



Research paper

Evaluation of a multiplex flow cytometric immunoassay to detect PR3- and MPO-ANCA in active and treated vasculitis, and in inflammatory bowel disease (IBD)[☆]

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ABSTRACT

This study compared the performance of a flow cytometric immunoassay for antineutrophil cytoplasmic antibodies (ANCA) directed against proteinase 3 (PR3) and myeloperoxidase (MPO), with indirect immunofluorescence (IIF) and ELISAs from 12 different manufacturers. Sera were from patients with active ($n=55$) or treated ($n=68$) small vessel vasculitis, or inflammatory bowel disease (IBD, $n=22$).

The immunoassay specificity was 88% compared with 96% for IIF and 94% (median, range 91–96%) for both ELISAs. Its sensitivity in treated disease was 82% compared with 84% for IIF and 69% (median, range 57–82%) for the ELISAs. The immunoassay's specificity was 88% which was the same as the median for both ELISAs (range 84–95%). The PR3- and MPO-ANCA immunoassay was almost as sensitive as IIF, and more sensitive than, but just as specific as, most ELISAs, in detecting ANCA in active and treated vasculitis. A major advantage of this assay is its ability to be further modified to simultaneously screen for a panel of autoantibodies relevant to vasculitis.

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1. Introduction

The 'International Consensus Statement on Testing and Reporting Antineutrophil Cytoplasmic Antibodies (ANCA)' requires sera from patients with suspected small vessel vasculitis to be screened by indirect immunofluorescence (IIF) on ethanol-fixed neutrophils, and all IIF-positive sera to be confirmed in antigen-specific ELISAs (Savage et al., 1999).

The IIF assay is highly sensitive for ANCA but also labour-intensive, expensive, subjective and lacking in specificity (Savage et al., 1998). The direct and capture ELISAs are less sensitive and more specific than IIF for small vessel vasculitis (Csernok et al., 2002; Csernok et al., 2004), but their performance is varied in treated disease where antibody levels are low (Trevisin et al., 2008). The usefulness of both IIF and the ELISAs is further limited by their inability to demonstrate antibodies directed against minor ANCA antigens and other vasculitis-associated antibodies, including antglomerular basement membrane (GBM) antibodies. The early detection of both ANCA and antiGBM antibodies is critical in patients with rapidly progressive glomerulonephritis where renal function recovers with timely therapeutic intervention.

The multiplex flow cytometric immunoassay for vasculitis (FIDIS, Biomedical Diagnostics, France) uses a novel technique

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to detect and quantitate multiple autoantibodies simultaneously in a single reaction (Vercammen et al., 2007; Yiannaki et al., 2004; Tozzoli et al., 2006). This technology is fast, easy-to-use, and operator-independent. Microspheres covalently-conjugated with the major ANCA targets, proteinase (PR3), myeloperoxidase (MPO) (Goldschmeding et al., 1989; Falk and Jennette, 1988) are incubated with diluted patient serum and subsequently phycoerythrin-conjugated anti-human IgG, and the resulting fluorescence intensity measured in a flow cytometer (Luminex Corporation, Texas, USA).

This study compared the performance of the PR3- and MPO-ANCA flow cytometric immunoassays with IIF and 12 different PR3- and 12 MPO-ANCA ELISAs in the diagnosis of active small vessel vasculitis, and in detecting low ANCA levels in treated vasculitis and inflammatory bowel disease (IBD) (Saxon et al., 1990).

2. Patients and methods

2.1. Patients

2.1.1. Small vessel vasculitis

All sera were from patients with biopsy-proven Wegener's granulomatosis, microscopic polyangiitis, or necrotizing vasculitis that could not be classified further. 'Active' sera ($n=55$) were collected within 3 months of presentation ($n=36$, 65%) or relapse ($n=19$, 35%) from patients with Wegener's granulomatosis ($n=20$), microscopic polyangiitis ($n=26$) or necrotising vasculitis ($n=9$). 'Treated' or 'inactive' sera ($T, n=68$) were collected at least 3 months after presentation or relapse and in the absence of obvious ongoing clinical activity from patients with Wegener's granulomatosis ($n=38$), microscopic polyangiitis ($n=24$) or necrotising vasculitis ($n=6$). IIF-positive sera were mainly selected to compare the assays' abilities to detect positive and especially low-positive antibodies.

2.1.2. IBD

Sera were collected from patients diagnosed with IBD by their treating clinicians ($n=22$ for the PR3-ANCA and $n=23$ for the MPO-ANCA assays). Again IIF-positive sera were usually selected.

2.1.3. Controls

Controls included consecutive hospital patients referred for ANCA testing with suspected vasculitis who were subsequently shown to have a non-vasculitic disease ($n=34$), patients with IBD described above or with SLE diagnosed using the ARA criteria (Tan et al., 1982) and positive anti-double stranded DNA antibodies ($n=20$), as well as normal healthy blood donors ($n=33$).

All participants provided signed informed consent, and their sera were stored in aliquots at -70°C , and thawed once only. This study was approved by the Human Research Ethics Committee of Austin Health.

2.2. Methods

2.2.1. IIF and PR3- and MPO-ANCA ELISAs

Results of testing sera in the immunoassay for PR3- and MPO-ANCA sera were compared with those seen with IIF and

Table 1
PR3- and MPO-ANCA ELISAs

Kit	Kit name	Manufacturer
1	Bindazyme	The Binding Site, Birmingham, UK
2	ORG 518	ORGENTEC, Mainz, Germany
3	Immunoscan	Euro-Diagnostics, Malmö, Sweden
4	Varelisa	Pharmacia Diagnostics, Freiburg, Germany
5	Wielisa PR3 capture MPO direct	Wieslab, Lund, Sweden
6	Rainbow ELISA	Bio-Diagnostics Ltd, Worcestershire, UK
7	Euroimmun	Euroimmun, Seekamp, Germany
8	Kallestad	Bio-Rad Laboratories, California, USA
9	QUANTA Lite	INOVA Diagnostics Inc., California, USA
10	Aeskulisa	Aesku, Diagnostics GmbH, Wendelsheim, Germany
11	Medical Innovations	Analytica Ltd, New South Wales, Australia
12	In-house, PR3- and MPO-ANCA	In-house

the 12 commercial and in-house direct and capture ELISAs described previously (Table 1) (Trevisin et al., 2008).

