A novel enzyme-linked immunosorbent assay using a mixture of human native and recombinant proteinase-3 significantly improves the diagnostic potential for antineutrophil cytoplasmic antibody-associated vasculitis

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ABSTRACT

Background: Antineutrophil cytoplasmic antibodies (ANCA) with a C-ANCA or P-ANCA pattern are detected in ANCA-associated vasculitis (AAV). While in most patients with AAV a C-ANCA pattern is due to reactivity with proteinase-3 (PR3)-ANCA, some C-ANCA-positive sera do not react with PR3.

Objective: The development and evaluation of a direct enzyme-linked immunosorbent assay (ELISA) for PR3-ANCA with increased sensitivity.

Methods: A mixture of human native (hn) and human recombinant (hr) PR3 was used as antigen coating. The resulting ELISA (anti-PR3-hn-hr) was compared with ELISAs using directly coated hn-PR3 or hr-PR3, as well as with a hn-PR3 capture ELISA. Assay characteristics were determined in patients with AAV (n = 248), with special attention for those patients with C-ANCA (n = 132), as well as disease controls (n = 585) and healthy controls (n = 429). Additionally, for prediction of relapses serial samples of 46 patients with PR3-AAV were analysed.

Results: At a predefined specificity of 99% both ELISAs containing hr-PR3 revealed a substantial increase in sensitivity. For the prediction of relapses by rises in PR3-ANCA titres the capture ELISA was most optimal (odds ratio 12.5). With an odds ratio of 8.9 the novel anti-PR3-hn-hr ELISA was second best.

Conclusions: Owing to the very high sensitivity of the novel anti-PR3-hn-hr ELISA for the detection of PR3-ANCA in C-ANCA-positive samples of patients with AAV this assay has an excellent diagnostic performance. This feature is combined with a good predictability of clinical relapses in patients with PR3-AAV. These characteristics challenge the dogma that, for detection of PR3-ANCA, capture ELISAs are superior for diagnosis and follow-up.

Antineutrophil cytoplasmic antibodies (ANCA) are associated with Wegener's granulomatosis (WG), microscopic polyangiitis, Churg–Strauss syndrome, and necrotising crescentic glomerulonephritis (NCGN), referred to collectively as ANCA-associated vasculitis (AAV).[1] ANCA react with constituents of primary granules of neutrophils. Basically, two immunofluorescent ANCA patterns are distinguished by indirect immunofluorescence (IIF), ie, a granular cytoplasmic fluorescence with central interlobular accentuation (C-ANCA) and a perinuclear pattern (P-ANCA). All other neutrophil-specific IIF reactivity is considered "atypical" ANCA. Only ANCA directed to proteinase 3 (PR3-ANCA) or myeloperoxidase (MPO-ANCA) are relevant for the diagnosis AAV. Therefore it is recommended that sera positive for ANCA by IIF, irrespective of the pattern, are assayed for PR3- and MPO-ANCA.[2] Obviously, the combination C-ANCA/PR3-ANCA or P-ANCA/MPO-ANCA results in high specificity for AAV,[3 4] but PR3- or MPO-ANCA combined with another ANCA IIF pattern does not exclude AAV.

The correlation between C-ANCA and PR3-ANCA depends on correct interpretation of IIF pattern and the test sensitivity of the antigen-specific immunoassay. If the classical granular cytoplasmic fluorescence is not distinguished from diffuse flat cytoplasmic fluorescence the correlation is reduced by the inclusion of ANCA directed to bactericidal/permeability-increasing protein.[5] Additionally, depending on the methods used, test sensitivity for PR3-ANCA detection differs in samples with C-ANCA. Capture enzyme-linked immunosorbent assays (ELISAs) are considered the best and this has been attributed to better conformational representation of the antigen as compared with direct ELISAs.[6]

In the ongoing search for optimising PR3-ANCA detection we have explored the application of human recombinant (hr) PR3. A PR3 expression construct was used essentially as described before.[7 8] For optimal post-translational processing and conformational identity to human native (hn) PR3, expression was established in human host cells (HEK293). Finally, for compensation of potential loss of epitopes the hr-PR3 was mixed with hn-PR3. This antigen mixture was used in a direct ELISA for determining the assay characteristics in patients with AAV (n = 248), with special attention for patients with C-ANCA (n = 132), as well as in a large cohort of disease controls (n = 585) and healthy controls (n = 429). The assay performance was compared with a direct and capture ELISA using hn-PR3, and a direct ELISA using hr-PR3. Finally, for the prediction of relapses, PR3-ANCA were measured in samples preceding relapse in patients with PR3-AAV and in matched patients with PR3-AAV without relapse.

