Kidney International, Vol. 53 (1998), pp. 743–753

Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis

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Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis. Anti-neutrophil cytoplasmic antibodies (ANCA) are widely used as diagnostic markers for Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), Churg-Strauss syndrome (CSS) and idiopathic rapidly progressive glomerulonephritis (iRPGN). The objective of this study was to evaluate the diagnostic value of ANCA measurement by the indirect immunofluorescence (IIF) test, and by anti-PR3 and anti-MPO ELISA performed in different locations, in patients with idiopathic small vessel vasculitis. Fourteen centers participated in a standardization study of ANCA assays, and entered a total number of 169 newly diagnosed and 189 historical patients with idiopathic systemic vasculitis or iRPGN. Patients were classified according to a pre-defined diagnostic classification system. Results were compared with those of 184 disease controls and 740 healthy controls. The IIF test was performed according to standard methodology; ELISAs had been standardized among the participants in a previous phase of the study. The sensitivities of assays in patients were as follows. The sensitivity in WG was: cANCA 64%, pANCA 21%, anti-PR3 66%, anti-MPO 24%. In MPA the sensitivity was: cANCA 23%, pANCA 58%, anti-PR3 26%, anti-MPO 58%. Sensitivity in iRPGN was: cANCA 36%, pANCA 45%, anti-PR3 50%, anti-MPO 64%. The specificity of assays (related to disease controls) was: cANCA 95%, pANCA 81%, anti-PR3 87%, anti-MPO 91%. When the results of the IIF test were combined with those of the ELISAs (cANCA/anti-PR3 positive, pANCA/anti-MPO positive), the diagnostic specificity increased to 99%. The sensitivity of the combination of cANCA + anti-PR3 or pANCA + anti-MPO for WG, MPA or iRPGN was 73%, 67% and 82%, respectively. From this study we conclude that the value of the IIF test for ANCA detection can be greatly increased by the addition of a well standardized antigen-specific ELISA. In a significant number of patients with idiopathic small vessel vasculitis, however, the ANCA test results (either in IIF or ELISA) are negative.

Anti-neutrophil cytoplasmic antibodies (ANCA) are now widely used as diagnostic markers for several forms of idiopathic systemic vasculitides and idiopathic rapidly progressive glomerulonephritis (iRPGN) [reviewed in 1, 2]. An indirect immunofluorescence test (IIF test) with ethanol-fixed neutrophils can be used to discriminate a cytoplasmic (cANCA) and a perinuclear (pANCA) staining pattern. The major target antigens for ANCA in idiopathic vasculitis have been characterized as enzymes present in the granules of the neutrophil. The cANCA pattern is strongly associated with antibodies against proteinase-3 (PR3) [3], and the pANCA pattern with a number of enzymes, among which myeloperoxidase (MPO) is the most frequent (others are lactoferrin, elastase, cathepsin-G, and bactericidal/permeability-increasing protein, some of which may cause an atypical staining pattern of neutrophils as well) [4–8].

A precise definition of the clinical value of ANCA has proven difficult. First, the unknown etiology of the systemic vasculitides has given rise to continuous debate about their classification. Without uniform classification, the diagnostic value of ANCA cannot be determined, and using ANCA as a disease-specific diagnostic criterion seems premature. Second, ANCA assays have not been standardized between laboratories. Standard procedures for the methodology and nomenclature of the IIF test for ANCA detection have been defined during ANCA workshops [9, 10]. A disadvantage of the IIF test for ANCA detection is that the assay is not antigen-specific. Characterization of the target molecules of ANCA makes antigen-specific detection in solid phase assays possible, but such assays have not been standardized until recently, and their clinical value has not been evaluated in a multi-center setting.

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[2] **See Editorial by Jennette, Wilkman, and Falk, p. 796.**

Key words: anti-neutrophil cytoplasmic antibodies, systemic vasculitis, ELISA, immunofluorescence test, proteinase-3, myeloperoxidase, Wegener's granulomatosis, microscopic polyangiitis, Churg-Strauss syndrome, rapidly progressive glomerulonephritis.

Received for publication August 5, 1997
and in revised form October 2, 1997
Accepted for publication October 2, 1997

The "European Commission/Measurement and Testing (DG XII, Science, Research and Development) (EC/BCR) Project for ANCA Assay Standardisation" is an international multi-center study that developed and standardized solid phase assays for ANCA detection. Over the past four years we have established standardized ELISAs for the detection of anti-PR3 and anti-MPO antibodies. The results of these development and standardization studies, which have defined the methodology and validation of these assays (and not sensitivity and specificity), have been reported elsewhere [11, 12].

The objective of the present study was to evaluate the diagnostic value of ANCA measurement by IIF tests and by antigen specific ELISAs in patients with idiopathic systemic vasculitis [Wegner's granulomatosis (WG), microscopic polyangiitis (MPA), classical polyarteritis nodosa (classical PAN) and Churg-Strauss syndrome (CSS)], classified according to the definitions of the Chapel Hill consensus conference on the nomenclature of systemic vasculitides [13]. For this purpose extensive clinical and histological data were collected from vasculitis patients, from clinically related disease controls and from healthy controls.

METHODS

Thirteen clinical centers with research laboratory facilities participated in the collection of patient data, biopsies and sera. Of the clinical centers, nine were previously involved in ANCA research. In addition, one commercial laboratory and one state reference laboratory participated in the serological study.

Patients

Each center was asked to include the last 20 consecutive patients with idiopathic systemic vasculitis seen in their center before June 1, 1991. These patients will be referred to as "historical patients." In addition, the first 15 consecutive patients with a similar systemic vasculitis, newly diagnosed or referred for the first time, after June 1, 1991, were included as "new patients." Patients fulfilling one of the following descriptions were included into the study.

(*1.*) Patients with clinically suspected WG, no histology required.

(*2.*) Patients with iRPGN, renal histology (including immunofluorescence) required. No underlying disease present.

(*3.*) Patients with histologically proven small vessel vasculitis and crescentic glomerulonephritis (MPA), histology required (renal or other site of vasculitis, negative or pauci-immune fluorescence).

(*4.*) Patients with classical PAN, CSS, polyangiitis overlap syndrome or unspecified small/intermediate sized artery vasculitis, histology required. Underlying diseases such as systemic lupus erythematosus, rheumatoid arthritis, mixed essential cryoglobulinemia, malignancy, serum sickness, drug induced vasculitis, lymphomatoid granulomatosis, Behçet's disease, Henoch-Schönlein purpura, and relapsing polychondritis were excluded.

