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Diagnosis of rheumatoid arthritis: multivariate analysis of biomarkers

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Abstract

Objective. To test if a combination of biomarkers can increase the classification power of autoantibodies to cyclic citrullinated peptides (anti-CCP) in the diagnosis of rheumatoid arthritis (RA) depending on the diagnostic situation. **Methods.** Biomarkers were subject to three inclusion/exclusion criteria (discrimination between RA patients and healthy blood donors, ability to identify anti-CCP-negative RA patients, specificity in a panel with major non-rheumatological diseases) before univariate ranking and multivariate analysis was carried out using a modelling panel (n = 906). To enable the evaluation of the classification power in different diagnostic settings the disease controls (n = 542) were weighted according to the admission rates in rheumatology clinics modelling a clinic panel or according to the relative prevalences of musculoskeletal disorders in the general population seen by general practitioners modelling a GP panel. **Results.** Out of 131 biomarkers considered originally, we evaluated 32 biomarkers in this study, of which only seven passed the three inclusion/exclusion criteria and were combined by multivariate analysis using four different mathematical models. In the modelled clinic panel, anti-CCP was the lead marker with a sensitivity of 75.8% and a specificity of 94.0%. Due to the lack in specificity of the markers other than anti-CCP in this diagnostic setting, any gain in sensitivity by any marker combination is off-set by a corresponding loss in specificity. In the modelled GP panel, the best marker combination of anti-CCP and interleukin (IL)-6 resulted in a sensitivity gain of 7.6% (85.9% vs. 78.3%) at a minor loss in specificity of 1.6% (90.3% vs. 91.9%) compared with anti-CCP as the best single marker. **Conclusions.** Depending on the composition of the sample panel, anti-CCP alone or anti-CCP in combination with IL-6 has the highest classification power for the diagnosis of established RA.

Keywords: *Rheumatoid arthritis, diagnosis, anti-CCP, marker combinations, multivariate analysis*

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder of unknown aetiology which primarily affects the joints, but can lead to a variety of extra-articular manifestations (Feldmann et al. 1996). The revised criteria for the classification of RA from 1987 by the American College of Rheumatology (ACR criteria) are the accepted 'gold standard' to assess the status rather than to diagnose the disease (Arnett et al. 1988). A multitude of biomarkers for RA has been described, targeting analytes found in the different compartments. In addition, biomarkers are described for general processes associated with RA such as synthesis and degradation of cartilage, inflammation or autoimmune events (Poole & Dieppe 1994, Sharif et al. 1998, Garnero et al. 2000, Nakamura 2000, Young-Min et al. 2001).

A new dimension in regard to sensitivity and specificity has been added to the diagnostic tool box by Schellekens and his colleagues (1998) identifying citrullination as an essential antigenic determinant recognized by RA-specific autoantibodies. The development of assays targeting citrullinated proteins followed by first-generation anti-cyclic citrullinated peptide (CCP) tests based on filaggrin-derived cyclic peptides up to the second-generation anti-CCP tests with citrullinated peptides selected from specific peptide libraries have recently been reviewed in detail (Zendman et al. 2006). A systematic literature review covering 68 interpretable datasets revealed that the second-generation anti-CCP assay has an overall sensitivity of $68 \pm 15\%$ in the combined RA groups with a specificity of $95 \pm 5\%$ in a heterogeneous control group consisting of healthy controls, rheumatic diseases other than RA and non-rheumatic diseases (Avouac et al. 2006). Using these summary data as a guideline, there seems to be a sensitivity gap of some 30% in the classification rate of RA when anti-CCP is used as a stand-alone marker and the high specificity ($\sim 95\%$) is retained. In this study, we evaluated additional biomarkers qualifying for multivariate analysis by a set of inclusion/exclusion criteria to test the hypothesis that marker combinations using anti-CCP as a lead marker can result in increased overall sensitivities at pre-set high specificities and thus can lead to an improved classification of RA as a disease entity.

Materials and methods

Study population

Patients. Patients were enrolled in six European centres in a prospective longitudinal study following unified standard operational procedures (SOP). The participating clinical centres are identical to the authors' affiliations given above. While samples from patients with osteoarthritis (OA) were collected in Bristol, UK, samples from patients with RA and control subjects were collected in the remaining five centres. Details on a sub-collective of the OA patients have been published recently (Sharif et al. 2004, 2006, 2007). In this report, only samples at the time of enrolment were used. Inclusion criteria for the RA cohort were the presence of a minimum of four ACR criteria plus a retrospective confirmation of the diagnosis after 2 years (Hochberg et al. 1992). All RA patients and controls had established disease with a positive diagnosis confirmed by the respective centre. Controls were collected at clinical sites with additional OA patients coming from general practitioners' offices. Ethics approval for this study was given by the ethic committees of each of the participating centres.

Case report forms for RA patients. Standardized case report forms (CRF) either in German (study sites Frankfurt, Vienna, Chur) or in English (study sites Stoke-on-Trent and Nijmegen) registered: age, sex, ACR criteria, disease duration, X-rays of hands and forefeet, joint surgeries and comorbidities, tender and swollen joint count (66 joints), the functional status of the patient, past and present medication, biomarkers (rheumatoid factor, C-reactive protein (CRP)) and the SF-36 health survey (Ware et al. 1993). The English CRF contained the Health Assessment Questionnaire (HAQ) (Fries et al. 1980), while the German CRF contained the 'Funktionsfragebogen Hannover' (FFb-H-P) (Raspe et al. 1990).