MATERIALS AND METHODS

Cloning of cDNA coding for ΔAE-PR3(S176A)-His

Generation of the PR3 expression construct was performed as described before.[7 8] Table 1 summarises the DNA oligonucleotide primers used. Full-length cDNA encoding PR3 was generated by OneStep-RT-polymerase chain reaction (Qiagen, Hilden, Germany) using primers 1 and 2 and total RNA isolated from HL-60-cells as template. After cleavage with *Esp*3I and *Xho*I the cDNA was ligated with pET24d (Novagen, Bad Soden, Germany) linearised with *Nco*I and *Xho*I. The resulting construct pET24d-PR3-His was used as template to generate the double modified cDNA lacking the propeptide coding sequence and the active centre serine due to substitution by alanine (S176A). The required mutagenesis was performed in two steps using splicing by overlap extension method.[9] In the first step, three overlapping cDNA fragments were produced by polymerase chain reaction using primers 3–4, 5–6 and 7–8 respectively. Subsequently, ΔAE-PR3(S176A)-His cDNA was generated in a polymerase chain reaction with these overlapping amplification products as a common template and primers 3 and 8. The final cDNA was cleaved with *Hin*dIII and *Sal*I and ligated with linearised pEP4 (*Hin*dIII and *Xho*I) resulting in pEP4-ΔAE-PR3(S176A)-His. pEP4 was derived from pCEP4 (Invitrogen, Karlsruhe, Germany) by cleaving off the *EBNA1* gene using *Eco*91I and *Bsg*I followed by Klenow modification and religation of the vector fragment.

Expression and purification of recombinant ΔAE-PR3(S176A)-His

For expression of the hr-PR3 1.2×10^8 HEK293 cells in 400 ml high glucose DMEM (Invitrogen) supplemented with 10% fetal calf serum (v/v) were seeded into 1750 cm^2 roller bottles (Nunc, Roskilde, Denmark) 3 h before transfection. Transfection was performed using ExGen 500 (Fermentas, St Leon-Rot, Germany) according to the manufacturer's recommendations. The cells were then cultivated for 6 days at 37°C, 8.5% CO_2 and 95% humidity. Supernatants corresponding to a flask surface of 3500 cm^2 containing the secreted form of ΔAE-PR3(S176A)-His were pooled, supplemented with 1 mol/l Tris–HCl (pH 8.0), 1 mol/l imidazole (pH 8.0) and 200 mmol/l $MgCl_2$ to give final concentrations of 10, 5 and 50 mmol/l respectively and adjusted to pH 8.0 with 1 mol/l HCl. ΔAE-PR3(S176A)-His was enriched by immobilised metal-chelate affinity chromatography using TALON sepharose (Clontech, Mountain View, California, USA) and 5 mmol/l Tris–HCl, 300 mmol/l NaCl, 150 mmol/l imidazole (pH 8.0) as eluent. Subsequently, the pooled eluate was dialysed against 20 mmol/l MES (pH 6.0) (buffer M-IEX) with a 6–8 kDa cut-off membrane (Spectrum Europe, Breda, The Netherlands) to prepare it for cation exchange chromatography. After application to POROS HS (Applied Biosystems, Foster City, California, USA) using M-IEX as running buffer residual contaminants were washed away with 300 mmol/l NaCl, and ΔAE-PR3(S176A)-His was finally eluted with 500 mmol/l NaCl. Protein concentrations were determined by spectrophotometry at 280 nm (Eppendorf, Hamburg, Germany). Protein analysis was performed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis using the NuPAGE system according to the manufacturer's manual (Invitrogen) and immunoblotting using a monoclonal antibody (mAb) specific to hexahistidine (Merck Biosciences, Darmstadt, Germany). The purified protein migrated consistent to its calculated mass of 26 kDa when separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and was recognised by the hexahistidine specific mAb on the immunoblot (data not shown).