2.2.2. Flow cytometric immunoassay

The PR3- and MPO-ANCA immunoassay (FIDIS vasculitis kit, Biomedical Diagnostics, Cedex, France) was performed according to the manufacturer's instructions using the reagents provided. The assay incorporated microspheres coated with PR3 and MPO, that were incubated with a 1/201 dilution of serum in phosphate-buffered saline Tween (PBS/T) buffer for 30 min at room temperature. Plates were washed twice with PBS/T and then incubated with prediluted phycoerythrin-labelled anti-human IgG for 30 min at room temperature, and the fluorescence intensity determined using a flow cytometer, the Luminex 100™ system (Luminex Corporation, Texas, USA).

All sera were tested in single wells, and dilution, washing, and dispensing of reagents were performed using an automated workstation (Biomedical Diagnostics, France). A positive and negative control, a single point calibrator and an external commercial QC control (ImmunoTrol I, Biomedical Diagnostics) were included in each run. Only assays where all controls behaved appropriately were included in the data analysis. Results were calculated using the single point calibrator and reported in units provided by the manufacturer (AU/ml). Binding less than 20 AU/ml was considered 'negative', 20–25 AU/ml 'borderline' and >25 AU/ml 'positive'. Borderline results were included in the 'positives'. All analyses except where indicated otherwise were performed using SPSS 13.0 (SPSS, Chicago, IL, USA).

2.2.3. Evaluation protocol

2.2.3.1. Precision. Within-run reproducibility was determined using 2 dilutions that resulted in low- and moderately-positive binding for each of PR3- and MPO-ANCA. These were each tested 30 times in a single run, and the coefficients of variation (CV) determined for each assay.

2.2.3.2. Binding characteristics and linearity

2.2.3.2.1. Binding levels. Binding levels of sera from active and treated Wegener's granulomatosis, microscopic polyangiitis and necrotising vasculitis were compared using one-way analysis of variance.

2.2.3.2.2. Linearity and dilution curves. Two strongly-binding sera that demonstrated linear binding with serial dilutions in the in-house PR3- and MPO-ANCA ELISA respectively were studied further. Serial dilutions in normal human serum (neat, 1/2, 1/5, 1/10, 1/50, 1/100) were tested and the 'expected' results calculated by dividing the neat serum binding by the dilution factor of each sample, and plotted against 'actual' binding. The slope (measure of standard error), y-intercept (measure of constant error), and R^2 value (coefficient of determination) were then calculated from the lines of best fit. A gradient of 1.00 ± 0.3 was considered acceptable.

2.2.3.3. Diagnostic performance

2.2.3.3.1. Sensitivity for small vessel vasculitis. The sensitivities of the PR3- and MPO-ANCA immunoassay for active small vessel vasculitis (Wegener's granulomatosis, microscopic polyangiitis, and necrotising vasculitis) were determined and compared with the sensitivities of IIF, and the ELISAs using the kappa statistic (k). This measured the agreement between 2 results beyond that expected by chance (Altman, 2005).

2.2.3.3.2. Specificity for small vessel vasculitis. The specificities of the PR3- and MPO-ANCA immunoassays for small vessel vasculitis were determined from patients with active small vessel vasculitis and those with suspected vasculitis, IBD, SLE and normal blood donors ($n=109$ for PR3- and 110 for MPO-ANCA).

2.2.3.3.3. Receiver operating characteristic (ROC) analysis. ROC analysis for active small vessel vasculitis was performed using hospital patients suspected of having vasculitis but subsequently shown to have another disease. ROC values for the immunoassay were used to calculate the areas under the curve (AUC) using the 'cut-offs' provided by the manufacturer. The AUC indicated the amount of separation between affected and unaffected individuals with '1' representing complete separation. Pairwise comparisons of ROC curves from the immunoassay and PR3- and MPO-ANCA ELISAs were then performed (MedCalc software, Mariakerke, Belgium).

3. Results

3.1. Precision

The within-run CVs were 5% for the low- and 3% for the moderately-positive PR3-ANCA, and 4% for both the low- and moderately-positive MPO-ANCA samples in the immunoassay.

3.2. Binding characteristics and linearity

3.2.1. Binding levels

3.2.1.1. Active and treated Wegener's granulomatosis. The mean binding of PR3-ANCA in the immunoassay in active Wegener's granulomatosis was 594 AU/ml (range 6–1160) and 30–287 Kit units in the ELISAs. Mean binding in treated disease was 320 AU/ml (range 0–1197 AU/ml) and 11–204 Kit units in the ELISAs (Fig. 1).