To avoid a bias towards a positive test result for ANCA, patients were selected on clinical and histological criteria only, not on serology. The result of an ANCA test was not taken into account for entry of patients. To avoid a bias in severity of disease, all consecutive patients with clinically suspected WG were included, even if the classical histological entities (granulomatous vasculitis and/or glomerulonephritis) were not present in a biopsy.

Because ANCA serology may become negative after the initiation of immunosuppressive therapy, a separate analysis was performed for patients who had not received any therapy before the serum was drawn versus patients who did have immunosuppressive therapy.

Controls

Two groups of controls were recruited.

Disease controls. A list of possible diagnoses for disease controls was provided, included secondary vasculitis, various kinds of glomerulonephritis or granulomatous disease. The patients were selected on clinical criteria and not on ANCA result. The diagnoses in these patients was based on solid clinical evidence, preferably with histology. Fifteen consecutive patients were included from each center.

Healthy controls Each center included 50 healthy controls, with sera derived from the local blood transfusion service. The first 35 healthy controls of each center were age and sex matched with vasculitis patients entered from that center. The rest were random donor samples.

Patient data collection

For each subject entered in the study, a patient record book was completed by the participating center, including age, sex, diagnosis and date of diagnosis. For vasculitis patients, data collection at entry consisted of clinical symptoms and signs of disease activity, laboratory data including autoimmune and viral serology, radiology data, histology data, data on active infections and therapy, including the time interval between the initiation of immunosuppressive drugs and the date the test-serum was drawn. For historical patients, an accumulation of clinical and histological data available from the period between the date of diagnosis (usually after 1989) and the date of entry in the study was scored.

Classification of patients

After entry of patients according to the above entry criteria, all patients were classified based on data from the record books. For patient classification a system was designed based on the diagnostic names and definitions adopted by the "Chapel Hill consensus conference on the nomenclature of systemic vasculitides" [13].

The following diagnostic groups were formed:

Wegener's granulomatosis. The first group, Wegner's granulomatosis, was divided into the following subgroups: (*a*) histologically proven vasculitis with granuloma and/or giant cells in a biopsy (with or without nephritis); (*b*) patients with clinical evidence of at least one airway symptom or sign, compatible with WG (pulmonary nodules or fixed infiltrates, sinusitis, purulent or bloody discharge from the nose, saddle nose, otitis media, orbital pseudotumor, tracheal stenosis). Renal histology in these patients showed crescentic and/or necrotizing glomerulonephritis with few or no immune deposits. (*c*) Patients with clinical evidence of airway symptoms compatible with WG (see b), but with a histology of any organ showing vasculitis with no evidence for glomerulonephritis. (*d*) Patients had clinical evidence of airway symptoms compatible with WG (see b). No histological evidence of the vasculitis nature of the disease was available.

Microscopic polyangiitis. Patients in this group had histologically-proven crescentic and/or necrotizing glomerulonephritis with few or no immune deposits, or had histologically proven vasculitis

of small vessels. Systemic manifestations of disease were compatible with vasculitis. No airway symptoms were compatible with WG (see above). All patients were hepatitis B antigen negative.

Idiopathic rapidly progressive glomerulonephritis. Patients with histologically-proven crescentic and/or necrotizing glomerulonephritis with few or no immune deposits, without systemic disease manifestations comprised this group.

Classical polyarteritis nodosa (classical PAN). Patients in this group had proof of arterial vasculitis (either by angiography or by biopsy). Criteria of small vessel vasculitis at any biopsy location (according to Jennette et al [13]) or crescentic glomerulonephritis at renal biopsy moved the patient into a diagnosis of MPA. All patients were hepatitis B antigen negative, and none had pulmonary involvement.

Churg-Strauss syndrome. Patients with histologically proven vasculitis or crescentic glomerulonephritis or giant cells/granuloma formation in combination with asthma and eosinophilia comprised this group.

Pathology review

Renal biopsies. Sections from renal biopsies were reviewed by a team of four pathologists. Each biopsy was scored independently by two pathologists according to a standard protocol [14]. The following items were scored: extra-capillary proliferation, fibrinoid necrosis of the glomerular tuft, vasculitis of interstitial vessels, interstitial granulomas. In case of discrepancies between the data provided by the reviewing pathologists and those in the patient record books, the reviewers' data were used.

Respiratory tract biopsies. All respiratory tract biopsies available were centrally reviewed by two pathologists. The presence of vasculitis and of granulomas and/or giant cells was used for classification of the patients. In case of discrepancies between the data provided by the reviewing pathologists and those in the patient record books, the reviewers' data were used.

Muscle biopsies. Muscle biopsies were reviewed by one pathologist. The presence of vasculitis and the size of the involved blood vessels were scored. There were no discrepancies between the reviewer and the record book data with respect to the presence or absence of vasculitis, but additional data were added with regard to vessel size.

Serum collection and storage

For each patient entered in the study, a 4 ml serum sample was drawn at the time of entry. From historical patients an additional sample was requested, if available, preferably from the date of the initial diagnosis. One sample of 2 ml, was sent to the coordinating center (Leiden) for central storage (−20°C) and testing; one sample was stored locally at −20°C until testing. All samples were sent in frozen condition.

Indirect immunofluorescence test

Guidelines for the methodology of the IIF test were provided to all participating centers [15], but were not obligatory. Earlier investigations in this project indicated that results with the locally used IIF methodology were comparable between centers with respect to the ANCA pattern scored [12]. All centers used an IgG-specific FITC-conjugate. The IIF test was scored as cytoplasmic staining (cANCA), perinuclear staining (pANCA), atypical, or negative staining using the cut-off screening dilution routinely used in each laboratory. Atypical and negative staining were combined as negative for specificity calculations. Because titration results between laboratories showed incomparable results in an earlier phase of this project [11], titration of sera was not performed.

ELISAs for anti-PR3 and anti-MPO antibodies

Sera were tested for the presence of anti-PR3 and anti-MPO antibodies in an ELISA format. For anti-PR3 antibody detection, three antigens, isolated from human neutrophils by three different methods, were used (Copenhagen PR3, Raisdorf PR3, and Leiden PR3) [11]. For anti-MPO detection one antigen was used (Copenhagen MPO). ELISAs were performed in a standardized manner that previously had shown acceptable results and reproducibly low variation between the participating laboratories (for anti-PR3 assays: intra-assay coefficient of variability (CV) between 12% and 14%, intra-center CV between 15% and 22%, between centers CV between 16% and 20%; anti-MPO assay: intra-assay CV 8%, intra-center CV 21%, between centers CV 34%) [12].