Table 1 Primers used for the generation of the cDNA coding for ΔAE-PR3(S176A)-His

Primer number	Sequence (5′–3′)
1	attacgtctcccatggctcaccggccccccagcc
2	taatctcgaggggcggccttggcctccacacggcgc
3	tttaagcttatcccgcgaaattaatacgactcactatag
4	cccacgatagcacgggcagcaccgctcagcag
5	gctgcccgtgctatcgtgggcgggcacgaggc
6	gggccacccgcgtctccgaagcagatgccgg
7	ttcggagacgcgggtggccccctgatctgtga
8	atagtcgacagcttcctttcgggctttgttagc

Human sera

Sera of three AAV cohorts (patients with renal biopsy-proven AAV, outpatients with AAV, and patients with WG) and five control cohorts (outpatient cohort with other vasculitides, rheumatoid arthritis (RA), systemic lupus erythematosus, Sjögren's syndrome and healthy controls) were used. This study was performed in accordance with the 1997 Declaration of Helsinki of the World Medical Association. From healthy controls informed consent was obtained. For the analyses on patient material serum was obtained for diagnostic and follow-up purposes. As rest-serum was used in an anonymous way, ethical approval was not necessary according to the Dutch guidelines.

Patients with renal biopsy-proven AAV (n = 115) were retrospectively identified in our renal biopsy registry[10] as diagnosed with pauci-immune NCGN between 1989 and 2006. Sera were obtained at the time of kidney biopsy. The outpatient cohort with AAV (n = 86) was registered with AAV (including WG, microscopic polyangiitis, Churg–Strauss syndrome and the renal limited form of AAV) in the outpatient clinic of the Department of Clinical and Experimental Immunology, University Hospital Maastricht. Sera were diagnostic samples (n = 57) or follow-up samples (n = 29). The latter samples were preferentially selected at the time that a capture ELISA was performed for confirmation of ANCA-specificity (n = 21); otherwise, the first sample available was included (n = 8). Twelve samples of this outpatient cohort with AAV were also included in the renal biopsy-proven cohort with AAV. Finally, sera of 59 patients with WG were kindly provided by Dr E Csernok (Department of Rheumatology, University Hospital of Schleswig-Holstein, Lübeck, Germany). These samples were previously used in a multicentre study for PR3-ANCA detection in WG.[6] For all patients with AAV, the clinical diagnosis was established according to the definitions of the 1992 Chapel Hill Consensus Conference.[11]

As disease controls we included samples from patients with RA (230), systemic lupus erythematosus (100) and Sjögren's syndrome (200). Except for 30 patients with RA (kindly provided by Dr E Csernok) that were part of the previously mentioned multicentre study,[6] all these control patients were diagnosed at the Department of Rheumatology and Clinical Immunology, Charité University of Medicine Berlin, according to internationally recognised criteria.[12–14] The outpatient cohort with other vasculitides (non-AAV) included patients (n = 55) that were registered in the outpatient clinic of the Department of Clinical and Experimental Immunology, University Hospital Maastricht. The diagnoses included: Henoch–Schönlein purpura (n = 9), polyarteritis nodosa (n = 8), giant-cell arteritis (n = 7), cryoglobulinaemia (n = 5), leucocytoclastic vasculitis (n = 5), Bürger's disease (n = 3), Behçet's disease (n = 3), retinal vasculitis

(n = 3), Goodpasture syndrome (n = 3), Takayasu's disease (n = 2) and miscellaneous (n = 7). Finally, 429 serum samples of healthy controls were included. Except for 29 samples (kindly provided by Dr E Csernok) that were part of the previously mentioned multicentre study,[6] all samples were obtained from the University Hospital of Lübeck.

To study whether quantification of PR3-ANCA titres can predict relapses of AAV, sera were analysed that were previously used in other test evaluations.[15–17] ANCA levels were measured in relation to relapse during follow-up in a cohort of patients with PR3-ANCA-positive AAV with (n = 23) or without (n = 23) relapse. Samples were obtained at the time of relapse and 3, 6, 9 and 12 months before the relapse; samples of controls were from age- and sex-matched AAV controls that neither experienced a relapse during the sampling period nor within the 12 months following this period. Clinical relapse was defined as described previously.[18 19] Complete remission was defined as the complete absence of symptoms or signs attributable to active vasculitis. Patients were either followed by JWCT or PVP (University Hospital Maastricht) or by CAS (University Medical Center Groningen).