3.2.1.2. Active and treated microscopic polyangiitis. The mean binding of MPO-ANCA in the immunoassay in active micro-

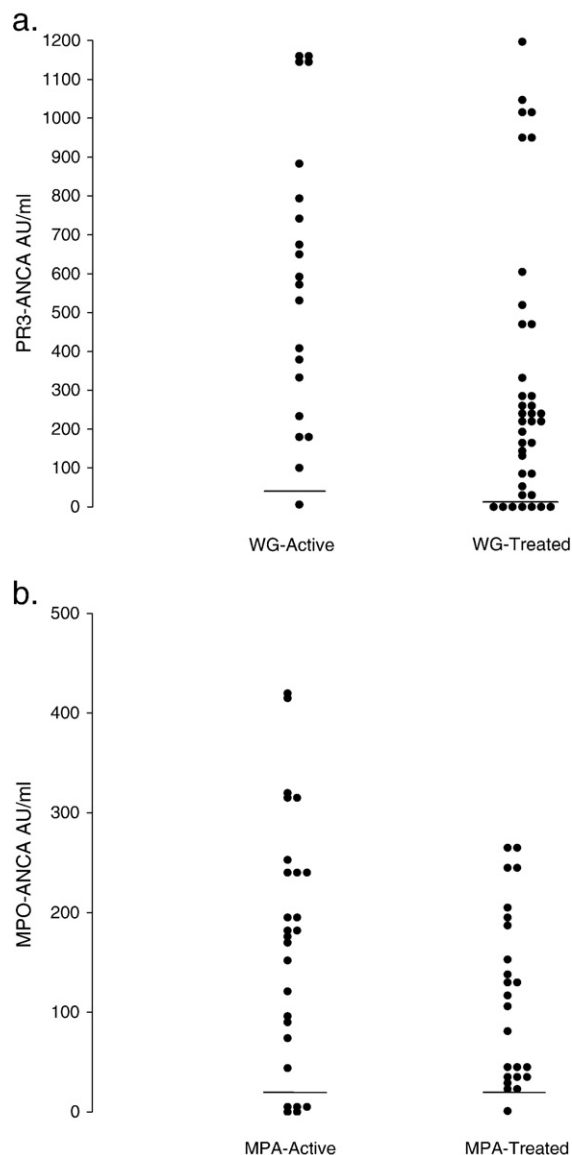


Fig. 1. Binding of (a) PR3- and (b) MPO-ANCA in the immunoassay in active and treated small vessel vasculitis. Sera binding above the solid lines were considered positive. Binding was expressed in individual Kit units.

scopic polyangiitis was 170 AU/ml (range 0–420) and 32–152 Kit units in the ELISAs. Mean binding in treated disease was 117 AU/ml (range 1–265) and 14–77 Kit units in the ELISAs.

3.2.2. Linearity

The undiluted PR3-ANCA test serum bound at 582 AU/ml, and binding of serial dilutions was linear in the PR3-ANCA immunoassay. The plot of 'expected' versus 'actual' results for these dilutions produced a gradient of 1.03, y-intercept of -42.37 and a R^2 of 0.97, which were similar to the values for the ELISAs. The y-intercept was less than for the ELISAs. The immunoassay (and most of the ELISAs) demonstrated acceptable linearity where binding increased proportionally with increased antibody levels (Table 2, Fig. 2).

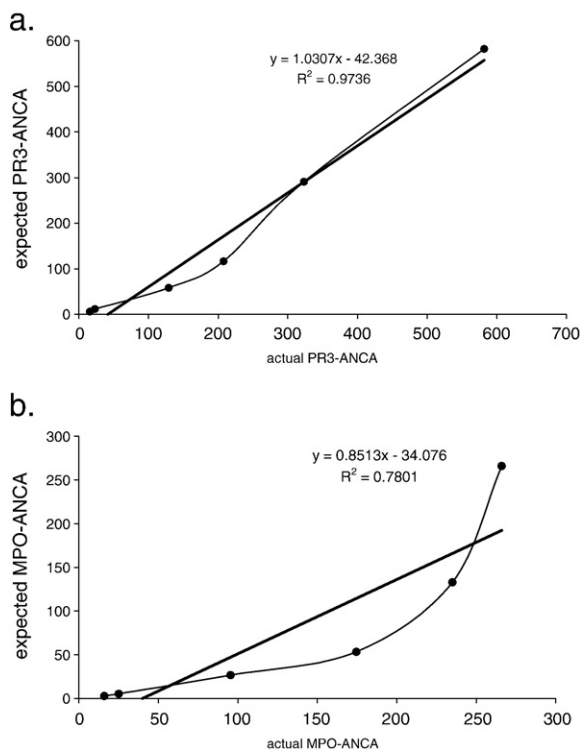
Table 2

Binding characteristics of the PR3- and MPO-ANCA immunoassay and ELISAs

Kit	PR3-ANCA				MPO-ANCA			
	Neat serum mean value (kit units)	Slope	y-intercept	R ²	Neat serum mean value (kit units)	Slope	y-intercept	R ²
1	103	1.01	1.23	0.99	46	1.01	-1.72	0.99
2	146	1.01	2.55	0.99	10	1.25	-2.51	1.00
3	131	1.01	0.57	1.00	124	1.06	-13.33	0.97
4	62	1.02	-3.49	0.99	60	0.98	-2.99	0.98
5	568	0.95	-26.67	0.96	179	0.96	-11.06	0.96
6	245	1.03	-1.28	0.99	100	0.88	-7.68	0.89
7	209	0.99	-8.08	0.98	100	0.96	-5.88	0.96
8	27	0.98	-0.75	0.98	19	0.95	-0.14	0.98
9	126	0.99	-15.47	0.93	30	1.14	-7.90	0.93
10	1386	0.96	109.53	0.96	95	0.95	-1.79	0.98
11	42	0.90	-8.44	0.74	34	0.91	-6.32	0.77
12	72	1.06	-4.64	0.99	60	0.97	-13.77	0.80
FCIA	582	1.03	-42.37	0.97	266	0.85	-34.08	0.78

PR3 – proteinase 3; MPO – myeloperoxidase; FCIA – flow cytometric immunoassay.

The undiluted MPO-ANCA test serum bound at 266 AU/ml, and binding of serial dilutions was not linear but tended to an asymptote after 235 AU/ml in the immunoassay. The plot of 'expected' versus 'actual' results for these dilutions produced a gradient of 0.85, y-intercept of -34.08 and R² of 0.7801. Thus the gradient and y-intercept for the immunoassay were less than for all 12 MPO-ANCA ELISAs. The low R² value was similar to one ELISA (Kit 11) which also used a single point calibrator. Thus the immunoassay performed acceptably in the linearity studies but not as well as the ELISAs.

