyclic peptides, containing the epitopes, to avoid collinear reactivity. We characterized the analytical performance of the immunoassay and then validated it in 3 independent rheumatoid arthritis (RA) cohorts (n = 2,110), Swedish age- and sex-matched healthy controls, and patients with osteoarthritis (OA), patients with psoriatic arthritis (PsA), and patients with systemic lupus erythematosus (SLE).

Results. Screening assays showed 5 peptide antigens that discriminated RA patients from healthy controls with 99% specificity (95% confidence interval [CI] 98–100%). In our validation studies, we reproduced the discriminatory capacity of the autoantibodies in 2 other RA cohorts, showing that the autoantibodies had high discriminatory capacity for RA versus OA, PsA, and SLE. The novel biomarkers identified 22.5% (95% CI 19–26%) of early RA patients seronegative for anti–cyclic citrullinated peptide and rheumatoid factor. The usefulness of the biomarkers in identifying seronegative RA patients was confirmed in validation studies using 2 independent cohorts of RA patients and cohorts of patients with OA, PsA, and SLE.

Conclusion. A multiplex immunoassay with peptides from disease-related proteins in joints was found to be useful for detection of specific autoantibodies in RA serum. Of note, this immunoassay had high discriminatory capacity for early seronegative RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease that results in inflammation of synovial joints followed by disability. With a prevalence of 0.5–1%, RA is the most common autoimmune rheumatic disease worldwide. Although advances in treatment during the past 2 decades have been tremendous, up to 60–70% of patients do not respond to the first line of treatment.

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Early diagnosis and effective treatment, however, provide the best chances to achieve remission (1). It is thus important to discover novel biomarkers that can predict RA development very early and deepen the understanding of the heterogenicity of the disease, potentially allowing prediction of prognosis and treatment response.

The current state-of-the art methods for serologic classification of RA are rheumatoid factor (RF) and anti–cyclic citrullinated peptide (anti-CCP) tests, which are both included in the 2010 American College of Rheumatology (ACR)/EULAR classification criteria for RA (2). The criteria have been shown to be sensitive in detecting RA cases among various populations, but their modest discriminatory capacity (i.e., pooled specificity of 0.61) means that a significant amount of non-RA patients may be classified as RA, leaving room for improvements (3). Today, the disease is classified as either seropositive, having either RF or anti-CCP antibodies, or seronegative. The anti-CCP2 test has a sensitivity of 57% and specificity of 95% in early (<2 years) RA, and RF has about the same sensitivity but lower specificity (86%) (4). More than 30% of early RA patients are seronegative (RF and anti-CCP negative) (4). Other serologic biomarkers have been described but have not been firmly established in the clinical setting. Although the presence of RF and/or anti-CCP antibodies, especially at high levels, are factors for poor prognosis (5), information about the RA subtype at the individual level is lacking.

The anti-CCP2 test with cyclic citrullinated peptides of undisclosed identity detects anti–citrullinated protein antibodies (ACPAs), but not all specificities are captured. Analyses of ACPA fine specificities in seronegative RA patients have identified patients with similar disease severity and evolution as seropositive RA (6,7). ACPAs appear in the serum decades before RA onset and increase in titer around the onset of overt RA (8,9). The contribution of different ACPAs to disease onset via their epitope specificity and their involvement in promotion and/or protection are largely unknown (10).

According to a recently proposed concept, promiscuous ACPAs bind citrulline independent of the remaining part of the epitope, whereas private ACPAs recognize citrulline and proximal amino acids, enabling specific protein–protein interactions (10). Although no evidence exists to our knowledge that promiscuous ACPAs induce arthritis, some private ACPAs interacting with citrullinated type II collagen (COL2) have been shown to do so (11–13). Interestingly, COL2 in arthritic cartilage can be citrullinated (14), and protein arginine deiminase type 4 (PADI4)–deficient mice are less susceptible to arthritis (15,16). Studies in mice have shown the pathogenicity of antibodies that bind specific epitopes on the cartilage (17); however, so far, only antibodies reactive with glucose-6-phosphate isomerase (GPI), COL2, type XI collagen, and cartilage oligomeric matrix protein (COMP) and antibodies that bind citrullinated COL2, targeting joint cartilage, have been shown to reproducibly induce or enhance arthritis and pain (11–12, 17–22).

In this study, we developed and characterized a bead-based multiplex flow immunoassay that detected autoantibodies against peptides representing epitopes on the selected disease-related proteins (i.e., GPI, COL2, COMP, and PADI4) and that demonstrated an ability to detect early RA and a discriminatory capacity to distinguish early RA against osteoarthritis (OA), psoriatic arthritis (PsA), and systemic lupus erythematosus (SLE).