Sera were tested at an initial dilution of 1:50 and the optical density (OD) value was compared to a 10 point standard curve included on each ELISA plate. If the optical density was above 90% of the maximal optical density of the standard curve, a serial dilution was performed. Values were expressed as units/ml derived from the standard curve. All calculations were performed centrally in the co-ordination center (for further details see [12]). Cut-off values for assay positivity were derived from receiver operating characteristic curves, displaying sensitivity versus 1-specificity for various cut-off points. Receiver operating characteristic (ROC) curves were calculated for each ELISA, comparing results of sera from new patients (WG, MPA, iRPGN, classical PAN, CSS) with those of the disease controls and the healthy controls.

Re-testing

Quality control was performed by re-testing 273 sera in three antigen providing laboratories (Copenhagen, Raisdorf and Leiden). The re-tested samples consisted of 108 new patient sera, 97 historical patient sera, 20 healthy control sera and 12 disease control sera. The sera from each patient category were randomly selected. The re-test results in the central laboratories were compared with the results obtained from the local centers.

Double positive sera

Sera that were recorded as positive in at least one of the three anti-PR3 assay and in the anti-MPO assay ("double positive sera") were re-tested centrally in Leiden in the Leiden anti-PR3 assay and the anti-MPO assay. Fluid phase inhibition experiments were done on all double positive sera. Sera were pre-incubated with PR3-Leiden (10 μg/ml) and MPO-Copenhagen (50 μg/ml) for 30 minutes at 37°C. These antigen concentrations were determined by dose response experiments as being the concentration of antigen that induced maximal ($> 80\%$) inhibition of the positive standard serum at a 1:50 dilution. The further ELISA methods were similar to that described earlier. Inhibition was considered positive if the difference in units/ml was $> 10\%$ between the inhibited and uninhibited assay. In addition, all these sera were tested for non-specific binding by testing on plates coated with human serum albumin instead of antigen.

Double positivity for anti-PR3 and anti-MPO was confirmed by immunoprecipitation experiments. Immunoprecipitation of ^{125}I-labeled PR3 (Leiden) and ^{125}I-labeled MPO (Copenhagen) was

Table 1. Patient characteristics

	New patients	Historical patients	Disease controls	Healthy controls
Number included in study	169	189	184	740
Serum available	169	106	184	740
Age (years) median (*range*)	59 (15–86)	59 (17–90)	46 (7–90)	47 (15–86)
Male: female ratio	1.1:1	1.5:1	1:1.3	1:1.1

	New patients		Historical patients	
Diagnosis	No therapy	All	No therapy	All
All diagnoses	126	169	59	106
Wegener's granulomatosis (all)	73	97	37	75
a WG, granulomas in biopsy	21	31	13	26
b WG, glomerulonephritis in biopsy	37	44	14	28
c WG, vasculitis in biopsy, no GN	7	9	4	8
d WG, no histology support	8	13	6	13
Microscopic polyangiitis	34	44	13	19
Idiopathic RPGN	10	12	3	6
Classical polyarteritis nodosa	5	10	3	3
Churg-Strauss syndrome	4	6	3	3

Abbreviations are: WG, Wegener's granulomatosus; RPGN, rapidly progressive glomerlulonephritis.

performed to further establish the reactivity of double positive sera. To this end replicate tubes containing 200,000 counts per minute of ^{125}I-labeled PR3 or ^{125}I-labeled MPO in phosphate buffered saline (PBS) were incubated with 25 μl dilutions of patient or control sera for two hours at 4°C. This was followed by addition of 10 μl of a 10% suspension of protein G-sepharose (Pharmacia). After incubation for one hour at 4°C, the mixtures were centrifuged and washed extensively with PBS. The final pellet was re-suspended in 50 μl PBS and 75 μl SDS sample buffer and boiled for three minutes. One hundred microliters of supernatant was subsequently subjected to SDS-PAGE analysis. After drying autoradiograms of the gels were made, and analyzed for specific immunoprecipitation. As controls, sera known to react solely with PR3 and MPO were used as well. Anti-PR3 sera exhibited specific precipitation of ^{125}I-labeled PR3 and no precipitation of ^{125}I-labeled MPO while anti-MPO sera only precipitated ^{125}I-labeled MPO.

RESULTS

Patients characteristics

Table 1 shows patient characteristics of the four groups of patients. A total number of 169 new patients were included in the study. A total number of 124 patients did not receive immunosuppressive drugs prior to entry in the study, 24 received short duration therapy (<21 days). Sera from all of these patients were available for evaluation. Of 189 historical patients, 106 sera were available for testing. Details of diagnoses are shown in Table 1.

A total number of 184 disease controls were included, histological confirmation was obtained in 141 (77%). The majority of these patients had ongoing (active) disease at the time the sample was drawn. Eighty-three disease controls received some sort of immunosuppressive therapy at the time the serum was drawn. Details of diagnoses are given in Table 2. The total number of healthy controls was 740.

Table 2. Disease control patients

Diagnosis	*N*
Temporal arteritis	13
Takayasu arteritis	3
Rheumatoid arthritis with vasculitis	7
Systemic lupus erythematosus	45
Mixed essential cryoglobulinemia	9
Henoch-Schönlein purpura	14
Other glomeruloephritis (GN)	
Membranous nephropathy	5
IgA nephropathy	13
MPGN	10
Minimal lesions	4
FSGS	2
Crescentic GN (not pauci-immune)	2
Chronic sclerosing GN	6
Immune-complex GN	1
Post-streptococcal GN	2
Anti-GBM disease	6
Tuberculosis	4
Sarcoidosis	14
Ulcerative colitis	7
Crohn's disease	6
Other vasculitides	
Serum sickness	1
Infective endocarditis	3
Visceral sepsis	1
Behçet's disease	3
Scleroderma/MCTD with vasculitis	3
Total	184

Table 3. Sensitivity and specificity of the indirect immunofluorescence test in patients with systemic vasculitis

		Sensitivity %		
	N	cANCA	pANCA	cANCA or pANCA
New patients				
Wegener's granulomatosis	97	62 (64)	20 (21)	82 (85)
Microscopic polyangiitis	44	10 (23)	26 (58)	36 (81)
Idiopathic RPGN	12	4 (36)	5 (45)	9 (81)
Classical polyarteritis nodosa	10	1 (10)	3 (30)	4 (40)
Churg-Strauss syndrome	6	2 (33)	2 (33)	4 (66)
Historical patients				
Wegener's granulomatosis	75	42 (56)	20 (27)	62 (83)
Microscopic polyangiitis	19	2 (13)	11 (69)	13 (82)
Idiopathic RPGN	6	0 (0)	4 (67)	4 (67)
Classical polyarteritis nodosa	3	0 (0)	0 (0)	0 (0)
Churg-Strauss syndrome	3	1 (33)	1 (33)	2 (66)
		Specificity %		
Control patients				
Disease controls	184	9 (95)	35 (81)	44 (76)
Healthy controls	740	15 (98)	30 (96)	45 (94)

A total of 212 renal biopsies were taken from the included vasculitis patients, of which 193 biopsies were available for review.