Panel composition for diagnostic settings. The composition was modelled to mirror two different diagnostic settings with different medical needs. The composition of the modelling panel ($n = 906$) is shown in Table I.

The representation of the different disease groups likely to be encountered by a general practitioner (modelled GP panel) is based on the relative prevalence data of musculoskeletal diseases in the general population of the USA (Lawrence et al. 1998) and is only modestly preselected. Positive results will lead to a referral of the respective patients to a specialized clinic.

The relative distribution of the selected disease groups (taken as 100%) in the modelled clinic panel is based on the absolute numbers of the admission rates in rheumatology centres in Germany (Zink A, Huscher D, Thiele, K, Weber, C, Topsch

Table I. Composition of the modelling panel ($n = 906$) and representation of the disease groups in the modelled GP panel according to prevalences and in the modelled clinic panel based on admission rates.

Disease group	No. of samples	Modelled GP panel (%)	Modelled clinic panel[a] (%)
Rheumatoid arthritis[b]	364	100.0	100.0
Spondylarthropathies[c]	50	1.8	38.4
Ankylosing spondylitis	19	0.46	17.5
Reactive arthritis	6	0.07	1.4
Psoriatic arthritis	25	1.25	19.5
Connective tissue diseases	52	1.8	21.9
SLE	10	0.34	12.2
Polymyositis	9	0.02	1.4
CREST	9	0.10	2.0
Systemic sclerosis	15	0.02	2.0
Sjögren syndrome	9	1.30	4.3
Vasculitis	10	0.7	10.9
Lower back pain	104	45.1	2.0
Fibromyalgia	21	5.2	10.1
Crystal related arthropathies	24	3.0	1.4
Gout	12	–	–
Chondrocalcinosis	12	–	–
Osteoarthritis	281	42.4	15.3
All controls	542	100.0	100.0

[a]Based on 10 772 patients in the 'Kerndokumentation' Germany 2002. [b]Ratio male/female, 128/236; mean age 59.6 years; mean disease duration 6.05 years. [c]Individual diseases were combined for the multivariate analysis into disease groups to have a minimum number of samples in each group for splitting into training set and test set. SLE, systemic lupus erythematosus; CREST, calcinosis, Raynaud's syndrome esophageal dysmotility, sclerodactyly, telangiectasia.

D, Otto S *et al*. Rheumatologische Kerndokumentation der Regionalen Kooperativen Rheumazentren in den Jahren 2001 und 2002. Berlin, Epi-Report No 17, 2004, unpublished). The patients in this setting are highly preselected as shown in Table I. Since the control group includes a significant fraction of autoimmune diseases the differential diagnosis of RA is much more demanding.

Enzyme-linked immunoassays

The enzyme-linked immunoassay (ELISA) tests were measured in duplicate in serum (unless stated otherwise) at Roche Diagnostics GmbH, Penzberg, Germany or at a service laboratory (Microcoat GmbH, Bernried, Germany). To exclude lot to lot variance within panels a single lot of each assay was used for all samples wherever feasible and appropriate controls were run. These assays were: aggrecan (BioSource, Nivelles, Belgium); anti-CCP (Axis-Shield Diagnostics Ltd, Dundee, UK); MRP14 (S100 A9; BMA Biochemicals AG, Augst, Switzerland); MRP8/14 heterodimer (S100 A8/A9; Bühlmann AG, Allschwil, Switzerland); beta-CrossLaps (degradation fragments of C-terminal telopeptides of collagen type I; Roche Diagnostics GmbH, Mannheim, Germany); circulating immune complexes (CIC; Bühlmann AG, Allschwil, Switzerland); C-natriuretic peptide (NT-proCNP, in EDTA plasma; testing service, Endolab, Christchurch, New Zealand); Col 2–3/4 (neoepitope by cleavage of collagen type II; IBEX Technologies Inc., Montreal, Canada); cartilage oligomeric matrix protein (COMP; AnaMar Medical A.B., Uppsala, Sweden); CPII (C-terminal peptide of collagen II synthesis; IBEX technologies Inc., Montreal, Canada); CRP (Roche Diagnostics GmbH, Mannheim, Germany); CTXII (C-terminal telopeptide of collagen type II; testing service, Ostex International Inc., Seattle, USA); human neutrophil defensin (HNP1–3; Hycult Biotechnology, Uden, the Netherlands); human melanoma inhibitory activity (MIA; Roche Diagnostics GmbH, Mannheim, Germany); human vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, USA); hyaluronic acid (Corgenix Ltd, Peterborough, UK); ICTP (cross-linked epitope of collagen I; Orion Diagnostica OY, Espoo, Finland); interleukin (IL)-1β (R&D Systems, Minneapolis, USA); IL-6 (Roche Diagnostics GmbH, Mannheim, Germany); matrix metalloproteinase 1 (pro-MMP1; The Binding Site Ltd., Birmingham, UK); matrix metalloproteinase 3 (pro-MMP3; The Binding Site Ltd); osteocalcin (Roche Diagnostics); P1NP (aminoterminal propeptide of collagen type I; Roche Diagnostics); pyridinoline crosslinks (PYD, in urine; Quidel Corporation, San Diego, CA, USA); soluble CD14 (IBL Gesellschaft für Immunchemie und Immunbiologie GmbH, Hamburg, Germany); total RF-factor (measuring IgG, IgM, IgA; Roche Diagnostics); RF isotype IgM, (TheraTest Labs, Lombard, USA); RF isotype IgA, (TheraTest Labs); RF isotype IgG (TheraTest Labs); serum amyloid A (BioSource, Nivelles, Belgium); and tumour necrosis factor-α (TNF-α; R&D Systems). The assay targeting calgranulin C (S100 A12) was an in-house development of Roche Professional Diagnostics using polyclonal antibodies from rabbits immunized with recombinant S100A12. The antibodies were biotinylated or digoxigenylated to give a sandwich ELISA on streptavidin-coated microtitre plates with anti-digoxin-peroxidase conjugate and ABTS development (Thierolf et al. 2007).