PATIENTS AND METHODS

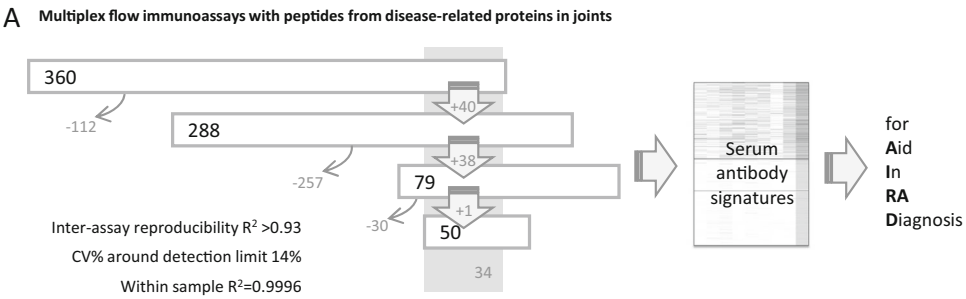
Assay antigens, sample acquisition, quality control, the monoclonal antibodies with human Fc region used as assay calibrators, and the characteristics of our analytical experiments are described in Supplementary Data 1, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.42463. Baseline characteristics and further descriptions of study groups are shown in Supplementary Data 2, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42463.

Patients and healthy donors. Patients with untreated early RA were included in several cohorts, as shown in Figure 1B. Patients with untreated early RA who met the ACR/EULAR 2010 criteria were included in the early RA patient cohort. Serum samples for early RA patients from the Better Anti-Rheumatic Pharmacotherapy (BARFOT) cohort (23) and the Timely Intervention in RA 2006–2009 cohort (TIRA-2) (24) had been collected at diagnosis. We also collected serum samples from patients from the Clinical Lupus Register in Northeastern Gothia (KLURING) cohort (25), which included patients diagnosed as having SLE according to the 1982 ACR criteria for SLE (26) and/or the 2012 Systemic Lupus International Collaborating Clinics (SLICC) criteria (27). All patients were consecutively recruited.

We collected serum samples from patients with established RA, patients with PsA, and patients with OA (Figure 1B) in the JointID study. Patients were diagnosed as having established RA according to the ACR 1987 classification criteria for RA (28) or the ACR/EULAR classification criteria for RA (2), and patients were diagnosed as having PsA according to the Classification of Psoriatic Arthritis Study Group Criteria for PsA (29). Patients treated with B cell depletion therapy were excluded. For primary OA, we included patients scheduled for prosthesis surgery of hip or knee and without any inflammatory rheumatic disease.

We obtained serum samples from healthy controls matched by age and sex to the BARFOT and TIRA-2 cohorts from the population-based health survey study Malmö Förebyggande Medicin-Återundersökning and from the Western Region Initiative to Gather Information on Atherosclerosis study (30). Healthy controls matched by age and sex to the early RA patient cohort were also recruited. All healthy control individuals were confirmed free from inflammatory rheumatic diseases at the time of sample collection.

A



B

		Multiplex immunoassay and the number of tested subjects (N)			
Condition	**Cohort/Study**	**360-plex***	**288-plex***	**79-plex***	**50-plex***
HC	WINGA	267			
RA	JointID	45			
OA	JointID	29			
PsA	JointID	21			
SLE	KLURING		259		
ueRA	TIRA-2		96		502
HC	ERAp			27	
ueRA	ERAp			70	
HC	MFM ÅUS				935
ueRA	BARFOT				1493
TOTAL		**362**	**355**	**97**	**2930**

Figure 1. **A**, Representation of multiplex flow immunoassays, consisting of 360, 288, 79, or 50 bead-coupled antigens, to detect antibodies and ultimately to identify the aid in RA diagnosis (AIRAD) peptide set, with usefulness in rheumatoid arthritis (RA). Numbers with minus and plus signs represent noninformative antigens excluded and new antigens included, respectively. Shaded area represents the antigen overlap. **B**, Patient and healthy control (HC) cohorts and studies from which serum samples were obtained for the multiplex screening assays. Supplementary Table 1, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42463, further defines the composition of the groups. * = Number of bead-coupled antigens multiplexed in the same reaction. CV% = coefficient of variation percentage; OA = osteoarthritis; PsA = psoriatic arthritis; SLE = systemic lupus erythematosus; ueRA = untreated early RA; WINGA = The Western Region Initiative to Gather Information on Atherosclerosis; KLURING = Clinical Lupus Register in Northeastern Gothia; TIRA-2 = Timely Intervention in RA in 2006–2009; ERAp = early RA patient cohort; MFM ÅUS, Malmö Förebyggande Medicin-Återundersökning; BARFOT = Better Anti-Rheumatic Pharmacotherapy.

This study was conducted in compliance with the Declaration of Helsinki and was approved by the relevant research authorities.