Indirect immunofluorescence test

Table 3 shows data obtained with the IIF test. The sensitivity of cANCA for WG in new patients was 64%. Perinuclear ANCA

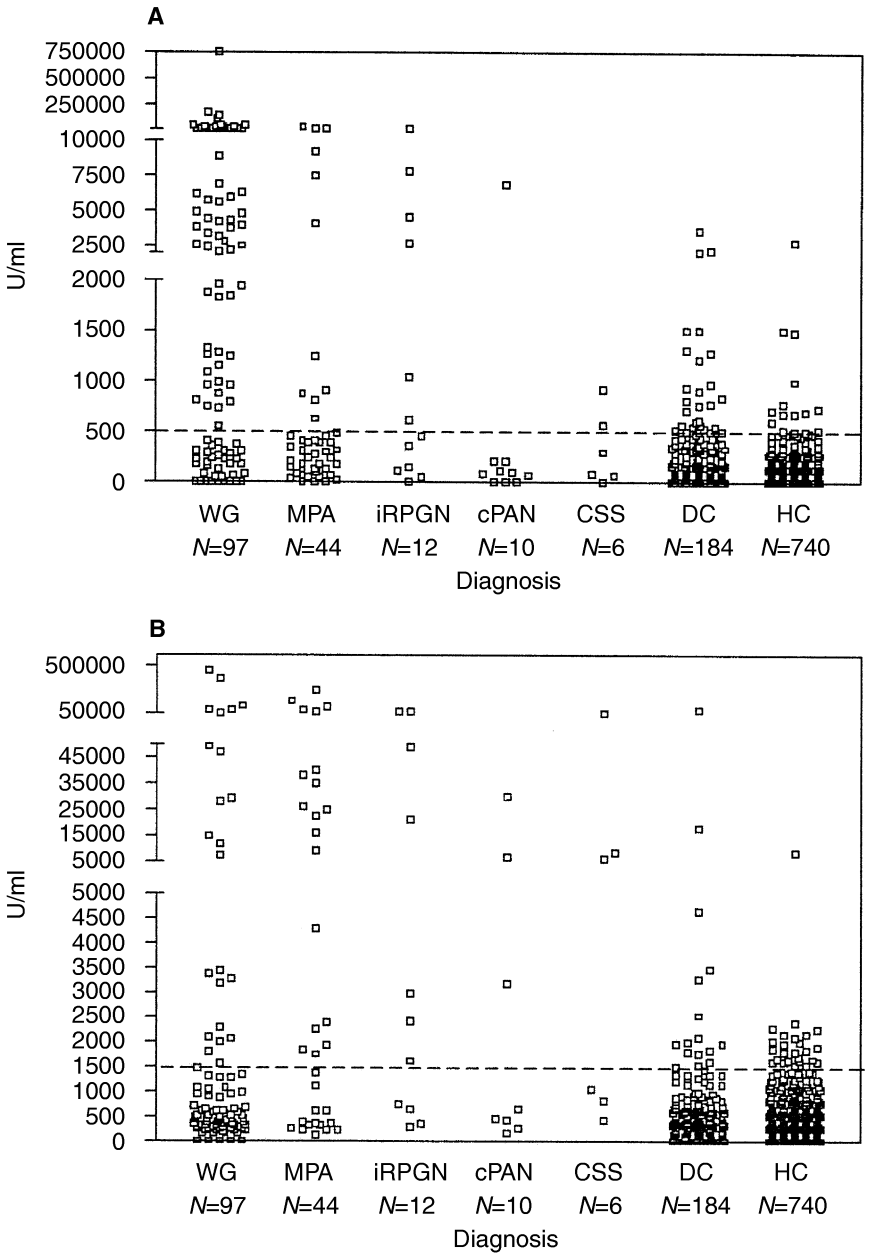

Fig. 1. ELISA results from all new patients obtained with the Copenhagen anti-PR3 ELISA (*A*) and anti-MPO ELISA (*B*). Results with anti-PR3 ELISAs from Leiden and Raisdorf were comparable to that obtained with the Copenhagen ELISA. Abbreviations are: WG, Wegener's granulomatosis; MPA, microscopic polyarteritis; iRPGN, idiopathic rapidly progressive glomerulonephritis; cPAN, classical polyarteritis nodosa; CSS, Churg-Strauss syndrome; DC, disease controls; HC, healthy controls.

(pANCA) were present in 21% of WG patients. The combination of either a cANCA or a pANCA therefore reached a sensitivity of 85%. The sensitivity for cANCA in WG patients with renal disease was similar to the WG patients without renal disease (both 64%). The pANCA was more prevalent in patients with histologically proven glomerulonephritis (30%) than in patients without renal disease (10%).

The pANCA were more prevalent than cANCA in MPA (58% vs. 23%) and iRPGN patients (45% vs. 36%). The sensitivity of cANCA + pANCA was 81% in MPA and 82% in iRPGN. Ten new patients with classical PAN were included, four were ANCA positive. Six CSS patients were included, four were ANCA positive.

The results obtained with sera from historical patients showed results comparable to those of new patients (Table 3). There was no significant difference in sensitivity between treated or untreated patients.

Of the disease controls 35 (19%) patients were positive for the pANCA pattern [15 SLE, 5 ulcerative colitis, 4 Crohn's disease, 3 temporal arteritis, 2 mixed cryoglobulinemia, 2 Goodpasture syndrome, 1 rheumatoid vasculitis, 1 Henoch-Schönlein purpura (HSP), 1 sarcoid and 1 IgA nephropathy patient]. Eight patients had a cANCA pattern (2 SLE, 2 sarcoid, 1 temporal arteritis, 1 HSP, 1 membraneous nephropathy, 1 IgA nephropathy). Of the healthy control sera 2% were recorded as cANCA positive, 4% as pANCA positive, resulting in a specificity of the IIF test (cANCA and pANCA together) of 76% towards disease controls and 94% towards healthy controls.