Detection of antineutrophil cytoplasmic antibodies by indirect immunofluorescence

IIF ANCA detection was performed on the BIOCHIP Mosaic (EUROIMMUN, Lübeck, Germany) using ethanol- and formalin-fixed granulocytes, HEp-2 cells and primate liver as substrates. Samples were diluted 1/10 in phosphate-buffered saline (PBS). A fluorescein isothiocyanate-conjugated goat antihuman IgG antibody (EUROIMMUN) was used to detect bound IgG antibodies. All slides were evaluated by two independent observers; in case of a difference in opinion a third observer was decisive. Based on the ethanol-fixed granulocytes, three staining patterns were distinguished: perinuclear (P-ANCA), granular cytoplasmic (C-ANCA), and diffuse homogeneous cytoplasmic (atypical ANCA).

Proteinase 3-antineutrophil cytoplasmic antibody enzyme-linked immunosorbent assays

For the production of anti-PR3-hn-hr ELISA, microtitre plates (Nunc) were coated at 4°C, first with 1 μg/ml PR3 prepared from human granulocytes in 200 mmol/l Na_2CO_3 (pH 9.6; coating buffer) for 3 h; this was followed by 1.5 μg/ml ΔAE-PR3(S176A)-His in coating buffer supplemented with 1 mmol/l PMSF overnight, washed with PBS 0.05% (v/v) Tween-20 and blocked for 2 h with PBS 0.1% (w/v) casein. The anti-PR3-hr ELISA was only based on ΔAE-PR3(S176A)-His; the other procedures remained similar. After washing, sera diluted 1:100 in PBS 0.1% (w/v) casein were incubated for 30 min. Bound antibodies were detected using peroxidase-conjugated antihuman IgG and stained with tetramethylbenzidine (EUROIMMUN). All steps were carried out at room temperature. The optical density (OD) was read at 450 nm using an automated spectrophotometer (Spectra Mini, Tecan, Crailsheim, Germany). A highly positive index patient serum was used to generate a standard curve consisting of three calibrators (2, 20 and 200 relative units (RU)/ml). RU were calculated by this standard curve. The cut-off was optimised either by receiver operating characteristics (ROC) curve analysis (maximal sum of sensitivity plus specificity) or by pre-defined specificities. Commercially available anti-PR3 ELISA (hn-PR3) and anti-PR3 Capture ELISA (hn-PR3 captured by a PR3-specific mAb) were used as a reference (both from EUROIMMUN) and performed following the manufacturer's instructions. All samples, irrespective of the fluorescence pattern, were analysed by all four PR3-ANCA ELISAs.

Statistics

Diagnostic significance of the tests was assessed by ROC curve analysis and the area under the curve (AUC) was calculated. Statistical analyses were performed using the EUROStat statistical package (EUROIMMUN). Fischer's exact tests were performed for determining if rises in ANCA enable prediction of relapses by the distinct methods. $p<0.05$ was considered statistically significant. Definition of the relevant increase was determined by ROC curve analysis.

RESULTS

Overall diagnostic performance of proteinase 3-antineutrophil cytoplasmic antibody enzyme-linked immunosorbent assays

Results as obtained by the distinct ELISAs are presented in fig 1. To determine the optimal cut-off values of these PR3-ANCA ELISAs, ROC curve analyses were performed based on C-ANCA-positive sera. For sensitivity calculations we included the renal biopsy cohort (n = 115; 58 C-ANCA), the outpatient AAV cohort with exclusion of those patients (n = 12) that were already included in the renal biopsy cohort (n = 74; 27 C-ANCA), and the patients with WG (n = 59; 47 C-ANCA). For specificity calculations we only included the disease controls (n = 585), irrespective of the IIF pattern. The anti-PR3-hr and anti-PR3-hn-hr ELISAs appeared superior in terms of the maximal sum of sensitivity and specificity, as compared with the anti-PR3-hn and anti-PR3-c ELISAs (table 2). For comparison, however, it is better to determine sensitivities at a predefined specificity. Both new assays revealed a substantial increase in sensitivity at a specificity of 98% as well as 99% (table 2).

Diagnostic performance of proteinase 3-antineutrophil cytoplasmic antibody enzyme-linked immunosorbent assays in antineutrophil cytoplasmic antibody-associated vasculitis cohorts

As the three study cohorts were diverse in terms of type of AAV and severity of disease, we analysed sensitivity within each cohort, again within the C-ANCA-positive samples, at a predefined specificity of 99% (table 3).