Multiplex bead-based flow immunoassays. Both cyclic and triple helical peptides were used for coating beads in a multiplex assay for assays of serum antibodies. We used the original COL2A1 antigens to represent the known triple-helical and cyclic B and T cell epitopes for both variant (e.g., citrulline or homocitrulline) and native sequences. Other antigens from COL2A1 and from GPI, COMP, and PADI4 were similarly selected to represent the possible sites of posttranslational modifications.

Antigens were coupled to magnetic beads, and assays were performed as previously described (31). Samples were 1:100-diluted and incubated with preblocking solution of 3% BSA (Roche), 5% skim milk (Merck), 100 μg/ml Neutravidin (Thermo Scientific) in phosphate buffered saline containing Tween 20 (0.05%). Thereafter, the samples were incubated with antigen-coupled beads on 96-well plates. After additional washes, beads were incubated with R-phycoerythrin–conjugated anti-human IgG Fcγ (Jackson Immunodiagnostics) and then washed again; we quantified results of bound antibodies on each antigen-specific bead using median fluorescence intensity.

Power calculations. Under the condition that the flow immunoassay should achieve a sensitivity of 80%, specificity of 99%, 80% power, and a 5% type I error, we determined that 196 participants (98 cases and 98 healthy controls) would be sufficient to detect patients with early-onset RA using the immunoassay.

Statistical analyses. We normalized the median fluorescence intensity results and conducted the statistical analyses using SAS version 9.4 (SAS Institute). For identification of the best peptides, we filtered out those that captured highly collinear antibody responses. For analyses, we selected peptides with the highest potential to distinguish untreated early RA patients from healthy controls (individual area under the curve [AUC] ≥65%

and volcano plot inspection to narrow down peptides having the highest diagnostic ability and to limit the number of peptides tested downstream). We used multivariate logistic regression models to find the antibodies differentiating untreated early RA patients from healthy controls.

A sample was deemed positive for a given peptide when the antibody levels exceeded a median of +5 median absolute deviation (MAD) in healthy controls. For tests on serum samples to be classified as positive, patient serum samples were required to show reactivity to at least 2 of the serologic markers in the model. We calculated test performance versus the actual status shown in untreated early RA patients versus healthy controls as the gold standard. Statistical methods are further described in Supplementary Data 2, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42463.

RESULTS

Identification of epitope-specific IgG antibodies with relevance to RA. We aimed to identify antigens as potential biomarkers for RA. To do this, we synthesized >350 peptides from disease-related proteins in joints shown in mouse models to bind antibodies that could induce or regulate arthritis. Peptides resembled the epitopes in conformation and/or included post-translational modifications, most often citrulline but also homocitrulline, hydroxylysine, and others (Supplementary Table 1, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42463).

Peptides were coupled to magnetic beads with a unique fluorescent label for each antigen for simultaneous detection of up to 360 antigens in a single sample. After we screened patient and healthy control samples for IgG antibodies that bind these, we filtered out the nonreactive and/or noninformative peptides and kept peptides with potential relevance for RA and added a few new ones for assays with smaller numbers of antigens (Figure 1A). Representative peptides were selected from a group of peptides with highly collinear reactivity in RA serum; we excluded those without additional value and prioritized those with functional relevance in the mouse models.