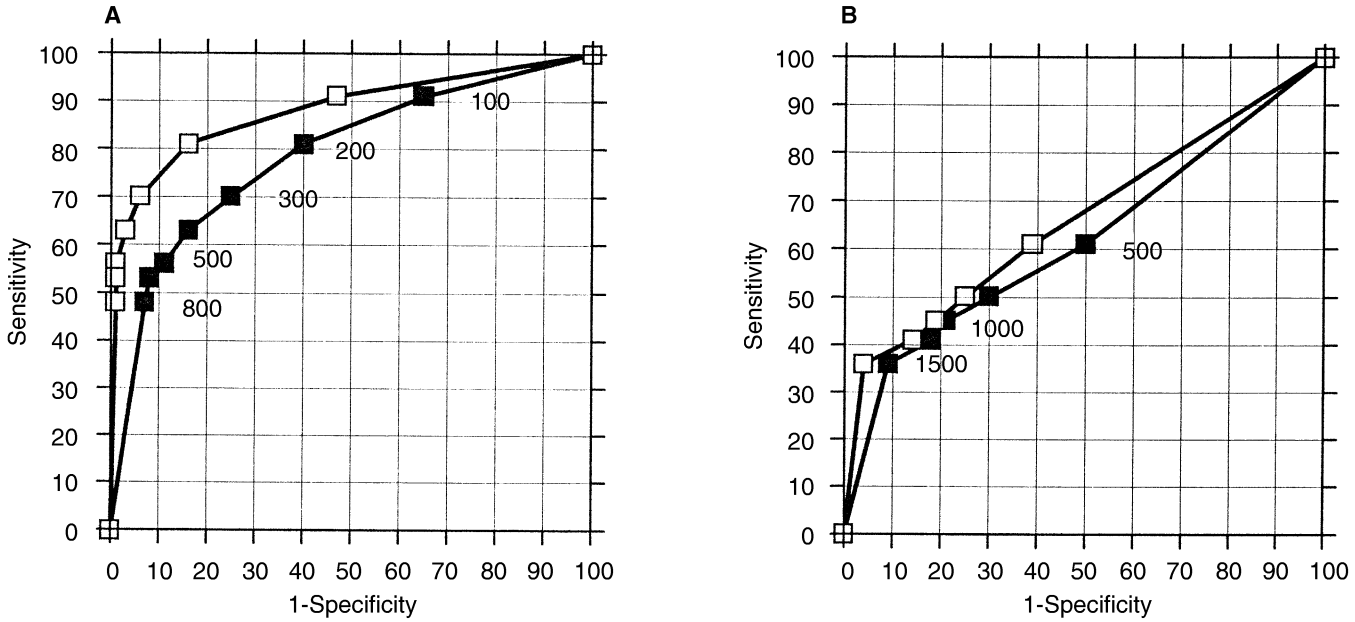

Fig. 2. Receiver operating characteristic (ROC) curves of Raisdorf ELISA for the detection of anti-PR3 antibodies and the anti-MPO ELISA. Symbols are: (□) values for healthy controls; (■) disease controls. (*A*) Anti-PR3 ELISA. (*B*) Anti-MPO ELISA. Each point represents the relation between sensitivity and 1-specificity for one cut-off value. The results obtained with the Copenhagen and Leiden anti-PR3 assays were comparable with the Raisdorf anti-PR3 assay. Abbreviations are: DC, disease control, HC, healthy control.

Table 4. Sensitivity and specificity of anti-PR3 and anti-MPO ELISAs in patients with systemic vasculitis, disease controls and healthy controls

		Sensitivity %			
		Anti-proteinase 3			
	N	Copenhagen	Raisdorf	Leiden	Anti-myeloperoxidase
New Patients					
Wegener's granulomatosis	97	63 (65)	64 (66)	65 (67)	23 (24)
Microscopic polyangiitis	44	11 (25)	12 (27)	12 (27)	26 (58)
Idiopathic RPGN	12	6 (50)	7 (55)	7 (58)	8 (64)
Classical polyarteritis nodosa	10	1 (10)	12 (13)	2 (20)	4 (38)
Churg-Strauss syndrome	6	2 (33)	2 (33)	1 (17)	3 (50)
Historical patients					
Wegener's granulomatosis	75	41 (55)	43 (58)	47 (63)	22 (29)
Microscopic polyangiitis	19	5 (26)	9 (46)	5 (26)	10 (54)
Idiopathic RPGN	6	2 (33)	1 (20)	1 (17)	5 (80)
Classical polyarteritis nodosa	3	1 (33)	0 (0)	0 (0)	0 (0)
Churg-Strauss syndrome	3	1 (33)	3 (100)	2 (67)	0 (0)
		Specificity %			
Control patients					
Disease controls	184	26 (86)	20 (89)	24 (87)	17 (91)
Healthy controls	740	15 (98)	7 (99)	15 (98)	30 (96)

Cut-off values were 500 U/ml for anti-PR3 assays and 1500 U/ml for anti-MPO assay. RPGN is rapidly progressive glomerulonephritis. New patients were newly diagnosed after the start of the study, historical patients were analyzed retrospectively.

Atypical fluorescence, defined as fluorescence of neutrophils that does not resemble the typical cytoplasmic or (peri)nuclear pattern, was considered negative for analysis.

ELISA results

Figure 1 shows the results of all evaluable sera from new patients in the anti-PR3 ELISA (Copenhagen) and in the anti-MPO ELISA. There were large differences in the concentration of antibodies against PR3 and MPO in the patients as measured by ELISA. Disease controls were never more than tenfold higher than the normal limit in anti-PR3 assays, and (with one exception) in the anti-MPO assay.

To ensure optimal sensitivity and specificity, receiver operating characteristic (ROC) curves were calculated for each ELISA (Fig. 2). A specificity approximating 90% towards disease control patients was considered the minimum required. This corresponded to a cut-off point between "normal" and "abnormal" values of 500 U/ml for the anti-PR3 assays, and 1500 U/ml for the

anti-MPO assay, as compared with the anti-PR3 and anti-MPO standard sera used (these were both set at an arbitrary level of 10,000 U/ml).