In the renal biopsy cohort, 58 patients (50.4%) had C-ANCA. The great majority of these patients had pauci-immune NCGN with systemic vasculitis (ie, generalised AAV). The disease was active at the time of biopsy and serum collection. In this cohort all four PR3-ANCA ELISAs shared high sensitivity. Nevertheless, the anti-PR3-hr and anti-PR3-hn-hr ELISAs had the best sensitivity (both 95%). In the 57 C-ANCA-negative samples, four samples revealed low-level PR3-ANCA positivity in one or two of the PR3-ANCA ELISAs. As all four samples were P-ANCA and MPO-ANCA positive in several MPO-ANCA ELISAs (data not shown), we considered these PR3-ANCA results false-positive. The combination of P-ANCA, MPO-ANCA and PR3-ANCA is often associated with drug-induced vasculitis. As all four samples were negative for Elastase-ANCA, we consider it unlikely that our patients with this combination were suffering from drug-induced AAV. In the renal biopsy cohort atypical ANCA patterns were not observed.

In the outpatient AAV cohort (n = 86), 35 patients (40.7%) had C-ANCA. The sensitivities of the anti-PR3-hn ELISA (83%)

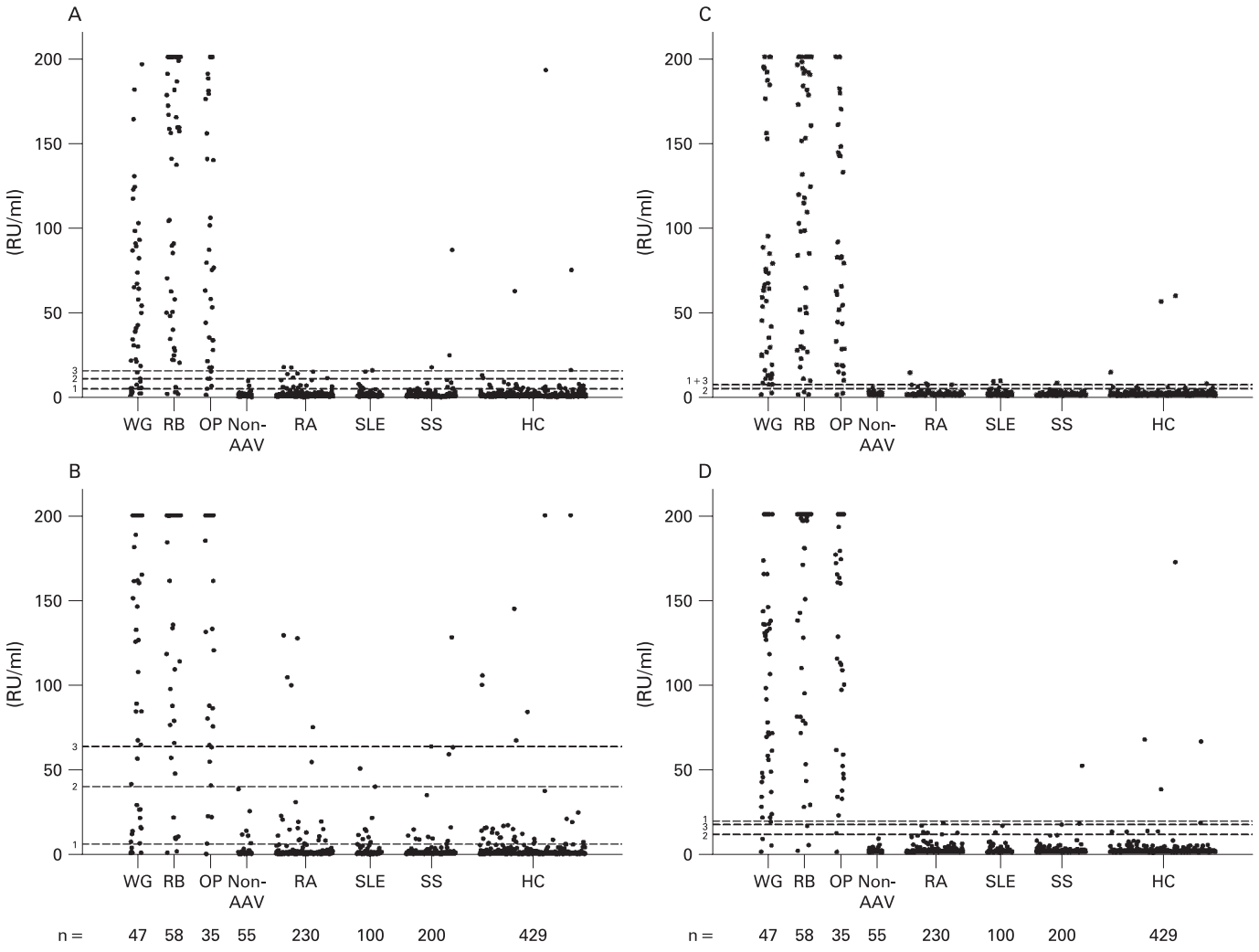

Figure 1 Diagnostic performance of the distinct EUROIMMUN ELISAs for detection of PR3-ANCA. (A) Data of the direct anti-PR3 ELISA with human native PR3; (B) data of the capture anti-PR3 ELISA with human native PR3; (C) data of the direct anti-PR3 ELISA with human recombinant PR3; and (D) data of the novel direct anti-PR3 ELISA with a mixture of human native and recombinant PR3. The different disease categories are: WG (C-ANCA-positive samples of the Wegener's granulomatosis cohort; n = 47); RB (C-ANCA-positive samples of the renal biopsy cohort; n = 58); OP (C-ANCA-positive samples of the outpatient AAV cohort; n = 35); non-AAV (outpatient cohort with vasculitides other than AAV; n = 55); RA (rheumatoid arthritis; n = 230); SLE (systemic lupus erythematosus; n = 100); SS (Sjögren's syndrome; n = 200); and HC (healthy controls; n = 429). Each symbol represents the result of a single serum sample. The dotted horizontal lines represent the distinct cut-offs as presented in table 2: (1) the optimised cut-offs based on ROC-curve analysis; (2) the cut-offs at a pre-defined specificity of 98%; and (3) the cut-offs at a pre-defined specificity of 99%. ELISA, enzyme-linked immunosorbent assay; ANCA, antineutrophil cytoplasmic antibody; AAV, ANCA-associated vasculitis.