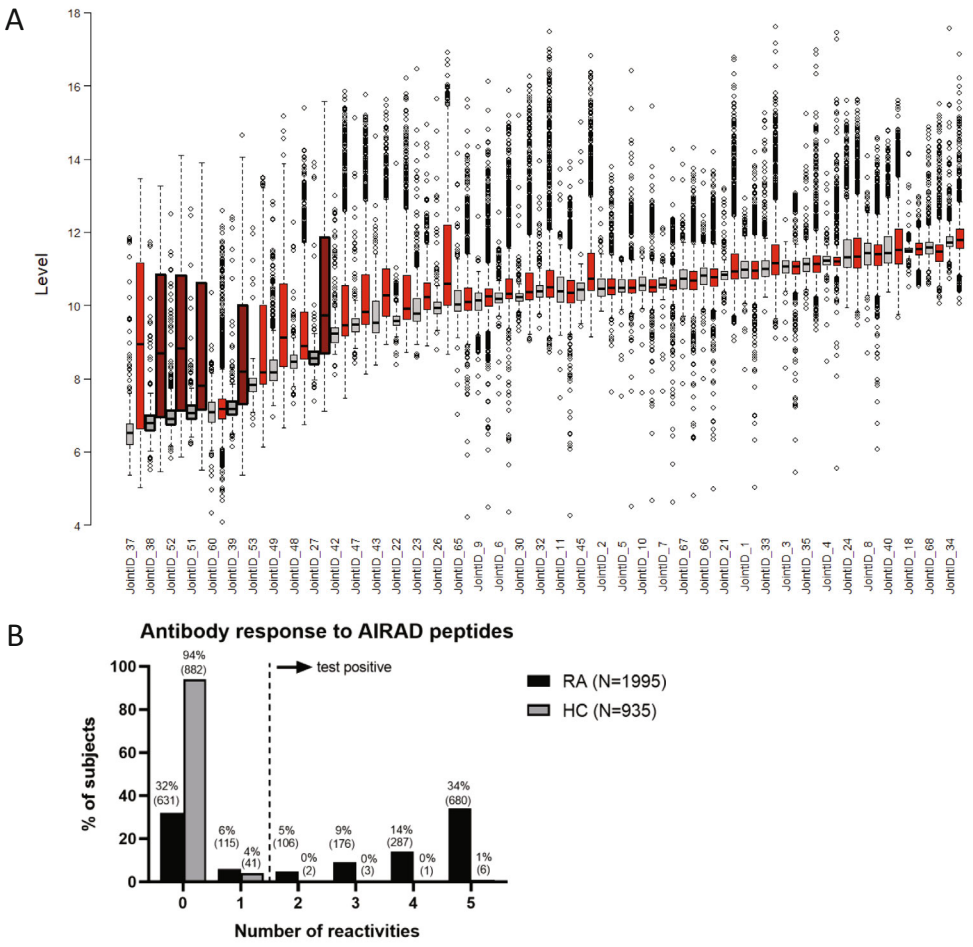
Verification of the reliability of the multiplex immunoassay. We confirmed the analytical performance of the multiplex immunoassay with a series of tests (Supplementary Data 1, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42463). We found reproducibility (expressed as median R^2) to be >0.93 (Figure 1A and Supplementary Table 3, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42463). The inter-assay variation was largest close to the limit of detection (LoD) but decreased when the assays were performed over a shorter period (Supplementary Figure 2, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42463). The coefficient of variation percentage (CV%) for repeatability around the LoD ranged from 10% to 27%, with median CV% of 14%. The correlation between the antibody responses against a peptide from 2 manufacturers was very high (R^2 = 0.9996), with minimal bias above LoD (Supplementary Figure 3, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42463) as previously observed (31).

Identification of an autoantibody signature that distinguishes early RA from healthy controls. Although anti-CCP and RF, the current serologic markers, are useful for classification of RA patients, a rather large proportion of patients with early RA are negative for both markers (4). We aimed to identify autoantibodies that could be informative alone or in combination with anti-CCP and/or RF. To discover the peptides with the highest diagnostic potential, we assayed serum samples from the BARFOT and TIRA-2 cohorts and the healthy controls (Figure 1B). The relative autoantibody levels for all specificities in the 50-plex assay in both untreated early RA patients and healthy controls are shown in Figure 2A. Five peptides (hereinafter named AIRAD, for aid in RA diagnosis) separated the untreated early RA patients from healthy controls. We specified that having serum reactivity to at least 2 of the peptides in AIRAD is a requirement to be classified as test positive (Figure 2B).

The antibody signature that included the AIRAD peptides (the AIRAD test) detected 63% of the untreated early RA patients with a very high (99%) specificity, and the receiver operating characteristic (ROC) analysis showed an AUC of 83% (Figure 2C). To investigate how the AIRAD and anti-CCP performed together, anti-CCP was included in the model. The combination of AIRAD and anti-CCP showed an ROC AUC of 84% and Tjur's coefficient of discrimination of 39% (Figure 2C), whereas anti-CCP alone showed an ROC AUC of 80% and Tjur's coefficient of 32% (Figure 2D).

When the antibody responses to AIRAD peptides were combined with the information from anti-CCP and RF (the classic seropositivity tests), sensitivity was 69%, whereas, without the AIRAD test, sensitivity was 66%, and the specificity remained similar (Figure 2D). The odds ratios for the AIRAD peptide responses are listed in Supplementary Table 6, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42463. Although the AIRAD test detected most of the early RA patients, the clinical utility of this test as an adjunct to anti-CCP and RF is limited.

Detection of autoantibodies that identify a population of seronegative untreated early RA patients. To find the best markers to identify the seronegative (anti-CCP2– RF–) RA patients, we performed a subset analysis of the BARFOT and TIRA-2 cohorts and healthy controls (Figure 1B). We identified a set of 5 peptides (hereinafter named SeNe, for seronegative RA diagnosis), which contained a different but partly overlapping set of peptides compared to AIRAD (used for identification of all untreated early RA patients) (Table 1). The SeNe peptides captured a serum antibody signature that



C

Tests	Test positive / Total		Performance					
	ueRA N / N	Controls N / N	Sensitivity (%) *	Specificity (%) *	AUC(ROC) (%)	Tjur's† (%)	NPV (%) *	PPV (%) *
AIRAD	1249/1995	12/935	63 (61 to 65)	99 (98 to 100)	83	35	55 (53 to 58)	99 (99 to 100)
AIRAD and aCCP	1205/1995	13/935	60 (58 to 63)	99 (98 to 99)	84	39	54 (52 to 56)	99 (99 to 100)