**Fig. 2.** Linearity curves for the (a) PR3- and (b) MPO-ANCA immunoassay.

3.3. Diagnostic performance

3.3.1. Sensitivity in active small vessel vasculitis

3.3.1.1. Wegener's granulomatosis. Nineteen (95%) of the 20 sera from patients with active disease were positive for PR3-ANCA in the immunoassay compared with 18 (90%, median, range 17–19) in the ELISAs. The serum with P-ANCA was negative in the PR3-ANCA immunoassay and all 12 ELISAs (Tables 3, 4a, 4b).

3.3.1.2. Microscopic polyangiitis. Twenty-one (81%) of the 26 sera from patients with active disease were positive for MPO-ANCA in the immunoassay compared with 22 (85%, median, range 21–22) in the ELISAs. Four sera (15%) did not bind in the MPO-ANCA immunoassay and were also negative in all the ELISAs. All 3 sera with C-ANCA bound PR3-ANCA in the immunoassay, and 2 bound in all ELISAs and the other in 8.

3.3.1.3. Necrotising vasculitis. Four (44%) of the 9 sera from patients with active disease bound PR3-ANCA in the immunoassay, 3 in all of the ELISAs and 1 (11%) in only 4. Four sera (44%) were negative in this immunoassay and all ELISAs, and one was negative in the immunoassay and 11 ELISAs.

Table 3

IIF positivity and patterns in disease groups

	C-ANCA			P-ANCA			ANA	IIF-negative
	3+	2+	1+	3+	2+	1+		
Active WG (n=20)	10	4	4	1				1
Active MPA (n=26)	3			22				1
Active NV (n=9)	2	2		5				0
Inactive WG (n=38)	4	10	13	3				8
Inactive MPA (n=24)				8	13	2		1
Inactive NV			1		3			2
IBD (n=23)		3	5	3	3	8	1	0
Suspected vasculitis (n=35)		1	8	2	1	1	2	8
SLE (n=20)	1			6	2	3	7	1
Normal blood donors (n=33)		1	1	2	1	1		27

WG – Wegener's granulomatosis, MPA – microscopic polyangiitis, NV – necrotizing vasculitis, IBD – inflammatory bowel disease, SLE – systemic lupus erythematosus, ANA – antinuclear antibodies, ANCA – antineutrophil cytoplasmic antibodies. ANA includes atypical ANCA.

Table 4a

Sensitivities, specificities and ROC analysis of the immunoassay and ELISAs for PR3- and MPO-ANCA in active and treated vasculitis

Kit	PR3-ANCA			MPO-ANCA		
	Number of sera positive (sensitivity, %)		Specificity (%; 95% CI)	Number of sera positive (sensitivity, %)		Specificity (%; 95% CI)
	WG – A (n=20)	WG – T (n=38)		MPA – A (n=26)	MPA – T (n=24)	
1	18 (90)	29 (76)	88 (82–94)	22 (85)	15 (63)	98 (93–100*)
2	18 (90)	28 (74)	94 (89–99)	21 (81)	11 (46)	93 (88–98)
3	18 (90)	26 (68)	96 (91–99*)	22 (85)	21 (88)	86 (80–93)
4	18 (90)	28 (74)	91 (86–96)	22 (85)	22 (92)	90 (85–96)
5	18 (90)	31 (82)	95 (89–98*)	21 (81)	17 (71)	97 (92–100*)
6	18 (90)	29 (76)	88 (82–94)	22 (85)	21 (88)	95 (89–98*)
7	17 (85)	25 (66)	92 (87–97)	22 (85)	17 (71)	95 (90–99*)
8	18 (90)	28 (74)	95 (90–99*)	21 (81)	12 (50)	99 (95–100*)
9	19 (95)	29 (76)	95 (90–99*)	22 (85)	12 (50)	99 (95–100*)
10	18 (90)	27 (71)	95 (90–99*)	22 (85)	17 (71)	98 (94–100*)
11	18 (90)	30 (79)	88 (82–94)	22 (85)	21 (88)	96 (91–99*)
12	18 (90)	29 (76)	93 (88–98)	22 (85)	17 (71)	99 (95–100*)
FCIA	19 (95)	30 (79)	91 (83–95)	21 (81)	23 (96)	96 (90–99)

PR3 – proteinase 3; MPO – myeloperoxidase; WG – Wegener's granulomatosis; MPA – microscopic polyangiitis; NV – necrotising vasculitis; A – active; T – treated; ROC – receiver operating characteristic; AUC – area under the curve; FCIA – flow cytometric immunoassay.

* Indicates 95% confidence interval calculated using binomial approximation.

Five sera (56%) bound MPO-ANCA in the immunoassay and in all ELISAs, and 4 sera (44%) did not bind in this immunoassay or the ELISAs.

In summary, the sensitivity of the PR3-ANCA immunoassay for active Wegener's granulomatosis was 95% compared with a sensitivity of 90% for IIF, and a median sensitivity of 90% (range 85–95%) for the corresponding ELISAs. The sensitivity of the MPO-ANCA immunoassay for active microscopic polyangiitis was 81% compared with 85% for IIF, and 85% (range 81–85%) for the corresponding ELISAs. Thus the sensitivity of the combination of the PR3- and MPO-ANCA immunoassays for active small vessel vasculitis (Wegener's granulomatosis, microscopic polyangiitis and necrotising vasculitis) was 96% (86–99%, 95% CI) compared with 96% for IIF and 94% (median, range 91–96%) for both ELISAs.