Statistical methods

First inclusion/exclusion filter for initial marker evaluation. A ratio of 2.0 of the median concentration in RA patients over the median concentration in healthy blood donors (black-and-white panels) was applied as cut-off for the inclusion of markers in further evaluation.

Second inclusion/exclusion filter for initial marker evaluation. The diagnostic performance of the markers was assessed in an anti-CCP-negative panel using the area under the curve (AUC) of a receiver operating characteristics (ROC) analysis (Zweig & Campell 1993) for the discrimination between anti-CCP-negative RA patients ($n = 83$, cut off ≤ 5 U ml^{-1}) and a control group composed of healthy blood donors ($n = 35$) and OA patients ($n = 35$). An AUC > 0.6 qualified for inclusion in additional evaluation.

Third inclusion/exclusion filter for initial marker evaluation. The specificity of the markers was evaluated in a specificity panel that included eight major diseases ($n = 301$) with cut-offs determined at 90% specificity in healthy blood donors ($n = 200$). A specificity of $< 25\%$ led to the exclusion from further assessment.

Analysis of the modelling panel: preprocessing of measurement value. To account for non-Gaussian distribution of concentration values the concentrations were log-transformed for analysis and visualization.

Analysis of the modelling panel: univariate analysis. The empirical criterion for the evaluation of the discriminatory power of each of the seven biomarkers was the pair of medians of the estimated sensitivities/specificities in 100 runs of a Monte-Carlo cross-validation. In each run, the cut-off was determined on two-thirds of the samples and the sensitivity/specificity was estimated on the remaining third of the samples. Samples were split randomly into the two sample sets at each of the 100 runs.

Analysis of the modelling panel: multivariate analysis. In multivariate analysis overfitting is a serious pitfall. To account for this the steps of model selection, parameter estimation and performance estimation were implemented in a nested cross-validation design with regularized discriminant analysis (RDA) (Friedman 1989) as the basic algorithm (Figure 1). RDA allows for combination of marker log-values that take into account their main effects (linear combination) and their interaction (quadratic terms), and allows the systematic optimization of the trade-off between the potential overfit due to the increased model complexity of main effects + interaction terms and the potential bias, when ignoring important interactions.

In each loop of the external cross-validation (ECV) a set of markers was chosen in the corresponding internal cross-validation (ICV) loops (Figure 1). The marker combinations chosen most often in the ECV were selected for final evaluation on the test set. The empirical criterion for the discriminatory power of the RDA rules on different marker sets was the estimated pair of sensitivity/specificity on the test set with a cut-off determined on the training data. The sensitivity was estimated by the percentage of correctly classified RA patients in the test set and the specificity by the prevalence-weighted sum of the percentages of correctly classified patients in the test set in each of the disease subgroups. The weights for the individual disease groups in the modelled clinic panel and in the modelled GP panel are given in Table I. When

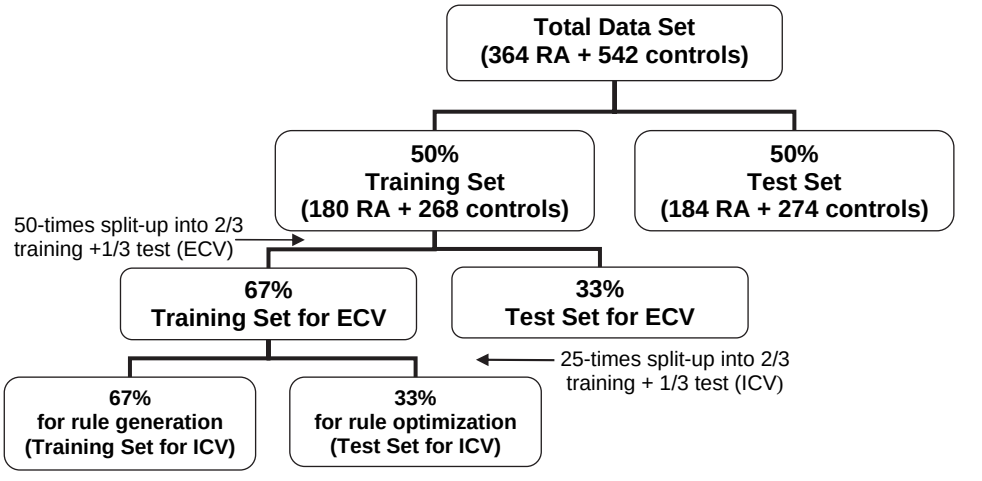

Figure 1. Schematic representation of training and validation using RDA for the selection of marker combinations.

focusing on the diagnostic setting at the general practitioner, the cut-off was determined such that prevalence-weighted specificity was at least 90%. For the diagnostic setting in rheumatology clinics the required prevalence weighted minimum specificity was 95%.