D

Tests	Test positive / Total		Performance					
	ueRA N / N	Controls N / N	Sensitivity (%) *	Specificity (%) *	AUC(ROC) (%)	Tjur's† (%)	NPV (%) *	PPV (%) *
aCCP	1141/1995	12/935	61 (58 to 63)	99 (98 to 99)	80	32	56 (53 to 58)	99 (99 to 100)
RF	1210/1995	51/935	61 (59 to 63)	95 (93 to 96)	78	28	54 (51 to 56)	96 (95 to 97)
aCCP+ and/or RF+§	1318/1995	62/935	66 (64 to 68)	93 (92 to 95)	84	38	56 (54 to 59)	96 (94 to 97)
aCCP+ and/or RF+§ and AIRAD	1385/1995	73/935	69 (67 to 72)	92 (90 to 94)	86	42	59 (56 to 61)	95 (94 to 96)

Figure 2. Identification of biomarkers for early untreated RA using serum samples from the untreated early RA cohorts (BARFOT and TIRA-2) and healthy controls. Samples were assayed with the 50-plex assay consisting of JointID peptides to capture the antibody reactivities. **A**, Relative antibody levels of JointID peptides in healthy controls (gray bars) and early RA patients (red bars). JointID peptides are listed in increasing order of their median antibody level in healthy controls. Relative antibody levels are shown as box plots, with each box representing the upper and lower interquartile range. Lines inside the box represent the median, and whiskers show minimum to maximum range among nonoutlier RA patients and healthy controls (symbols). Dark red indicates the AIRAD peptides. **B**, Percentage of RA patients and healthy controls (of total numbers in parentheses) who showed serum reactivity to the 5 AIRAD peptides. A positive test (dashed line) is defined as serum reactivity to ≥2 of the 5 peptides. **C** and **D**, Results of tests to determine performance of the AIRAD test for diagnosis of untreated early RA, alone and in combination with the anti–cyclic citrullinated peptide (anti-CCP) test (**C**), and performance of the AIRAD test in different combinations with the classic seropositivity tests (anti-CCP+ and/or rheumatoid factor [RF+]) (**D**). Untreated early RA patients were from the BARFOT and TIRA-2 cohorts. Status of anti-CCP and RF (positive/negative) was simulated among the healthy controls without inflammatory rheumatic diseases. Percentages were rounded to the nearest integer. * = Sensitivity, specificity, NPV, and PPV are with 95% confidence interval in parentheses. † = Coefficient of discrimination (49). § = Anti-CCP+ and/or RF+ status represented classic seropositivity. AUC = area under the curve; ROC = receiver operating characteristic curve; NPV = negative predictive value; PPV = positive predictive value; see Figure 1 for other definitions.

Table 1. The top JointID peptides identified in the multiplex flow immunoassay panels, including peptides for detection of patients with untreated early RA (AIRAD set) and for detection of RA patients who were seronegative on the classic anti-CCP and RF tests (SeNe set)*

	Peptide set					Flow immunoassay				
JointID peptide	AIRAD	AIRAD4	SeNe	Protein	Peptide	360-plex	288-plex	79-plex	50-plex	Reference
JointID-52	+	+	+	GPI	GPI_C_19_CIT	+	+	+	+	–
JointID-38	+	+	–	PADI4	PADI4_C_13_CIT	+	–	+	+	–
JointID-27	+	+	–	GPI	GPI_C_11_CIT	+	+	+	+	–
JointID-51	+	+	+	PADI4	PADI4_C_14_CIT	+	–	+	+	–
JointID-39	+	–	–	PADI4	PADI4_C_19_CIT	–	–	+	+	–
JointID-18	–	–	+	COL2A1	C_(F4)_[R]-R	+	+	+	+	19,50,51
JointID-30	–	–	+	GPI	GPI_C_20_CIT	+	+	+	+	–
JointID-5	–	–	+	COL2A1	T_U1_R-CIT	+	+	+	+	51

* Symbols +/– indicate presence/absence. RA = rheumatoid arthritis; AIRAD = aid in RA diagnosis 5-peptide set; AIRAD4 = AIRAD 4-peptide set; SeNe = seronegative RA diagnosis peptide set; GPI = glucose-6-phosphate isomerase; PADI4 = protein arginine deiminase type 4; COL2A1 = α1 chain of type II collagen.

identified 22.5% (n = 125 of 556) of CCP2–RF– patients with 99% specificity. The ROC AUC was 67% (Figure 3), and the odds ratios were between 2.8 and 11 for the individual peptides in the SeNe set (Supplementary Table 7, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42463).