3.3.2. Sensitivity in treated small vessel vasculitis (Tables 3, 4a, 4b)

3.3.2.1. Wegener's granulomatosis. Thirty (79%) of the 38 sera from patients with treated disease were positive for PR3-ANCA in the immunoassay compared with 29 (76%, median, range 25–31) in the ELISAs. One serum with 2+ P-ANCA was positive for MPO-ANCA in the immunoassay and also bound MPO-ANCA at low levels in 7 ELISAs.

3.3.2.2. Microscopic polyangiitis. Twenty-three (96%) of the 24 sera from patients with treated disease were positive for MPO-ANCA in the immunoassay compared with 17 (71%, median, range 11–22) in the ELISAs. All sera bound MPO-ANCA less than 300 AU/ml. One serum with P-ANCA 2+ bound MPO-ANCA at low levels in the immunoassay but was negative in all ELISA; another that bound MPO-ANCA in the immunoassay was negative in 10 ELISA; and the IIF-negative serum did not bind MPO-ANCA in the immunoassay, was negative in 6 ELISAs and bound at low-positive levels in the other 6.

3.3.2.3. Necrotising vasculitis. Of the 6 sera from patients with treated disease, the serum with C-ANCA bound PR3-

ANCA in the immunoassay and 6 ELISAs. Two of the 3 sera with P-ANCA did not bind MPO-ANCA but were positive at borderline levels in 3 ELISAs.

In summary, the sensitivity of the PR3-ANCA assay for treated Wegener's granulomatosis was 79% compared with 71% for IIF, and 76% (median, range 66–82%) for the ELISAs. The sensitivity of the MPO-ANCA assay for treated microscopic polyangiitis was 96% compared with 96% for IIF, and 71% (median, range 46–92%) for the ELISAs. The median sensitivity of the combination of PR3- and MPO-ANCA immunoassays for treated small vessel vasculitis (Wegener's granulomatosis, microscopic polyangiitis and necrotising vasculitis) was 82% compared with 84% for IIF, and 69% (median, range 57–82%) for both ELISAs.

Table 4b

Sensitivities and specificities of the immunoassay and both ELISAs from a single manufacturer for PR3- and MPO-ANCA in active vasculitis

Combined assays		
Kit	Sensitivity% (95% CI)	Specificity % (95% CI)
	Active WG, MPA or NV (n=55)	(n=110)
1	93 (82–98*)	87 (81–94)
2	91 (80–97*)	87 (81–94)
3	93 (82–98*)	84 (77–91)
4	95 (85–99*)	84 (77–91)
5	96 (88–100*)	93 (88–98)
6	96 (88–100*)	86 (80–93)
7	93 (82–98*)	89 (83–95)
8	91 (80–97*)	95 (89–98*)
9	96 (88–100*)	95 (89–98*)
10	93 (82–98*)	94 (87–97*)
11	95 (85–99*)	86 (80–93)
12	95 (85–99*)	92 (87–97)
FCIA	96 (86–99*)	88 (80–93)

PR3 – proteinase 3; MPO – myeloperoxidase; WG – Wegener's granulomatosis; MPA – microscopic polyangiitis; NV – necrotising vasculitis; A – active; T – treated; ROC – receiver operating characteristic; AUC – area under the curve; FCIA – flow cytometric immunoassay.

* Indicates 95% confidence interval calculated using binomial approximation.

Table 5

Correlation of results of the PR3- and MPO-ANCA immunoassay and the ELISAs

ELISA	FCIA PR3-ANCA		FCIA MPO-ANCA	
	WG – A	WG – T	MPA – A	MPA – T
	k (95% CI)	k (95% CI)	k (95% CI)	k (95% CI)
1	0.64 (–0.04–1.33)	0.93 (0.78–1.07)	0.87 (0.61–1.12)	0.14 (–0.35–0.62)
2	0.64 (–0.04–1.33)	0.86 (0.66–1.05)	1.00 (1.00–1.00)	–0.08 (–0.45–0.28)
3	0.64 (–0.04–1.33)	0.73 (0.48–0.98)	0.87 (0.61–1.12)	–0.67 (–1.02–0.89)
4	0.64 (–0.04–1.33)	0.86 (0.66–1.05)	0.87 (0.61–1.12)	–0.59 (–1.18–1.06)
5	0.64 (–0.04–1.33)	0.75 (0.47–1.02)	0.76 (0.43–1.08)	–0.08 (–0.69–0.53)
6	0.64 (–0.04–1.33)	0.93 (0.78–1.07)	0.87 (0.61–1.12)	–0.07 (–1.02–0.89)
7	0.46 (–0.25–1.17)	0.68 (0.41–0.94)	0.87 (0.61–1.12)	0.18 (–0.37–0.74)
8	0.64 (–0.04–1.33)	0.86 (0.66–1.05)	1.00 (1.00–1.00)	0.08 (–0.32–0.48)
9	1.00 (1.00–1.00)	0.93 (0.78–1.07)	0.87 (0.61–1.12)	0.08 (–0.32–0.48)
10	0.64 (–0.04–1.33)	0.79 (0.56–1.02)	0.87 (0.61–1.12)	0.18 (–0.37–0.74)
11	0.64 (–0.04–1.33)	1.00 (1.00–1.00)	0.87 (0.61–1.12)	0.47 (–0.24–1.17)
12	0.64 (–0.04–1.33)	0.77 (0.53–1.02)	0.87 (0.61–1.12)	–0.08 (–0.69–0.53)

FCIA – flow cytometric immunoassay; PR3 – proteinase 3; MPO – myeloperoxidase; WG – Wegener's granulomatosis; MPA – microscopic polyangiitis; A – active, T – treated; k – kappa value.