Analysis of the modelling panel: challenging RDA with other statistical methods. The results obtained with RDA were challenged by applying other statistical methods, which differ in the mathematical algorithm and the optimization strategy: Bayes Logistic Regression (BLR), Bayes Linear Support Vector Machine (BL-SVM) and Bayes Kernel Support Vector Machine (BK-SVM) (Suykens et al. 2002, Genkin et al. 2007). For the BLR the BBR-Toolbox for Matlab of Genkin et al. (2007) and for BL-SVM and BK-SVM the LSSVMlab-Toolbox (Pelckmans et al. 2002, Suykens et al. 2002) were used. These methods were applied using the default settings of the respective software tools. The criterion for the comparison of methods common to all applied statistical methods is the median of estimated sensitivity and specificity in an ECV design on the training set. Since all applied methods gave similar results only the sensitivity and specificity estimations generated on the test set with RDA are shown.

Results

Preselection of markers

In total, 131 biomarkers were subject to a preliminary assessment of their potential to aid in the classification of RA patients (Figure 2). The markers under consideration were derived from three sources: (1) from the literature and search of patent databases ($n = 41$); (2) from proteomics efforts targeting synovial fluid of patients with either erosive or non-erosive RA and tracing the marker candidates in matched serum samples and sera of healthy subjects as controls ($n = 63$) conducted in collaboration with Millennium Pharmaceutical Inc., Boston, USA (Liao et al. 2004); (3) through

the identification of proteins by mass spectroscopy based on MHC-associated peptides ($n = 27$) in collaboration with A. Vogt, Roche Center for Medical Genomics, Basel, Switzerland (unpublished).

Published references to sensitivity and specificity of the markers in question, extended pathway analysis, direct measurements of selected marker candidates in serum by multiple reaction monitoring (Kuhn et al. 2004) or the reproducibility of protein identification based on the number of peptides found by mass spectroscopy led to the exclusion of 77 of the 131 initial marker candidates. To allow for the unbiased reproduction of the results presented in this study, only the 31 commercial assays available for the remaining 54 targets are referred to in addition to a single in-house development targeting S100A12, bringing the total number of biomarkers evaluated in this study to 32.

Inclusion/exclusion criteria

The fist inclusion/exclusion criterion was the classification power of the selected biomarkers between RA patients and healthy blood donors as controls in black-and-white panels. In this simplified scenario we calculated the ratio of the median value of each marker in the RA group and the control group and used an arbitrary ratio >2 as a threshold for inclusion (data not shown). Sixteen markers passed this initial filter: anti-CCP, CRP, hyaluronic acid, IL-6, pro-MMP1, pro-MMP3, PYD (sample: urine), SAA, S100 A12, S100 A8/A9, S100 A9, total RF, RF IgA, RF IgG, RF IgM and VEGF. Of these, 13 markers were reanalyzed in the anti-CCP-negative panel. Since no advantage of specific RF isotypes was apparent from our results for the discrimination of RA vs. healthy controls we preferred total RF over the three RF isoforms RF IgM, RF IgG or RF IgA. Remarkably, 14 of the remaining 16 commercial assays did not pass the hurdle, actually showing ratios ≤ 1 with the median values in the healthy control group being equal or higher than in the RA group, a configuration we considered as unfavourable for routine diagnostic assessments.

The second inclusion/exclusion criterion for the 13 markers carried forward (see Figure 2) was the ability to recognize anti-CCP-negative RA samples, defining negative as anti-CCP values ≤ 5 U ml^{-1} (anti-CCP-negative panel). We used an AUC of >0.6 in the ROC analysis as a rule-in criterion so as to ensure no early elimination of markers which could contribute in the multivariate analysis. Eleven of the 13 markers showed AUC values >0.6 leading to the elimination of VEGF and pro-MMP1 (Table II). Of the remaining candidates S100 A12 recognized virtually the same samples as S100 A9 and was chosen over the latter assay based on superior technical performance. Although PYD showed a rule-in AUC value of 0.75 (Table II) it was excluded due to extensive cross-reactivity with OA when previously tested in an extended OA panel (data not shown). Therefore, the four markers VEGF, S100 A9, PYD and pro-MMP1 were eliminated leaving nine marker candidates to be carried further. Note that 15 of the 83 anti-CCP-negative samples in the evaluation panel defined by the cut-off of ≤ 5 U ml^{-1} were recognized by anti-CCP itself when the cut-off was lowered to ≤ 1.6 U ml^{-1} to capture the borderline samples at a pre-set specificity of 90% (Table II).