and the anti-PR3-c ELISA (74%) were clearly reduced in comparison with the renal biopsy cohort, while the sensitivities of the anti-PR3-hr (94%) and anti-PR3-hn-hr ELISAs (94%) remained high. In the 51 C-ANCA-negative samples only two samples were PR3-ANCA positive. One sample had a formalin-sensitive P-ANCA pattern (MPO-ANCA negative) and was positive in the anti-PR3-hn, anti-PR3-c and anti-PR3-hn-hr ELISAs. The other sample, negative by IIF and for MPO-ANCA, was positive in the anti-PR3-hr ELISA. In this cohort atypical ANCA patterns were absent.

Finally, in the WG cohort (n = 59), 47 patients (79.7%) had C-ANCA. Again, the sensitivities of the new anti-PR3-hr and anti-PR3-hn-hr ELISAs are relatively high (94%) as compared with the anti-PR3-hn and anti-PR3-c ELISAs (64–66%). None of the 12 C-ANCA-negative samples, including 11 formalin-sensitive atypical ANCA samples, revealed PR3-ANCA by these ELISAs.

Diagnostic performance of proteinase 3-antineutrophil cytoplasmic antibody enzyme-linked immunosorbent assays in control cohorts

Using the 99% specificity cut-off values as determined in the total cohort of disease controls we analysed the specificity of each separate control cohort, as false-positive results might be clustered in one single cohort. No such clustering was observed (table 4).

Quantification of proteinase 3-antineutrophil cytoplasmic antibodies for prediction of relapses in patients with antineutrophil cytoplasmic antibody-associated vasculitis

To determine if quantification of PR3-ANCA by the distinct ELISAs enables appropriate prediction of relapses in patients with WG, rises in ANCA levels were related to the occurrence of relapse in individual patients. A rise had to occur in consecutive samples, that is, within a period of 3 months. As the samples

Table 2 Overall test characteristics

Test variables	PR3-hn	PR3-c	PR3-hr	PR3-hn-hr
AUC	0.975	0.961	0.982	0.984
95% CI	0.961–0.989	0.938–0.985	0.966–0.997	0.968–1.001
Maximal sum of sensitivity and specificity (cut-off*)	183.6% (5.1)	184.5% (6.5)	193.7% (6.8)	193.8% (18.4)
Sensitivity at a specificity of 98% (cut-off)	83.3% (11.0)	81.1% (40.1)	94.7% (5.0)	95.5% (11.0)
Sensitivity at a specificity of 99% (cut-off)	80.3% (15.1)	76.5% (64.8)	94.7% (6.8)	94.7% (16.1)