3.3.3. Correlation of results from the immunoassay and the ELISAs (Table 5)

3.3.3.1. Active Wegener's granulomatosis and microscopic polyangiitis. The results from the PR3-ANCA immunoassay correlated with only one ELISA (Kit 9) for sera from active Wegener's granulomatosis, $k=1.0$ (1.0–1.0, 95% CI). The immunoassay and ELISA both demonstrated PR3-ANCA in 19 sera (19/20, 95%) and the single serum that was positive in both assays bound PR3-ANCA at moderately high levels in the immunoassay but was borderline in one and negative in all the other ELISAs.

The results from the MPO-ANCA immunoassay correlated with the results from 2 MPO-ANCA ELISAs (Kits 2 and 8) in active microscopic polyangiitis, $k=1.0$ (1.0–1.0, 95% CI). The immunoassay and both ELISAs demonstrated MPO-ANCA in 21 sera (21/26, 81%). The immunoassay did not detect MPO-ANCA in the single serum that was borderline to moderately-positive in 10 ELISAs.

3.3.3.2. Treated Wegener's granulomatosis and microscopic polyangiitis. The results from the immunoassay correlated with those of only one PR3-ANCA ELISA (Kit 11) for the detection of antibodies in treated patients, $k=1.0$ (range 1.0–1.0, 95% CI). The immunoassay and this ELISA detected PR3-ANCA in 30 sera (30/38, 79%) in treated Wegener's granulomatosis. One serum bound PR3-ANCA at low levels in the immunoassay and at borderline levels in Kit 11 but was negative in the other 10 ELISAs.

The results from the MPO-ANCA immunoassay did not correlate with the results from any ELISA ($k<1.0$ for all assays). The immunoassay was 96% sensitive for treated vasculitis and there was no agreement (negative k values) for 6 ELISAs (Kits 2, 3, 4, 5, 6 and 12 with sensitivities of 46%, 88%, 92%, 71%, 88% and 71% respectively).

3.3.3.3. Sensitivity in IBD. The sensitivity of the PR3-ANCA immunoassay was 27% (6/22) for IBD compared with 15% (median 5, range 1–10, $n=22$) for the ELISAs. The sensitivity of the MPO-ANCA immunoassay was 17% (4/23) compared with 15% (median 4, range 1–7, $n=23$) for the ELISAs. The sensitivity of the combination of the PR3- and MPO-ANCA

immunoassays was thus 20% for IBD compared with 33% (range 13–52%) for both ELISAs (Table 6).

3.3.4. Specificity for active small vessel vasculitis (Tables 3, 4a, 4b)

3.3.4.1. Suspected vasculitis. Three of the 15 sera were positive for PR3-ANCA and none for MPO-ANCA in the immunoassays compared with 2 (median, range 1–4), and none (range 0–7, in Kits 3 and 4) respectively in the ELISAs. The immunoassay detected PR3-ANCA in a patient with 1+ C-ANCA who was negative in all PR3-ANCA ELISAs.

3.3.4.2. SLE. One serum (5%) was positive for PR3-ANCA in the immunoassay and 2 (10%) were positive in 4 ELISAs. One serum bound PR3-ANCA at borderline levels in the immunoassay and in 3 ELISAs (Kits 1, 6 and 11). No serum was positive in the MPO-ANCA immunoassay but one bound MPO-ANCA in 2 ELISAs (Kits 3 and 4).

3.3.4.3. Normal blood donors. None of these sera bound in the PR3- or MPO-ANCA immunoassays. Two (6%) bound in the

Table 6

Sensitivities of the PR3- and MPO-ANCA immunoassay and ELISAs for inflammatory bowel disease

	PR3-ANCA	MPO-ANCA
	Number of sera positive (sensitivity, %)	Number of sera positive (sensitivity, %)
	IBD ($n=22$)	IBD ($n=23$)
1	9 (39)	2 (9)
2	6 (26)	7 (30)
3	3 (13)	4 (17)
4	7 (30)	4 (17)
5	4 (17)	3 (13)
6	10 (43)	5 (22)
7	6 (26)	4 (17)
8	5 (17)	1 (4)
9	3 (13)	1 (4)
10	1 (4)	2 (9)
11	8 (35)	4 (17)
12	4 (17)	1 (4)
FCIA	6 (27)	4 (17)

PR3 – proteinase 3; MPO – myeloperoxidase; IBD – inflammatory bowel disease; FCIA – flow cytometric immunoassay.

PR3-ANCA ELISAs (Kits 7 and 11) and four (12%) in the MPO-ANCA ELISAs (Kits 2, 3, 6 and 7).

Thus the specificity of the PR3-ANCA immunoassay for Wegener's granulomatosis was 91% (83–95%, 95% CI) compared with 94% (median, range 88–96%) for the corresponding ELISAs. The specificity of the MPO-ANCA immunoassay for microscopic polyangiitis was 96% (90–99%, 95% CI) compared with 97% (median, range 86–99%) for the ELISAs. The specificity of the combination of PR3- and MPO-ANCA immunoassays for small vessel vasculitis was 88% (80–93%, 95% CI) and also 88% (median, range 84–95%) for the ELISAs.

3.3.5. ROC analysis in active small vessel vasculitis

The AUC value for the PR3-ANCA immunoassay was 0.993 which was more than for the ELISAs (median 0.951, range 0.929–0.979). There was no difference in the performance of the PR3-ANCA immunoassay and the 12 individual ELISAs ($p > 0.05$). Thus the PR3-ANCA immunoassay and the ELISAs

were highly sensitive for the diagnosis of small vessel vasculitis and distinguished satisfactorily between vasculitis and non-vasculitic disease (Table 7, Fig. 3).

The AUC value for the MPO-ANCA immunoassay was 0.945 which was more than for the ELISAs (median 0.881, range 0.846–0.962). The AUC for the immunoassay was different from that for Kit 6 ($p < 0.05$) which had the lowest value of all the MPO-ANCA ELISAs. Nevertheless the MPO-ANCA immunoassay and the ELISAs all distinguished satisfactorily between vasculitis and non-vasculitic disease.