Validation of autoantibodies distinguishing untreated early RA from healthy controls in independent cohorts. To validate the capacity of the AIRAD peptides to detect untreated early RA patients, we used the assay results from plasma samples collected from untreated early RA patients from the early RA patient cohort and matched healthy controls (Figure 1B). We found that AIRAD distinguished untreated early RA patients from healthy controls with 81% sensitivity (95% confidence interval 72–91%) and 100% specificity (Figure 4). When plasma samples were reassayed after 40 days, 95% (n = 90 of 95) of the positive and negative AIRAD test results were reproduced.

To validate the clinical significance of the antibody response captured by the 5 peptides in the SeNe set, we next determined the performance of the peptides in identifying the seronegative (CCP2–RF–) RA patients in the other 2 independent cohorts (the early RA patient and JointID cohorts). The SeNe test detected 14% and 22% of the seronegative patients with 93% and 97% specificity, respectively (Supplementary Figure 4, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42463). When the early RA patient cohort samples were reassayed after 40 days, 98% (n = 93 of 95) of the positive and negative SeNe test results were reproduced.

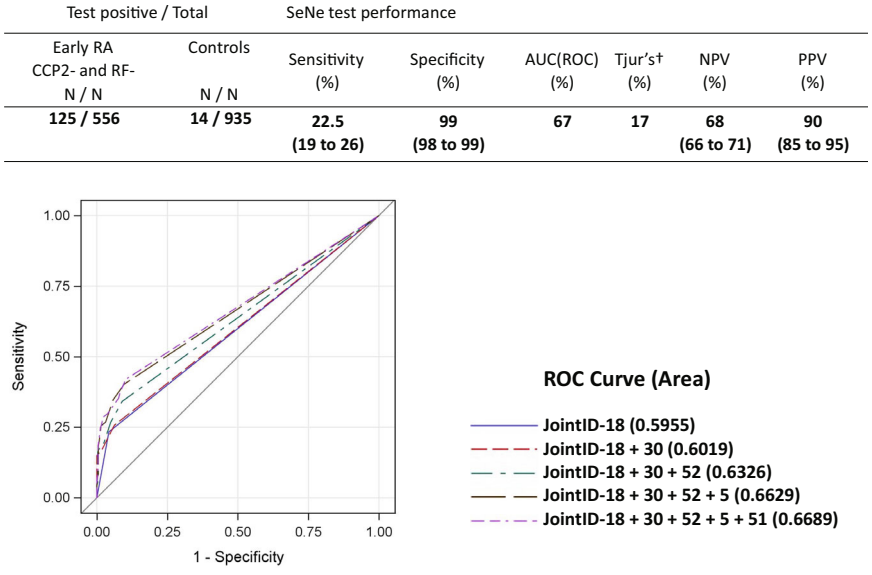
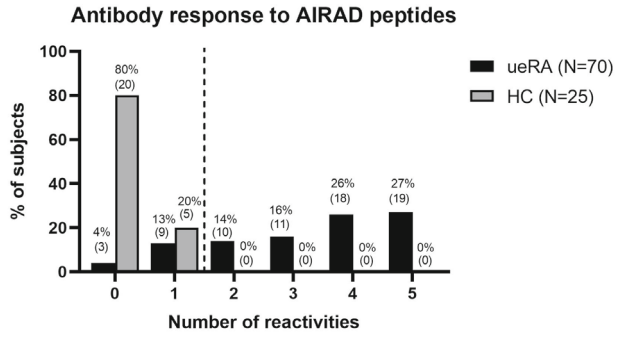


Figure 3. Sensitivity and specificity analysis of the seronegative early RA test (SeNe). The 5 peptides identified in the SeNe set detected seronegative (anti–cyclic citrullinated peptide negative [CCP2–] and rheumatoid factor negative [RF–]) early RA patients in the BARFOT and TIRA-2 cohorts (n = 556) compared with healthy controls (n = 935). Top, Performance of the SeNe test, with numbers in parentheses indicating 95% confidence interval. Bottom, Receiver operating characteristic (ROC) curves illustrating the contribution of each peptide to the combined performance. † = Coefficient of discrimination (49). AUC = area under the curve; NPV = negative predictive value; PPV = positive predictive value; see Figure 1 for other definitions.



Condition	Test positive, N (%)	Test negative, N (%)	Total N (%)
RA	**58 (81%; 72% to 91%) ***	**12 (19%)**	**70 (100%)**
HC	**0 (0%)**	**25 (100%)**	**25 (100%)**

Figure 4. Performance of the AIRAD test in distinguishing untreated early RA patients from healthy controls in the early RA patient cohort. Top, Percentage of patients and healthy controls (of total numbers in parentheses) who showed serum reactivity to the 5 AIRAD peptides. A positive test (dashed line) is defined as serum reactivity to ≥2 of the 5 peptides. Bottom, Performance of the AIRAD test in distinguishing untreated early RA patients from healthy controls. Percentages have been rounded to the nearest integer. * = 95% confidence interval. See Figure 1 for definitions.