As the third inclusion/exclusion criterion we ran a specificity check in major disease groups which may exist as comorbidities with RA (= specificity panel). The specificity panel revealed an outstanding overall specificity of 96% for anti-CCP and a perfect

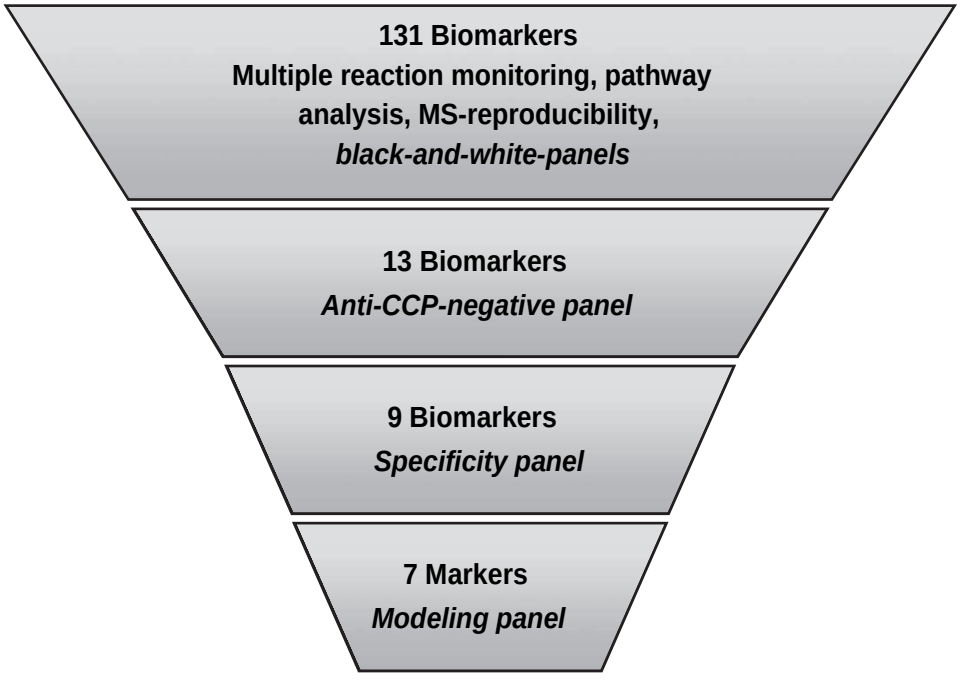

Figure 2. Evaluation process selecting markers for multivariate analysis.

specificity of this marker in diseases as EBV infection, mumps, cardiac infarction and congestive heart failure (Table III). In contrast, S100 A12, the marker with the highest discrimination potential for anti-CCP-negative RA samples in the second inclusion/exclusion criterion (Table II) cross-reacted extensively with the disease groups

Table II. Receiver operator characteristics in the anti-CCP-negative panel (cut-off ≤ 5 U ml^{-1}) ($n = 83$), healthy controls ($n = 35$) and osteoarthritis ($n = 35$) samples. An AUC > 0.60 qualified for further evaluation.

Marker	AUC	Sensitivity (%) at 90% specificity
S100 A12	0.87	65.1
Interleukin 6 (IL-6)	0.80	55.4
Hyaluronic acid (HA)	0.77	47.0
Pro-matrix metalloproteinase 3 (pro-MMP3)	0.75	55.4
Pyridinoline crosslinks (PYD)	0.75	47.0
Serum amyloid A (SAA)	0.74	49.4
Total rheumatoid factor (total RF)	0.69	34.9
Autoantibodies against cyclic citrullinated peptides (anti-CCP)[a]	0.69	18.1
S100 A8/A9	0.69	63.9
S100 A9	0.63	55.4
C-reactive protein (CRP)	0.63	28.9
Matrix metalloproteinase 1 (pro-MMP1)	0.60	18.1
Vascular endothelial growth factor (VEGF)	0.55	21.7

[a]Cut-off ≤ 1.6 U ml^{-1}. AUC, area under the curve in the receiver operator characteristics.

Table III. Specificity (%) of the nine markers remaining after applying the first two inclusion/exclusion criteria in a specificity panel including selected diseases ($n = 301$) with cut-offs used resulting in 90% specificity in a healthy control group ($n = 200$).

Specificity panel	Number of samples	Anti-CCP[a]	Total RF	HA	Pro-MMP3	SAA	IL-6	CRP	S100 A12	S100 A8/A9
Cancer[b]	105	95	79	50	86	69	33	25	6	6
Hepatitis A/B	51	92	78	87	57	59	69	41	16	20
EBV/mumps	29	100	100	92	100	55	79	77	38	7
Cardiac infarction	20	100	95	95	100	5	5	15	0	0
Congestive heart failure	20	100	90	84	89	50	55	65	20	10
Renal insufficiency	30	90	87	67	53	37	37	40	50[c]	0[c]
COPD	29	93	93	93	93	69	76	76	6[d]	0[d]
Liver cirrhosis	17	76	71	24	94	100	76	94	ND	ND
Total specificity	NA	96	84	72	69	51	44	28	13	8

[a]The markers are abbreviated as shown in Table II. [b]Composition of cancer group: breast, 32; colorectal, 26; pancreas 13; lung, 11; liver, 4; bile duct, 3; stomach, 2; ovarian, 2; cervical, 1; oesophagus, 1; metastasis with unknown primary tumour, 7. [c]Eight samples measured. [d]17 samples measured. EBV, Epstein–Barr virus; COPD, chronic obstructive pulmonary disease; ND, not done; NA, not applicable.

represented in the specificity panel, e.g. 99 out of 105 sera from various cancers were recognized by S100 A12 in addition to 90% of sera from patients with cardiac problems. Hence the overall specificity of S100 A12 was only 13%. The heterodimer S100 A8/A9 turned out to be even less specific (8%). Therefore, we eliminated S100 A12 and S100 A8/A9 from further evaluation.