4. Discussion

This study demonstrated that the flow cytometric immunoassay was highly sensitive and specific for the diagnosis of active vasculitis, and accurately distinguished between active vasculitis and non-vasculitic disease. The PR3-ANCA immunoassay was more sensitive than IIF and most ELISAs in

Table 7

Comparison of ROC analysis AUC values for the (a) PR3- and (b) MPO-ANCA immunoassay and ELISAs in patients with active small vessel vasculitis

a.													
PR3-ANCA ELISA	1	2	3	4	5	6	7	8	9	10	11	12	FCIA
1		0.846	0.764	0.934	0.918	0.404	0.699	0.749	0.464	0.574	0.751	0.794	0.201
2	0.846		0.885	0.863	0.805	0.286	0.564	0.890	0.270	0.669	0.629	0.911	0.151
3	0.764	0.885		0.798	0.747	0.284	0.527	0.980	0.287	0.771	0.537	0.985	0.126
4	0.934	0.863	0.798		0.880	0.318	0.615	0.794	0.380	0.572	0.669	0.815	0.182
5	0.918	0.805	0.747	0.880		0.509	0.834	0.733	0.557	0.589	0.907	0.752	0.285
6	0.404	0.286	0.284	0.318	0.509		0.580	0.277	0.973	0.184	0.538	0.286	0.540
7	0.699	0.564	0.527	0.615	0.834	0.580		0.483	0.617	0.370	0.902	0.540	0.270
8	0.749	0.890	0.980	0.794	0.733	0.277	0.483		0.240	0.791	0.552	0.969	0.498
9	0.464	0.270	0.287	0.380	0.557	0.973	0.617	0.240		0.214	0.577	0.258	0.130
10	0.574	0.669	0.771	0.572	0.589	0.184	0.370	0.791	0.214		0.410	0.744	0.104
11	0.751	0.629	0.537	0.669	0.907	0.538	0.902	0.552	0.577	0.410		0.596	0.192
12	0.794	0.911	0.985	0.815	0.752	0.286	0.540	0.969	0.258	0.744	0.596		0.149
FCIA	0.201	0.151	0.126	0.182	0.285	0.540	0.270	0.498	0.130	0.104	0.192	0.149	
b.													
MPO-ANCA ELISA	1	2	3	4	5	6	7	8	9	10	11	12	FCIA
1		0.580	0.422	0.444	0.852	0.205	0.331	0.115	0.500	0.078	0.478	0.467	0.226
2	0.580		0.917	0.834	0.474	0.770	0.087	0.042	0.136	0.039	0.935	0.982	0.077
3	0.422	0.917		0.759	0.501	0.623	0.143	0.051	0.184	0.028	0.988	0.904	0.091
4	0.444	0.834	0.759		0.255	0.910	0.092	0.028	0.166	0.030	0.773	0.827	0.066
5	0.852	0.474	0.501	0.255		0.316	0.428	0.154	0.648	0.191	0.517	0.457	0.314
6	0.205	0.770	0.623	0.910	0.316		0.091	0.024	0.146	0.018	0.568	0.696	0.048
7	0.331	0.087	0.143	0.092	0.428	0.091		0.474	0.715	0.589	0.181	0.127	0.777
8	0.115	0.042	0.051	0.028	0.154	0.024	0.474		0.302	0.808	0.049	0.045	0.652
9	0.500	0.136	0.184	0.166	0.648	0.146	0.715	0.302		0.342	0.244	0.191	0.503
10	0.078	0.039	0.028	0.030	0.191	0.018	0.589	0.808	0.342		0.047	0.029	0.838
11	0.478	0.935	0.988	0.773	0.517	0.568	0.181	0.049	0.244	0.047		0.937	0.079
12	0.467	0.982	0.904	0.827	0.457	0.696	0.127	0.045	0.191	0.029	0.937		0.075
FCIA	0.226	0.077	0.091	0.066	0.314	0.048	0.777	0.652	0.503	0.838	0.079	0.075	
						Significant ($p < 0.05$)							
						Not significant ($p > 0.05$)							

ROC – receiver operating characteristic; AUC – area under the curve; PR3 – proteinase 3; MPO – myeloperoxidase; FCIA – flow cytometric immunoassay.

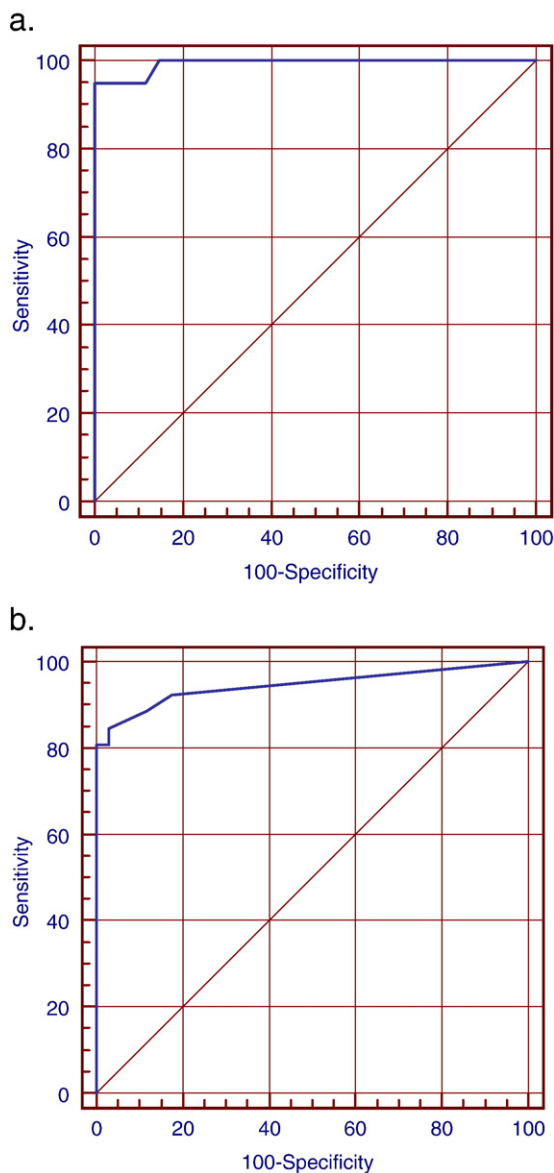


Fig. 3. ROC analysis curves of the (a) PR3- and (b) MPO-ANCA immunoassay.