Discrimination of RA from healthy controls and from patients with OA, PsA, or SLE by the detected autoantibodies. We next compared the disease specificity of the antibody signatures captured by the AIRAD and SeNe tests versus populations with other types of joint inflammation (baseline characteristics listed in Supplementary Table 5, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42463).

Data from the JointID cohort assayed earlier included 4 of the AIRAD peptides (Table 1); our results showed that the AIRAD test reached 71% sensitivity and 95% specificity for RA when the healthy control group (WINGA) was enriched with samples from patients diagnosed with OA or PsA (Table 2). Specificity for healthy controls without rheumatic diseases was 96%, and specificities for OA and PsA patients were 93% and 81%, respectively (Table 2). The specificity of the AIRAD test for RA increased from 93% to 97% relative to testing for OA samples and increased from 81% to 100% relative to testing of PsA samples without a remarkable drop in sensitivity (from 71% to 67%) when a more stringent overall requirement for test positivity was applied (Supplementary Table 8, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42463). Against SLE, the test reached 88% specificity (Supplementary Figure 5, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42463).

A clinically relevant question is whether patients with joint inflammatory diseases other than RA are negative for the 5-peptide SeNe set that we used to identify a proportion of the seronegative RA patients. Thus, we analyzed the JointID cohort, which included patients with OA and PsA, and found that SeNe had a specificity of 95–97% for OA and PsA, similar to that for the healthy controls without rheumatic diseases (Table 2). A more stringent cutoff further increased the specificity for RA but with an

Table 2. Capacity of the AIRAD4 and SeNe tests to distinguish among patients with RA, healthy controls, and patients with other types of joint inflammation*

Test, condition	RF+	Anti-CCP+	Positive on test; 95% CI	Negative on test; 95% CI	Total no./group
AIRAD4					
RA	27 (63)†	28 (65)†	32 (71); 58–84%	13 (29)	45
HC + OA + PsA	NA	NA	15 (5)	282 (95); 92–97%	297
HC	NA	NA	9 (4)	238 (96); 94–99%	247
OA	NA	NA	2 (7)	27 (93); 84–100%	29
PsA	1 (6)‡	1 (6)§	4 (19)	17 (81); 64–98%	21
SeNe					
RA	27 (63)†	28 (65)†	28 (62); 48–76%	17 (38)	45
HC + OA + PsA	NA	NA	9 (3)	288 (97); 95–99%	297
HC	NA	NA	7 (3)	240 (97); 95–99%	247
OA	NA	NA	1 (3)	28 (97); 90–100%	29
PsA	1 (6)‡	1 (6)§	1 (5)	20 (95); 86–100%	21
SLE	28 (27)¶	17 (7)#	40 (15)	219 (85); 80–89%	259
SLE anti-CCP+	6 (35)	17 (100)	5 (29)	12 (71); 49–92%	17
SLE anti-CCP−	22 (25)**	0 (0)	35 (14)	207 (86); 81–90%	242

* Except where otherwise indicated, values are the number (%) of patients or healthy controls (HC) per group. Percentages have been rounded to the nearest integer. RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide; 95% CI = 95% confidence interval; NA = not available; OA = osteoarthritis; PsA = psoriatic arthritis; SLE = systemic lupus erythematosus (see Table 1 for other definitions).
† Information missing for 2 of 45 subjects.
‡ Information missing for 4 of 21 subjects.
§ Information missing for 3 of 21 subjects.
¶ Information missing for 157 of 259 subjects.
# Information missing for 1 of 259 subjects.
** Information missing for 154 of 242 subjects.

unacceptable drop in sensitivity (Supplementary Table 9, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42463). For SLE (the KLURING cohort), we showed a specificity of 85% for SLE, with the anti-CCP–positive SLE patients captured more often by the SeNe set than patients who were negative for anti-CCP.

DISCUSSION

We developed and characterized a multiplex immunoassay for detection of serum autoantibodies reactive to disease-related proteins in joints for detection of patients with untreated early RA. The peptides from disease-related proteins in joints, used as assay antigens, were carefully selected to avoid collinearity and competition in antibody binding. The AIRAD test reached an ROC AUC of 83% for untreated early RA compared with the anti-CCP test, which had an ROC AUC of 80%. More importantly, ~20% the RA patients classified as seronegative were identified with the SeNe test. We also showed that both tests distinguished patients with RA from healthy controls and from patients with OA, PsA, and SLE.