AUC, area under the curve; hn, human native; c, capture; hr, human recombinant; PR3, proteinase 3.
*Cut-off values are presented (between brackets) in relative units/ml.

were gathered during the 12 months before relapse, rises could occur in any of the four periods involved. Relevant increases in PR3-ANCA levels were determined based on ROC curve analyses and the definition of an increase may differ because the standard curves for calculating relative units differ between distinct assays. These increases were 100% (anti-PR3-hn and anti-PR3-c ELISA), 150% (anti-PR3-hr ELISA) and 75% (anti-PR3-hn-hr ELISA). As concluded from the odds ratios (ORs) the anti-PR3-c ELISA (OR = 12.5) performs best in predicting relapses (table 5). The novel anti-PR3-hn-hr ELISA performance is slightly less predictive (OR = 8.9), but better than the other two direct ELISAs.

DISCUSSION

We have evaluated a novel PR3-ANCA ELISA, applying a mixture of hn-PR3 and hr-PR3 as coating, in patient samples that were identified as having C-ANCA by IIF. Compared with commercially available direct and capture ELISAs, the novel anti-PR3-hn-hr ELISA reveals a significant improvement in sensitivity. The addition of hn-PR3 does not seem to improve the test performance with respect to sensitivity and specificity (tables 2 and 3). In daily practice, however, it should be noted that the ELISA with only hr-PR3 revealed rather low OD values (data not shown). The mixture with hn-PR3 resulted in a more robust assay as required in routine laboratories. In addition, the anti-PR3-hn-hr ELISA also performs better in predicting clinical relapses based on rises in ANCA levels, when compared with other direct ANCA assays, including the anti-PR3-hr ELISA.

Because capture ELISAs better preserve the conformation of PR3, they are considered superior for PR3-ANCA detection.[6] There are, however, two major drawbacks of capture ELISAs: (1) the potential covering of important epitopes by the capturing mAb, and (2) in follow-up studies, high OD values

Table 3 Sensitivities in the distinct AAV cohorts

Panel	n (C-ANCA positive)	PR3-hn	PR3-c	PR3-hr	PR3-hn-hr
NCGN (renal biopsy)	58	53 (91.4%)*	50 (86.2%)	55 (94.8%)	55 (94.8%)
AAV (outpatient)	35	29 (82.9%)	26 (74.3%)	33 (94.3%)	33 (94.3%)
WG	47	31 (66.0%)	30 (63.8%)	44 (93.6%)	44 (93.6%)

AAV, ANCA-associated vasculitis; ANCA, anti-neutrophil cytoplasmic antibody; hn, human native; hr, human recombinant; NCGN, necrotising crescentic glomerulonephritis; PR3, proteinase 3; WG, Wegener's granulomatosis.
*Results are presented as number of patients positive in the respective test and (between brackets) the percentage within C-ANCA-positive patients. All data are based on a cut-off defined at 99% specificity for the total cohort of disease controls.

Table 4 Specificities in the distinct control cohorts

Panel	n	Patients with false positive results (n)	PR3-hn	PR3-c	PR3-hr	PR3-hn-hr
RA	230	7	2 (99.1%)*	5 (97.8%)	2 (99.1%)	2 (99.1%)
SLE	100	3	1 (99.0%)	0 (100.0%)	2 (98.0%)	0 (100.0%)
SS	200	5	3 (98.5%)	1 (99.5%)	2 (99.0%)	3 (98.5%)
HC	429	11	4 (99.1%)	7 (98.4%)	4 (99.1%)	5 (98.8%)
Non-AAV	55	0	0 (100.0%)	0 (100.0%)	0 (100.0%)	0 (100.0%)

AAV, ANCA-associated vasculitis; ANCA, antineutrophil cytoplasmic antibody; HC, healthy controls; hn, human native; hr, human recombinant; non-AAV, vasculitides other than AAV; PR3, proteinase 3; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome.
*Results are presented as number of patients positive in the respective test and (between brackets) the percentage of the respective controls. All data are based on a cut-off defined at 99% specificity for the total cohort of disease controls.