Wegener's granulomatosis. The sensitivity of anti-PR3 antibodies using the above mentioned normal limits for new patients with WG was 65 to 67% (Table 4). The sensitivity in patients with classical histology (granulomas) as compared to those with the clinical picture of WG, but with histological evidence only of vasculitis or crescentic nephritis, was similar (data not shown). Anti-MPO antibodies were present in 24% of all WG patients. Patients with biopsy-proven crescentic glomerulonephritis had anti-MPO more frequently than patients without renal disease (32% vs. 18%). Therapy with immunosuppression did not change the sensitivity of the anti-PR3 or anti-MPO assay. There was no difference between historical and new patients (Table 4).

Microscopic polyangiitis. A total of 58% of new patients with MPA had anti-MPO antibodies, and 25 to 27% had anti-PR3 antibodies. The percentages in historical patients were similar, but immunosuppressive therapy did not change the sensitivity (Table 4).

Idiopathic pauci-immune crescentic glomerulonephritis. Anti-PR3 antibodies were present in 45% to 55% of patients, anti-MPO antibodies were present in 64% (Table 4).

Classical PAN and Churg-Strauss syndrome. The number of patients with these diseases was low. Anti-PR3 antibodies were present in 1 of 10 new patients with classical PAN, and in 2 of 6 new patients with CSS. Anti-MPO were present in 4 of 10 new patients with classical PAN, 3 of 6 CSS patients were anti-MPO positive (Table 4).

Control subjects. The definition of the normal cut-off of the ELISAs was based on a specificity approximating 90% towards disease controls. This resulted in a specificity for the anti-PR3 assays of 86%, 87% and 89%, respectively, and for the anti-MPO assay of 91%. Towards healthy controls, the specificity was 98% to 99% for the anti-PR3 assays and 96% for the anti-MPO assay. There was an equal distribution among diagnoses of disease controls who were positive for anti-PR3 and anti-MPO ANCA. Six of 45 (13%) of sera from patients with SLE nephritis were positive for anti-PR3 antibodies and 7 of 45 for anti-MPO antibodies (Fig. 3).

Combination of IIF and ELISA

The results from the IIF test were combined with the ELISA results (Table 5). For the combination of cANCA with anti-PR3 antibodies, the sensitivity for new WG patients was 56 to 58%, and for pANCA with anti-MPO it was 16%. For MPA, the sensitivity of the combined assays was 12 to 16% for cANCA/anti-PR3 and 49% for pANCA/anti-MPO. For iRPGN the sensitivity was 36% and 46%, respectively. The specificity towards disease control patients increased to 99%, towards healthy controls to 100% after combining the IIF test with ELISA. (Table 5).

Re-testing of sera

A total of 237 sera were re-tested in the central laboratories. The correlation coefficient for the results obtained in the center of origin and the central laboratory for the assays was 0.94 (Copenhagen PR-3), 0.83 (Raisdorf PR3), 0.84 (Leiden PR3) and 0.84 (MPO) ($P < 0.0001$ for all assays). There was no significant difference between the test and re-test results (paired *t*-test, $P = 0.48$ for Copenhagen anti-PR3 ELISA, $P = 0.66$ for Raisdorf anti-PR3 ELISA, $P = 0.37$ for Leiden anti-PR3 ELISA, $P = 0.48$ for the anti-MPO ELISA).

Double positive sera

Of 275 historical and new patient sera, 184 disease control sera and 740 healthy control sera tested (total 1204 sera), 32 were positive in both the anti-PR3 Copenhagen ELISA and the anti-MPO ELISA, 35 in the Raisdorf anti-PR3 and anti-MPO ELISA, and 33 in the Leiden anti-PR3 and anti-MPO ELISA. This means that about 15% of all anti-PR3 positive sera were double positive (20% of all anti-MPO positive sera). All these 100 sera were re-tested in the co-ordinating laboratory (Leiden), after which only ten showed persistent double reactivity. Seven of these 10 sera showed predominant positivity against MPO with low-positive anti-PR3 values. Two had high anti-PR3 titers with low-positive anti-MPO. One was strongly positive in both assays. Fluid phase inhibition ELISA showed inhibition of anti-PR3 and anti-MPO reactivity in eight out of these ten. Only one of these ten showed nonspecific binding to the ELISA plate. Results obtained with the double positive sera are listed in Table 6.

Of the ten double positive sera nine came from patients with idiopathic systemic vasculitis, and one from a patient with systemic lupus erythematosus (SLE). Two sera could not be inhibited by fluid phase antigen, one from an SLE patient, the other from a patient with classical PAN. All other patients had histological evidence of glomerulonephritis, two patients were classified as WG, five as MPA and one as idiopathic RPGN (Table 6). The SLE serum also showed reactivity with plates coated with human albumin instead of antigen (non-specific binding).

DISCUSSION

This study investigated the diagnostic value of the IIF test and newly developed and standardized ELISAs for the detection of ANCA in patients with idiopathic systemic vasculitis in an international multi-center setting. The results indicate that, in comparison with the sole usage of the IIF test for ANCA detection, the combination of the IIF test with antigen specific ELISAs for anti-PR3 and anti-MPO antibodies results in an approximately 10% lower sensitivity. However, with this combination the specificity increased to 98% towards disease control patients. Control patients were selected on the basis of diagnosis with clinical signs and symptoms reminiscent of the proband primary vasculitis patients, and thus sometimes posed differential diagnostic problems.

Early reports on the IIF test for ANCA detection showed a high sensitivity and specificity for active WG and MPA [16–19]. These conclusions were drawn on the basis of results obtained with the standard IIF test, often in combination with solid phase assays for ANCA detection using purified antigens or partially purified neutrophil extracts. Others have questioned the diagnostic value of ANCA. Davenport et al reported a low specificity (77%) of ANCA in patients with respiratory tract symptoms [20, 21]. Results from a prospective cohort study among rheumatology patients suggested that the sensitivity of cANCA for WG was even lower (28% in patients with ACR-criteria defined WG) [22]. However, the performance of the diagnostic criteria used in this study (ACR criteria [23]) must be questioned since 19 of 25 (76%) of diagnoses assigned were proven to be wrong during later follow-up. A recent meta-analysis of all published literature on the diagnostic value of cANCA for WG revealed an overall sensitivity