active and treated Wegener's granulomatosis. The MPO-ANCA immunoassay was just as sensitive as IIF, and while it was less sensitive than most ELISAs in active microscopic polyangiitis, it was more sensitive than the ELISAs in treated disease.

All test sera were from patients in whom the diagnosis of vasculitis was confirmed histologically. Disease was classified as 'inactive' or 'treated' based on the time since presentation or relapse. This was to compare the ability of different assays to detect low but still positive antibody levels. IIF-positive sera from patients with inactive vasculitis or IBD were selected for the same reason.

The immunoassay was simple to use, did not require further dilutions of strongly-binding sera, and included only one calibrator and the minimum number of controls. Fewer reagents were required than for the ELISAs, and all manipulations were performed in a single well. The results were less

subjective than for IIF, the set-up was fully-automated which reduced errors and cost, and the time required to perform the assay was less than for an ELISA.

However the equipment required for the immunoassay was expensive. In addition the use of a single point calibrator meant binding levels were high, and results did not necessarily reflect a proportional increase in antibody levels. This is important where antibody levels are used to monitor the response to treatment.

The immunoassay performed well compared with IIF and the ELISAs. No data on precision were provided by the manufacturer but within-run coefficients of variation in this study were less than 10% for both low- and moderately-binding PR3- and MPO-ANCA samples. Binding levels were higher in the PR3- and MPO-ANCA immunoassays in both active and treated vasculitis, and levels were more varied in treated disease than in the ELISAs. Levels were sometimes markedly elevated many years after presentation when patients were in clinical remission. This was more pronounced than with the ELISAs and occurred despite our current understanding that ANCA are pathogenetic and their persistence implies ongoing disease activity (Xiao et al., 2002).

The immunoassay was as sensitive as IIF for the diagnosis of active vasculitis, and the PR3-ANCA immunoassay was more sensitive than the MPO-ANCA immunoassay. ROC analysis demonstrated that the immunoassay distinguished satisfactorily between active vasculitis and non-vasculitic disease. The AUC value for the PR3-ANCA assay was higher than for all the ELISAs indicating that it distinguished best between vasculitis and non-vasculitic disease. The PR3-ANCA immunoassay detected PR3-ANCA in one patient with relapsing Wegener's granulomatosis that was evident only with the capture ELISA. The increased sensitivity and higher binding of the MPO-ANCA immunoassay in these patients suggest that the immunoassay is not suitable for monitoring the response to treatment and results should not be compared with those obtained with an ELISA.

A previous evaluation of the immunoassay did not distinguish between active and treated vasculitis (Damoiseaux et al., 2005a,b). It found sensitivities of 92% and 97% for PR3- and MPO-ANCA in Wegener's granulomatosis and microscopic polyangiitis respectively compared with our findings of 95% and 81%. The corresponding specificities were 97% and 100% compared with 91% and 96% in our study but these differences probably resulted from using different controls. The previous study also found that the immunoassay was less sensitive in detecting relapse than the capture ELISA (Damoiseaux et al., 2005a,b) and although this was not our experience, our series was small.

The immunoassay was also used to detect low levels of ANCA in IBD. The sensitivity for the combination of PR3- and MPO-ANCA was 20% compared with 33% for both ELISAs. We do not advocate the use of any of these assays for the diagnosis of IBD, to distinguish subtypes, nor to monitor disease activity, but have demonstrated here the immunoassay's lesser sensitivity for what are usually low level ANCA compared with most ELISAs.

At least 2 other methodologies are also used to test for ANCA. Conventional flow cytometry uses isolated peripheral blood neutrophils (Yang et al., 1994). Antibodies are detected with a fluorescent-tagged antihuman IgG and result in

cytoplasmic staining. Positive sera are confirmed with IIF of neutrophil smears, and subsequently in assays for PR3- and MPO-ANCA. This assay is simple, rapid, and highly sensitive and specific, with a high negative predictive value for small vessel vasculitis (Kitching et al., 2004), but it cannot distinguish between different ANCA patterns, nor between ANCA and ANA, and, unlike the ELISAs and flow cytometric immunoassay described here, is not quantitative.

Another method is the fluorescent-enzyme immunoassay (EliA, Pharmacia Diagnostics, Freiburg, Germany) (Damoiseaux et al., 2005a,b), that uses highly-purified human antigens coated onto irradiated polystyrene cups that are incubated with serum, chromagen and substrate, and antibody binding is demonstrated fluorometrically. This assay analyses a number of samples simultaneously, does not require a standard curve with each run, and is fully-automated.

5. Conclusions

The flow cytometric immunoassay evaluated here is a sensitive and specific method to confirm the demonstration of PR3- and MPO-ANCA in active small vessel vasculitis. This assay has the advantage that it can be further adapted to simultaneously detect other vasculitis-associated antibodies and antibodies directed against additional ANCA antigens, without a significant increase in labour, time or cost. However the variation in binding levels between this and the more conventional assays highlights the difficulties with using different ANCA detection methods for monitoring disease, and emphasises the need for international ANCA standards and standardised binding units.

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