Univariate analysis: modelled clinic panel and modelled GP panel

The combined performance of the biomarkers with three inclusion/exclusion criteria (Tables II and III) left seven of the initial 32 candidates to be further assessed in the modelling panel (Table I). Note that we did not include 'healthy' controls in the modelling panel. Comparing the representation of the disease control groups in the modelled clinic panel and in the modelled GP panel, striking differences become apparent. Lower back pain and osteoarthritis make up 88% of the controls in the modelled GP panel as compared with only 17% in the modelled clinic panel. Conversely, diseases grouped under spondylarthropathies, connective tissue diseases and vasculitis represent 71% in the modelled clinic panel but only 4% in the modelled GP panel (see Table I).

Using a target specificity of 95% in the modelled clinic panel, anti-CCP correctly classified 76.9% of the 364 RA samples in the modelling panel, followed by total RF (sensitivity 62.1%) whereas the remaining five markers – due to the high cut-offs necessary to reach the target specificity – did not recognize the majority of the RA samples, as expressed in the low sensitivities ranging from 19.0% to 25.3% (Table IV).

In the modelled GP panel not only the reduced specificity target of 90% but also the lower representation of autoimmune diseases (see Table I) contributed to lower cut-off values thereby leading to an increased sensitivity of the seven assays as compared with the modelled clinic panel. Especially the inflammation markers pro-MMP3, SAA, IL-6 and CRP showed improved sensitivities ranging from 44.8% to 63.3% (Table IV). The impact of the diagnostic setting becomes very evident with IL-6 where the sensitivity increased threefold from 19.0% in the clinic setting to 63.3% in the GP setting. In contrast anti-CCP appeared to be independent of the panel composition since it showed a sensitivity of approximately 77% in both settings when compared at 95% specificity. The increase in sensitivity from 76.9% to 82.7% given in Table IV is attributable to the reduced specificity target in the modelled GP panel.

Multivariate analysis in the modelled clinic panel

RDA was applied to the modelled clinic panel. As a selection criterion for biomarker combinations we chose the frequency of selection in 50 rounds of external cross-validation at a preset specificity of 95%. Anti-CCP was selected as the best single marker and anti-CCP in combination with SAA as the best marker pair (Table V) giving a sensitivity of 80.1% in the test set. However, the high specificity of anti-CCP in the test set could not be retained in the combination of anti-CCP and SAA as documented by a drop in specificity of 7.4%. The selection of marker pairs other than anti-CCP and SAA was less stable with low specificities in the training set, rendering test set calculations superfluous (Table V). No marker triplet resulted in an increase in sensitivity compared with anti-CCP alone while maintaining the high pre-set specificity (data not shown). Considering the high sensitivity and the excellent

Table IV. Univariate analysis of the selected seven markers in the modelling panel ($n = 906$) with the representation of the different disease groups according to admission rates (modelled clinic panel) or prevalences (modelled GP panel). The specificity was preset to 95% in the modelled clinic panel and to 90% in the modelled GP panel. The sensitivity (%) is derived from the median of 100 cycles of ROC analysis.

	Clinic panel			GP panel		
Marker	AUC (p5, p95)[a]	Cut-off (p5, p95)[a]	Sensitivity (p5, p95)[a]	AUC (p5, p95)[a]	Cut-off (p5, p95)[a]	Sensitivity (p5, p95)[a]
Anti-CCP[b]	0.93 (0.93, 0.94)	2.2 U ml^{-1} (2.0, 2.8)	76.9 (74.5, 76.9)	0.96 (0.96, 0.97)	0.9 U ml^{-1} (0.7, 1.1)	82.7 (80.8, 86.0)
Total RF	0.87 (0.86, 0.87)	26.9 IU l^{-1} (18.4, 27.6)	62.1 (61.8, 67.9)	0.88 (0.87, 0.88)	11.6 IU l^{-1} (10.2, 12.8)	75.0 (73.4, 76.4)
HA	0.73 (0.72, 0.74)	130.4 ng ml^{-1} (119.2, 134.5)	21.4 (19.0, 24.5)	0.68 (0.67, 0.69)	102.5 ng ml^{-1} (93.4, 112.0)	31.6 (26.6, 34.3)
Pro-MMP3	0.72 (0.71, 0.73)	81.6 ng ml^{-1} (72.9, 82.4)	24.2 (24.2, 27.7)	0.81 (0.80, 0.82)	33.0 ng ml^{-1} (29.6, 36.1)	55.2 (51.2, 58.8)
SAA	0.71 (0.70, 0.72)	260.8 mg/l (210.6, 300.0)	25.3 (22.8, 26.1)	0.78 (0.77, 0.79)	52.7 mg/l (47.7, 58.7)	52.3 (49.5, 54.1)
IL-6	0.71 (0.69, 0.72)	24.2 pg ml^{-1} (19.2, 25.6)	19.0 (17.6, 21.7)	0.87 (0.87, 0.88)	4.7 pg ml^{-1} (4.0, 4.8)	63.3 (61.0, 65.5)
CRP	0.68 (0.66, 0.69)	25.6 mg/l (21.1, 27.1)	23.6 (23.1, 25.8)	0.70 (0.69, 0.72)	11.8 mg/l (10.5, 14.1)	44.8 (36.0, 48.2)

[a]p5, 5% percentile; p95, 95% percentile. [b]The markers are abbreviated as shown in Table II. AUC, area under the curve in the receiver operator characteristics.