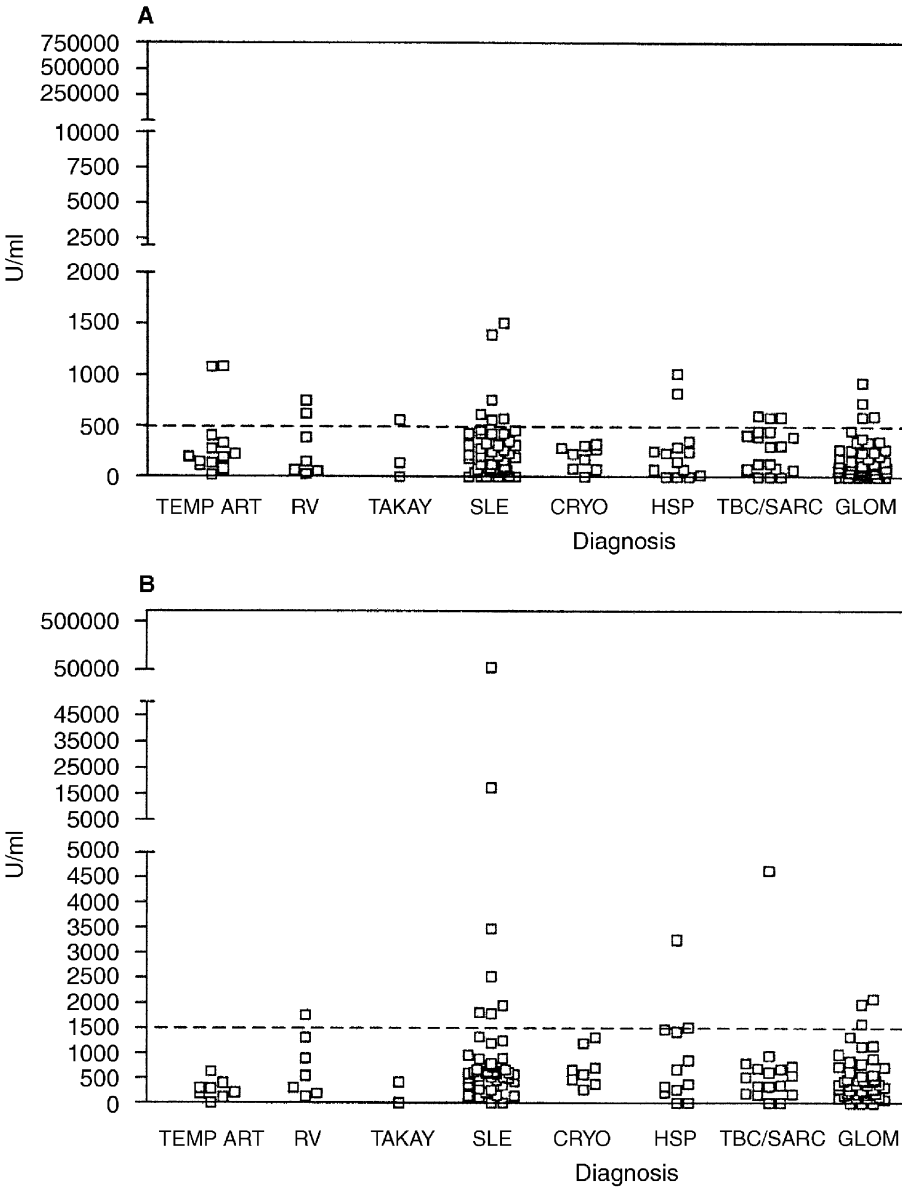

Fig. 3. Results of anti-PR3 and anti-MPO ELISA of disease control patients in (*A*) PR3, Leiden, and (*B*) MPO, Copenhagen. Abbreviations are: TEMP ART, temporal arteritit; RV, rheumatoid arthritis with vasculitis; TAKAY, Takayasu's disease; SLE, systemic lupus erythematosus; CRYO, essential mixed cryoglobulinemia; HSP, Henoch-Schönlein purpura; TBC/SARC, tuberculosis or sarcoid; GLOM, glomerulonephritis; other VASC, other kinds of vasculitis; IBD, inflammatory bowel disease. For patient numbers and further subclassifications, see Table 2.

of 66%, with values of up to 91% in patients with active disease [24]. The specificity of cANCA varied between 98% and 99%; however, the authors noted that large groups of individuals with a low pre-test probability for WG were used as controls [24].

We have tried to avoid several types of biases that could influence the results of the evaluation of ANCA assays. Selection of patients for severity of disease was avoided by including all consecutive patients. Referral bias of patients could not be prevented completely, because mainly specialized centers participated in this study, involving several subspecialties: internal medicine, nephrology, rheumatology and otolaryngology. Patients with WG often lack histological confirmation of the disease, and such patients were only included if the clinical picture was convincing. Patients were included on clinical grounds, not on the basis of ANCA results. All patients were subsequently classified using the names and definitions adopted from the "Chapel Hill consensus conference on vasculitis nomenclature." This classification was undertaken centrally, formally based upon objectively scored signs and symptoms, and upon centrally reviewed histology, in order to avoid differences in nomenclature between centers. All sera were tested in a blinded manner. In this way we tried to avoid some problems that have caused debate concerning the clinical value of ANCA in the past.

The identification of the target antigens most frequently recognized by ANCA in primary vasculitides has facilitated the development of solid phase assays [3, 19, 25–28]. A comparison of such assays at the start of this project showed comparable results among anti-MPO positive sera between centers, but large variability in results obtained with assays for the measurement of antibodies to cANCA-related antigen [11]. The main reason for this variability appeared to be differences in antigen purity and coating conditions. Therefore, a major point of this project was

Table 5. Sensitivity and specificity of the combination of IIF-test and ELISA results in patients with systemic vasculitis

		Sensitivity %				
		cANCA + anti-PR3			pANCA + anti-MPO	cANCA/PR3[a] or pANCA/MPO
	N	Copenhagen	Raisdorf	Leiden		
New patients						
Wegener's granulomatosis	97	55 (57)	56 (58)	54 (56)	16 (16)	71 (73)
Microscopic polyangiitis	44	7 (16)	5 (12)	7 (16)	22 (49)	30 (67)
Idiopathic RPGN	12	4 (36)	4 (36)	4 (36)	6 (46)	10 (82)
Classical polyarteritis nodosa	10	1 (10)	1 (10)	1 (10)	1 (10)	2 (20)
Churg-Strauss syndrome	6	2 (33)	2 (33)	0 (0)	2 (33)	3 (56)
Historical patients						
Wegener's granulomatosis	75	32 (43)	31 (41)	36 (48)	15 (19)	46 (62)
Microscopic polyangiitis	19	1 (5)	1 (6)	1 (6)	7 (36)	8 (42)
Idiopathic RPGN	6	0 (0)	0 (0)	0 (0)	4 (60)	4 (60)
Classical polyarteritis nodosa	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Churg-Strauss syndrome	3	1 (33)	0 (0)	1 (33)	0 (0)	1 (33)
		Specificity %				
Control patients						
Disease controls	184	1 (99)	1 (99)	1 (99)	2 (99)	3 (98)
Healthy controls	740	1 (100)	1 (100)	1 (100)	0 (100)	1 (100)

RPGN is rapidly progressive glomerulonephritis. New patients were newly diagnosed after the start of the study, old patients were analyzed retrospectively.