Multiplex screening assays led to the identification of 5 peptides (AIRAD) that detected antibodies with 99% specificity for untreated early RA and supported the diagnosis of RA in 63% of patients with untreated early RA. Several studies have been performed to increase the accuracy of diagnosis of RA patients, although these have not focused on antibodies to joint-related proteins that might be of importance for disease development. In a recent study, a panel of 4 biomarkers distinguished established RA patients from healthy controls with an AUC of >0.97 (32). Another study showed the ability of the RA-associated 14-3-3 η protein (33) to distinguish RA from healthy controls with an ROC AUC of >0.92 for RA according to the ACR 1987 classification criteria (34). However, when combined with anti-CCP and RF, the 30% false-positive rate (34) may limit the clinical utility.

The ability to distinguish between OA and other types of inflammatory arthritis is imperative as the treatment is fundamentally different between diseases. The AIRAD and SeNe tests were positive for 3–7% of OA patients and 0–7% of healthy controls, which are similar to previous reports on ACPA positivity (35,36). However, in contrast, we found that a larger proportion of patients with inflammatory arthritis (i.e., SLE and PsA) showed positivity for our set of AIRAD autoantibodies (12% and 19%, respectively) compared to prior studies, where the proportion of SLE and PsA patients with positivity for anti-CCP was 2–17% and 2–10%, respectively. This may be because the B cells that produce the AIRAD-captured autoantibodies are activated in response to antigens released from inflamed joints. Whether these antibodies are also pathogenic or regulatory is not yet possible to determine as direct experimental data in vivo are needed, as previously discussed (10). However, circumstantial evidence has shown that the presence of anti-CCP antibodies in SLE and PsA patients is associated with a more destructive disease (37–40). If a higher specificity for the AIRAD test is desired in the clinical setting, a more stringent cutoff can be used without significant loss in sensitivity.

The clinical challenge is to accurately diagnose RA patients negative for both anti-CCP and RF (41), and delayed diagnosis and treatment (42) may lead to worse prognosis (43). Both different immunoglobulin isotypes (8,44,45) and the specificity of autoantibodies as tools for improved diagnosis of RF-negative and/or ACPA-negative RA have been proposed, with antibody fine specificities showing 18–23% sensitivity and 83–87% specificity in this patient group (46,47). ACPA fine specificities identified 15.7% of anti-CCP2–/IgM RF– RA patients (6), and 10% sensitivity with 98% specificity was observed with 2 ACPAs (48). The 5 peptides in our SeNe set showed similar sensitivity in the 3 independent seronegative cohorts that we examined (14–22%), and the different composition of the multiplex assays used indicated technical robustness of the test. Considering the test's specificity (99%), we found the overall performance to exceed those shown in previous studies. This strongly supports the clinical use of SeNe as a tool to identify patients with anti-CCP-negative and RF-negative RA with a minimal false-positive rate in healthy controls and only 3% rate (97% specificity) when patients with OA and patients with PsA were included.

The SeNe test essentially detects new ACPA (cross)reactivities but also includes a peptide without citrulline. ACPAs bind to citrulline, allowing promiscuous binding, but they may have a variable degree of more private specificity, particularly in vivo (10). The captured fine specificities could reveal some surprises with regard to both the capacity of these antibodies to bind joints and the functional role of these antibodies, which are essentially different from those detected earlier (6,7,9). Among the peptides in the SeNe set, the best peptide (JointID-18) showed an ROC AUC of 60% for anti-CCP2-negative and RF-negative RA patients, making it a candidate for an enzyme-linked immunosorbent assay test as an aid in clinical decision making. The SeNe test could be feasible on the multiplex platform currently used for systemic autoimmune diseases in clinical laboratories.

Our study has several limitations. First, several screening assays were performed with smaller cohorts. The statistical power would not be sufficient to capture the rare antibody reactivities in these smaller cohorts, thus possibly overlooking these responses, which may explain the dominance of citrullinated peptides in the selection of SeNe and AIRAD. Second, although the triple helical peptides synthesized do offer conformational epitopes like the native protein, it is not known how the epitopes presented on cyclic peptides mimic epitopes of the native protein exposure in vivo.

The multiplex immunoassay is suitable for screening large patient cohorts for a wide spectrum of antibody specificities using a few microliters of serum or plasma. Antigens from disease-related proteins in joints open the possibility to search for

antibodies having specific roles in joint inflammatory diseases. The assay can be supplemented with additional peptides to understand pathogenesis of disease subtypes, to possibly predict clinical onset and severity, to identify and classify RA subtypes, or to predict treatment responses. We conclude that simultaneous detection of autoantibody specificities against epitopes on disease-related proteins in the joints has additional value in classifying RA patients negative for anti-CCP and RF. This study demonstrated the value of this test among patients with early RA, but its clinical utility in predicting disease onset or other outcomes, including treatment responsiveness, remains to be investigated.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Holmdahl had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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