Table V. Multivariate analysis using regularized discriminant analysis (RDA) in the modelling panel ($n = 906$) using admission rates (modelled clinic panel) and a preset specificity of 95% or the prevalences of control diseases (modelled GP panel) and a preset specificity of 90%. The results in the training set are based on 50 cycles of external cross-validation (ECV).

	Training set		Test set	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Marker combination in clinic panel				
Anti-CCP	76.9	95.2	75.8	94.0
Anti-CCP+SAA	82.0	93.0	80.1	86.6
Anti-CCP+pro-MMP3	79.4	85.6	NC	NC
Anti-CCP+hyaluronic acid	79.2	83.7	NC	NC
Marker combination in GP panel				
Anti-CCP	81.6	93.3	78.3	91.9
Anti-CCP+IL-6	85.0	92.7	85.9	90.3
Anti-CCP+IL6+SAA	86.1	92.7	84.8	89.2

NC, not calculated.

specificity of anti-CCP, it is the most discriminating marker in a panel reflecting the clinic situation.

Confirmation by different mathematical models applied to the modelled clinic panel

To test the stability of the multivariate results obtained by RDA in the modelling panel (Table V), we applied three additional mathematical models to the same sample set. In close correlation with RDA, the BK-SVM selected in all loops of the ECV anti-CCP alone (sensitivity 76.6%; specificity 95.8%) with no additional marker being able to increase these values. BLR selected a triple combination (anti-CCP, SAA and total RF) gaining 5% in sensitivity as compared with anti-CCP alone (82%) but at a slight loss in specificity (93%). Similarly, the BL-SVM selected a marker triplet (anti-CCP, SAA and pro-MMP3) resulting in an increased sensitivity of 82% at a corresponding reduced specificity of 93%. This consistent interrelation between gains in sensitivity and losses in specificity confirms that the sensitivity of anti-CCP alone cannot be significantly improved by any marker combination based on the seven markers under evaluation irrespective of the mathematical model used without compromising on the target specificity of 95%.

Multivariate analysis in the modelled GP panel

In contrast to the clinic setting above, we used in the GP setting a target specificity of 90%, reasoning that a general practitioner could tolerate a higher number of false-positive test results for the benefit of an increased sensitivity. Any misclassification could be corrected after referral to a clinic using the higher specificity target. The best single marker selected in the modelled GP panel by RDA was again anti-CCP (Table V). When anti-CCP was combined with IL-6 to give the best marker combination for the GP setting the sensitivity increased by 7.6% to 85.9% while reaching the specificity target with 90.3%. Triple combinations in the GP panel did not increase the sensitivity any further while retaining a 90% specificity level.

Discussion

Many arguments can be made in favour of assessing the ability of a biomarker to discriminate between the presence and absence of a given disease in unselected populations. Due to the low prevalence/incidence rates of some rheumatological disorders, a test panel truly reflecting the general population would lead to unmanageable sample numbers in a R&D setting. Therefore, we assessed biomarkers in highly characterized and thus selected sample panels taking prevalences/incidences and admission rates into account using appropriate mathematical models to reflect different diagnostic scenarios at a rheumatology clinic or at a general practitioners office.

At the time the longitudinal sample banks were started we decided to include only confirmed RA patients in the modelling panel to eliminate additional variables inevitably associated with the diagnosis of 'early RA'. We reasoned that in established RA the underlying disease-specific metabolic processes characterizing RA are either ongoing or fully expressed, increasing the chances to identify at least those markers present in sufficiently high concentrations (>20 pg ml^{-1}) to be traceable with optimized ELISA assays in peripheral fluids such as serum. This strategy is based on the assumption that markers found in clinically manifested RA patients will also be present in 'early arthritis' which is by far the medically and diagnostically more relevant question guiding the therapeutic intervention. Consequently, our panel selection carries the inherent theoretical risk that markers could be missed simply for the fact that they are present in the early disease stages but fall under the detection limit once the disease has manifested. However, it has been extensively documented that at least three markers under investigation (anti-CCP, RF and CRP) are present in early arthritis patients, sometimes even before symptoms appear (Forslind et al. 2004, van Gaalen et al. 2004, Vittecoq et al. 2004).

The final modelling panel used in this study comprises 906 samples (Table I). We deliberately excluded 'healthy controls' from this panel since in a real diagnostic situation, individuals with no symptoms will not undergo testing for rheumatological disorders. Consequently, the inclusion of healthy controls would only influence the ROC analysis unduly resulting in sensitivity/specificity values not representative of the true diagnostic situation.

In a first selection filter, we compared RA patients from panel A with healthy controls, reasoning that markers unable to even differentiate between patients with confirmed RA and healthy blood donors will not be able to discriminate correctly RA from closely related disorders, especially autoimmune diseases. RF isotypes formally passed this first selection filter with ratios >2.0 for the discrimination of RA vs. healthy controls. Since we did not see any advantage of RF isotypes over total RF with our results, which is in accordance with Visser et al. (1996), only total RF was considered for further evaluation. However, RF isotypes have attracted new attention in the prognosis of RA especially when combined with anti-CCP (Lindqvist et al. 2005, Berglin et al. 2006, Agrawal et al. 2007). These recent results might warrant additional studies investigating the prognosis of disease progression.