[a] Anti-PR3 result is average of the sensitivity of the 3 anti-PR3 ELISAs

Table 6. Results of consistently double positive sera

							Inhibition		Immuno-precipitation	
	Diagnosis	PR3-CO[a]	PR3-RS[a]	PR3-LE[a]	MPO[a]	IIF	PR3	MPO	PR3	MPO
1	WG	552	658	404	6956	pANCA	+	+	+	+
2	MPA	114	295	545	12729	atyp ANCA	+	+	±	−
3	MPA	519	597	378	5908	pANCA	+	+	−	+
4	MPA	332	533	495	15939	pANCA	+	+	−	+
5	MPA	1246	1566	599	263743	pANCA	+	+	−	+
6	MPA	349	563	545	25944	negative	+	+	nd	nd
7	WG	3804	3370	1971	2432	cANCA	+	+	−	−
8	iRPGN	4452	2532	1908	48621	pANCA	+	+	nd	nd
9	SLE[b]	556	893	313	17709	negative	−	−	nd	nd
10	classical PAN	6831	4963	2184	3178	cANCA	+	−	nd	nd

Sera were tested in ELISA and in the IIF-test. Inhibition was performed by fluid phase antigen (see text). All figures are in units/ml.

the isolation of purified PR3 for use in standardized assays. The results of the methods and standardization study have been reported elsewhere [12].

We confirm that ANCA detected by the IIF test (cANCA or pANCA) are a sensitive marker for WG, MPA and "pauci-immune" iRPGN, sometimes referred to as renal limited vasculitis with sensitivity scores ranging from 81% to 85%. The low specificity, however, (76%), which is mainly caused by positive pANCA in disease controls, is a strong disadvantage. pANCA in disease controls can be caused by the presence of anti-nuclear antibodies, especially in SLE patients. The use of ELISA using purified antigens as an alternative method could not improve the specificity without a significant loss in sensitivity. However, if the IIF test was combined with ELISA using PR3 and MPO, the specificity was improved to 98%, with a sensitivity loss of only 10%. Data from the retrospective part of this study showed comparable results to those of the prospective part of the study. In patients who had been on immunosuppressive therapy for more than three weeks, the sensitivity of the assays was only slightly lower than in patients with no or short duration therapy. The numbers of patients with classical PAN and CSS were too low to allow conclusions on the prevalence of ANCA in these types of vasculitis.

A significant number of sera were positive in both the anti-PR3 and the anti-MPO antibody assay. Most of these results were lower than twice the cut-off value. Re-testing of these sera in the co-ordinating laboratory indicated that only some double positive results could be confirmed. Antigen specific inhibition of most of these sera could be achieved by adding fluid phase antigen to the serum prior to incubation. It was less easy to demonstrate double positivity by immune precipitation analysis, presumably because the interaction in the fluid phase between PR3 and MPO with their respective antibodies may be different from the interaction with solid phase antigen. From this we can conclude that true double positivity is a rare event. However, in routine practice double positivity in ELISA is quite frequent, due to low positive results.

The use of disease controls with conditions that may show similarities to WG, MPA or iRPGN causes a drop in specificity compared to that observed with healthy controls for comparison

with the proband population. Since the only relevant control population is the former, cut-off values of the assays should not be defined on the basis of healthy donor values, but rather on values seen in the proper disease controls using ROC curve analysis, which allows selection of high diagnostic specificity over sensitivity.

From this study we conclude that direct ELISAs using purified PR3 are not in themselves more sensitive than the cANCA test. If a high specificity is selected for, cANCA is more specific at a comparable sensitivity level. However, by combining the IIF test with antigen specific ELISAs for PR3, ANCA serology remains a very specific tool for the diagnosis WG especially in newly diagnosed patients. The rather frequent occurrence of pANCA in Wegener's patients is somewhat unexpected, but may relate to the classification system used in this study. Patients with airway symptoms other than pulmonary hemorrhage were classified as WG, while in earlier studies some would have been diagnosed as MPA. The low specificity of pANCA alone was expected due to the known occurrence of pANCA in some of the disease control groups (rheumatoid arthritis, ulcerative colitis and infective endocarditis) as well as the presence of anti-nuclear antibodies in patients with systemic lupus erythematosus, which makes the IIF interpretation difficult. When positive pANCA was combined with presence of MPO-ANCA at a cut-off level of 1500 U/ml, a very high specificity for proband diagnoses was seen, both towards healthy controls and disease controls.

In conclusion, the value of ANCA tests for differential diagnosis of idiopathic necrotizing small vessel vasculitis can be optimized by combining two assay methods (the IIF test together with standardized antigen specific ELISAs choosing cut-off levels allowing a specificity of 90% towards disease controls). These measures are absolutely necessary to improve the diagnostic significance of ANCA, to heighten the quality of scientific communication regarding vasculitides and to avoid false interpretation of positive results and, hence, unnecessary therapeutic measures.

ACKNOWLEDGMENTS

This work was supported by grant no. 5383/1/6/352/90/07-BCR-NL [10] from the EC-BCR program, grant no. MAT1-CT92-0011 from the EC program "Measurement and Testing" and grant no. C94.1375 from the Dutch Kidney Foundation. Mr. C. Profilis is gratefully acknowledged for his continuing support from Brussels.

APPENDIX

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The following investigators participated in this study:

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Abbreviations

Abbreviations used in this article are: ANCA, anti-neutrophil cytoplasmic antibodies; cANCA, cytoplasmic or classical anti-neutrophil cytoplasmic antibodies; classical PAN, classical polyarteritis nodosa; CSS, Churg-Strauss syndrome; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; IIF test, indirect immunofluorescence test; iRPGN, idiopathic rapidly progressive glomerulonephritis; MPA, microscopic polyangiitis; MPO, myeloperoxidase; pANCA, perinuclear anti-neutrophil cytoplasmic antibodies; PR3, proteinase-3; WG, Wegener's granulomatosis; EC/BCR, European Commission/Measurement and Testing (DG XII, Science, Research and Development).

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