and ensuing unit values are often found requiring additional dilutions to obtain reliable ANCA levels (vide infra). Recent new technologies, including fluorescent-enzyme immunoassays and multiplex bead arrays, all have applied direct coating to the carrier structure.[16 17] Therefore, it is not surprising that these assays perform similar to direct ELISAs. Also recombinant PR3 has not been successfully introduced in clinical practice due to problems with expression and purification procedures.[20] A direct ELISA using recombinant, proteolytically inactive PR3 produced in the baculovirus expression system, appeared less sensitive than a direct ELISA using native PR3.[21] Recombinant PR3, however, is preferentially expressed in human cell lines that constitutively secrete the wild-type precursor, but are unable to generate active PR3 in vivo.[8 22] Purification of the wild-type pro-enzyme is facilitated by a C-terminal poly-histidine tag. These modifications have been applied in our anti-PR3-hn-hr ELISA. Although the poly-histidine tag was not purposely used for coating, another recently developed ELISA applies a bridging molecule for binding native PR3 to the solid phase.[23] This new "anchor ELISA", shares a similar high sensitivity with our anti-PR3-hn-hr ELISA.

The increased sensitivity of the novel anti-PR3-hn-hr ELISA was less apparent in the renal biopsy cohort. This is in line with previous evaluations of PR3-ANCA assays in the renal biopsy cohort.[16 17] The majority of these patients had generalised AAV, as can be expected, because renal-limited disease occurs predominantly in patients with MPO-ANCA.[24] Patients with generalised AAV might have increased antibody levels and/or more epitope spreading as compared with patients with more limited forms of AAV. Interestingly, also in the "anchor ELISA" the increased sensitivity was most apparent in patients with WG with only upper airways involvement.[23] Apparently, both assays share the high sensitivity of C-ANCA in IIF, and have the advantage of being PR3-specific. Most important, the increased sensitivity is not at the cost of specificity.

To predict relapses by rises in ANCA levels, we have previously compared methods with the same sample cohort as in the current study.[15–17] The anti-PR3-c ELISA has the best performance. The novel anti-PR3-hn-hr ELISA, together with another commercially available capture ELISA,[15] is second best. Capture ELISAs, however, have the disadvantage that they use mouse mAb for capturing the PR3 antigen. In particular, patients that receive mouse antihuman antibody treatment, may produce human antimouse (HAMA) or human anti-chimeric antibodies (HACA) and, therefore, present with falsely elevated ANCA levels. Although routine treatment of patients with AAV consists of cyclophosphamide and prednisolone,

Table 5 Prediction of clinical relapses by rises in PR3-ANCA in consecutive serial samples

Test characteristics	Anti-PR3-hn	Anti-PR3-c	Anti-PR3-hr	Anti-PR3-hn-hr
Rises in relapses patients (n = 23)	16	20	14	19
Rises in non-relapse patients (n = 23)	8	8	5	8
Sensitivity (%)	70	87	61	83
Specificity (%)	65	65	78	65
PPV (%)	67	71	74	70
NPV (%)	68	83	67	79
OR (95% CI)	4.3 (1.2 to 14.7)	12.5 (2.8 to 55.3)	5.6 (1.5 to 20.5)	8.9 (2.2 to 35.3)
p Value	0.038	0.001	0.016	0.002

c, capture; hn, human native; hr, human recombinant; NPV, negative predictive value; PPV, positive predictive value; PR3, proteinase 3.

clinical trials with biologicals are ongoing.[25] As already discussed,[16] another disadvantage of capture ELISAs is the limited range of the standard curve, indicating that multiple dilutions have to be tested for optimal quantification.

In conclusion, our novel anti-PR3-hn-hr ELISA has a very high sensitivity for the detection of PR3-ANCA in patients with AAV with C-ANCA. This increased sensitivity is most apparent in patients with limited disease. Second, this assay enables prediction of clinical relapses, although the positive predictive value remains too low to warrant treatment based only on ANCA levels.[26] Significant increases should prompt the clinician to monitor more closely the condition of the patient. Altogether, these test characteristics challenge the dogma that PR3-ANCA capture ELISAs are superior for diagnosis and follow-up.

Competing interests: CD, AR, CP and LK all are employees of EUROIMMUN AG, Lübeck Germany. W Schlumberger and W. Stöcker are board members of EUROIMMUN AG. The other authors have declared no conflict of interest.

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