As a second selection filter, we tried to fill the sensitivity gap still existing when anti-CCP is used as a stand-alone marker as reviewed elsewhere (Avouac et al. 2006). We challenged the 13 markers having passed the first inclusion/exclusion hurdle in a panel with anti-CCP-negative RA samples (Table II). With the test specifications used, S100 A12 identified two out of three anti-CCP-negative RA samples (65% sensitivity)

with the other members of the S100 family able to recognize approximately 55% and 64% of the anti-CCP-negative samples followed by three common inflammation markers (IL6, pro-MMP3, SAA) with sensitivities ranging from 49% to 55%. This indicates that those markers do have the potential to fill the sensitivity gap of anti-CCP. The number of RF-positive samples in the anti-CCP-negative RA group is with 35% (Table II) higher than the 19% previously reported based on 62 anti-CCP-negative samples (Sihvonen et al. 2005), also rendering RF a marker to be included in the multivariate analysis. Taken together, there are markers with a potential for identifying anti-CCP-negative RA samples, although to a different degree.

As a third selection filter we used the specificity of the remaining nine marker candidates in a specificity panel containing disease entities well known to be associated with the extra-articular disease manifestations of RA characterized by a high-inflammation potential. RA itself is associated with or leads to increased incidences of cardiovascular complications (Snow & Mikuls 2005, van Doornum et al. 2006), cancer and lymphomas in particular with EBV involvement (Kojima et al. 2005, Whelan 2006), renal complications (Kuroda et al. 2006a,b) and infections such as hepatitis (Buskila 2000, Lormeau et al. 2006). Yet anti-CCP can differentiate these disease groups from RA with an overall specificity of 96%. Even in the cancer group with an entire spectrum of different cancers ($n = 105$, Table III), anti-CCP is of superior diagnostic value (95% specificity) and more specific than total RF. The results with the disease groups in Table III confirm the notion that the second-generation anti-CCP tests (Zendman et al. 2006) are detecting autoantibodies highly specific for RA. Citrullination, a common post-translational modification present in a variety of diseases (György et al. 2006, Makrygiannakis et al. 2006) has been described in the context of apoptosis and regulation of histone transcription (Tarcsa et al. 1996, Asaga et al. 1998, Cuthbert et al. 2004, Wang et al. 2004). Yet the specific mechanism elucidating the high specificity of anti-CCP in RA remains a matter of debate. Models are proposed which describe a multifactorial chain of triggers ('multiple hit model for RA') of how the anti-CCP response might be generated, its relation to chronic inflammation and in the general pathogenesis of RA (van Gaalen et al. 2005). This general involvement of anti-CCP in the pathogenesis of RA is supported by recent findings in a mouse model (Kuhn et al. 2006).

The lower specificity of 28–69% by the known inflammation markers (SAA, IL-6, CRP, pro-MMP3, see Table III) was to be expected, making them – in spite of their ability to identify anti-CCP-negative RA samples – less promising candidates to support anti-CCP in the multivariate analysis once high specificity is used as a selection criterion.

The three acceptance/rejection criteria for marker candidates evaluated led to an attrition rate of 78%. From an initial 32 markers, only seven qualified for an assessment in the modelling panel which was modelled according to two major diagnostic scenarios (see Table I). This directly impacts on the marker selection and ability of biomarkers to support anti-CCP in different diagnostic settings. The best algorithm in the GP setting, which is characterized by a higher number of pain-related diseases (lower back pain and OA) and a lower number of autoimmune diseases, selects out of the group of inflammation markers IL-6 in support of anti-CCP. This selection is also driven by the lower specificity requirements (90%) attributed to a GP setting. Obviously, in this setting, the marker pair anti-CCP and IL-6 is able to best

capture the two biochemical hallmarks of RA, e.g. the presence of specific citrullinated proteins and the concurrent inflammatory events, with the remaining five markers, including RF, unable to add any additional classification power.

In contrast, in the clinic setting dominated by autoimmune diseases in combination with a high pre-set specificity of 95%, general inflammation markers will be rejected due to their specificity deficiencies. No marker combination could be identified by any of the four mathematical models used in which the gains in sensitivity were not offset by losses in specificity. The lack of specificity is the single critical feature as to why none of the biomarkers investigated is able to support anti-CCP in a clinical setting.

The true medical need in diagnosing RA is with early arthritis or even asymptomatic patients. In this study, the RA patients had a mean disease duration of six years receiving different medications. The size of our multicentre study did not allow forming statistically relevant subgroups to analyze the dependency of biomarker algorithms on disease duration and therapeutic regimen. Therefore, the inclusion/exclusion criteria used in this study for established RA will need to be re-applied to an early arthritis target population. Nevertheless, the assessment of the discrimination power of markers between RA, relevant controls in the context of RA and the disease groups in the specificity panel presented in this study with established RA can be transferred to the early RA scenario.

Independent of the status of the RA patients, it is apparent from this study that the marker selection for the diagnosis of RA will be critically dependent on the composition of the patient collective. Marker selection and algorithm development has to take into account the different diagnostic scenarios likely to be encountered by a general practitioner or reflecting the selected population seen by rheumatologists. Due to the pronounced lack in specificity of the markers other than anti-CCP, there will probably be no 'best universal diagnostic biomarker algorithm' for RA as a general disease entity, provided no additional markers become available matching or complementing the discrimination power of anti-CCP itself.

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and assays where run either at Roche Diagnostics GmbH, or Microcoat GmbH, Bernried, Germany.

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