# A novel high sensitivity ELISA for detection of antineutrophil cytoplasm antibodies against proteinase-3

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## ABSTRACT

**Objective.** *Conventional direct enzyme-linked immunosorbent assays (ELISA) for the detection of anti-neutrophil cytoplasm antibodies (ANCA) often lack sensitivity because epitopes of the target antigen are hidden by binding to the ELISA plate. This study was designed to evaluate a novel ELISA method for detection of ANCA against proteinase-3 (PR3) for the diagnosis of Wegener's granulomatosis (WG) using PR3 presented in its native form.*

**Methods.** *Sera from four subgroups of patients with a diagnosis of WG (n = 86), 80 healthy controls and 450 disease controls were tested for the presence of C-ANCA/PR3-ANCA by anchor ELISA, direct ELISA, capture ELISA, indirect immunofluorescence (IFT) and immunoblotting.*

**Results.** *In prospectively analysed consecutive patients, anchor ELISA showed the highest sensitivity for a diagnosis of WG of 96.0% (95% CI: 79.6-99.3), followed by IFT 92.0% (73.9-98.8), capture ELISA 72.0 (50.6 -87.9) and direct ELISA 60.0 (38.7 -78.8). Specificity was high for all methods and ranged from 98.5 (97.0 -99.4) to 95.5% (97.9-99.8). Receiver operating characteristics curve analysis revealed that the overall diagnostic performance of the anchor ELISA was significantly superior compared to the direct ELISA and the capture ELISA in patients with generalized WG, and also compared to IFT and immunoblotting in patients with localised WG.*

**Conclusion.** *Anchor ELISA is a novel highly sensitive and specific method for the detection of PR3-ANCA in patients with WG, which may replace the need for a combined analysis with IFT and ELISA in the future.*

## Introduction

Antineutrophil cytoplasm antibodies (ANCA) are found in a high percentage of patients with Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) and Churg-Strauss syndrome (CSS) and are used as diagnostic makers for these diseases, which are also coined the ANCA-associated vasculitides (AAV). On indirect immunoflourescence test (IFT) ANCA exhibit most commonly either a granular cytoplasmic pattern (C-ANCA) or a perinuclear pattern (P-ANCA). C-ANCA are a characteristic finding of patients with WG and are mostly directed against proteinase 3 (PR3), while P-ANCA that frequently occur in MPA are in general directed against myeloperoxidase (MPO). However, ANCA detected by IFT are not specific for vasculitis as C-ANCA or P-ANCA directed against other target antigens can occur in many other inflammatory or non-inflammatory conditions that may mimic clinical features of vasculitis (1, 2). Therefore, current consensus guidelines recommend to perform an enzyme-linked immunoassay (ELISA) in addition to IFT (3). However, several studies have shown that commercially available ELISA kits vary considerably in their performance characteristics and often lack sensitivity (4, 5). Even between reference laboratories substantial differences have been revealed in the performance characteristics of their in-house ELISAs (6). In previously used ELISAs the target antigen was immobilised by binding of mostly hydrophobe epitopes of PR3 to the plastic surface of the ELISA plate. This directed binding may protect those epitopes from binding to ANCA directed against those epitopes and thus may lead to false negative results. For testing of PR3 ANCA, capture ELISA has been shown to be superior in overall diagnostic performance compared to direct ELISA (6, 7). However, sensitivity of capture ELISA assays can also be impaired if the capturing antibody covers those epitopes of PR3 to which some PR3 ANCA bind.

A new technique to immobilise PR3 on the ELISA plate by using a bridging molecule as an "anchor" has been developed, thus preventing direct adhesion to the plastic surface and thereby preserving all epitopes for binding with ANCA. Therefore, this study was designed to test the diagnostic performance of this novel method of ANCA detection termed "anchor ELISA" in different clinical scenarios that make ANCA testing in clinical practice particularly challenging.

## Patients and methods

*Patients*

*Patients with WG:* The utility of the anchor ELISA for the diagnosis of WG was evaluated using four different scenarios that can represent a diagnostic challenge in clinical practice: Group I: unselected consecutive patients with a final diagnosis of WG; Group II: patients with generalized or early systemic WG who tested positive for C-ANCA on indirect IFT, but were negative on direct ELISA for PR3-ANCA, Group III: patients with generalized or early systemic WG and active disease who tested negative for C-ANCA/PR3-ANCA on all methods (IFT, ELISAs, immunoblotting), and Group IV: patients with localised WG. Patients from Group I consisted of prospectively assessed consecutive patients presenting at the vasculitis center with a diagnosis of WG. Patients from Groups II-IV were retrospectively analysed.

In all patients WG was diagnosed based on typical biopsy findings according to the Chapel Hill Consensus Definitions for WG (8) and/or the presence of surrogate parameters of systemic vasculitis (i.e. mononeuritis multiplex, rapid progressive glomerulonephritis etc.) and/or granulomatous inflammation (i.e. retro-orbital granuloma, destructive sinusitis, pulmonary nodules). Biopsies were examined in the German reference centre for vasculitis pathology (Department of Pathology, University of Schleswig-Holstein, Campus Lübeck) by two independent observers. In addition, all patients had to fulfil at least 2 of 4 of the American College of Rheumatology classification criteria for WG (9). The diagnosis of WG was made independent of the ANCA status. All patients were categorised into disease stages (localised, early systemic, generalised) according to definitions by the European Vasculitis Study Group (10). Disease activity was evaluated by a multidisciplinary team of the vasculitis center, as previously described (11). The disease extent index (DEI) score was calculated to assess extent and activity of WG (12).

*Control sera*. Sera from 80 probands without a history of a chronic inflammatory disorder were used as healthy controls. Prospectively collected sera from 30 patients with other forms of ANCA-associated vasculitis (CSS, n = 19; MPA, n = 11) and from 450 patients with inflammatory or non-inflammatory disorders which may mimic different features of the broad spectrum of clinical manifestations were analysed as disease controls. Among these were patients with giant cell arteritis (N = 28), systemic lupus erythematosus (N = 40), primary Sjögren's syndrome (N = 40), systemic sclerosis (N = 40), rheumatoid arthritis (N = 38), rheumatoid vasculitis (N = 29), nasal cocaine abuse (N = 2), infectious diseases (N = 111) and patients with diseases in single target organs of WG (N = 64) such as acute or chronic renal failure do to non-inflammatory causes, polyposis nasi, sinusitis, bronchial carcinoma, lung metastases, membraneous glomerolonephritis, or hemoptysis.

*Methods for ANCA detection*

Indirect IFT on ethanol-fixed leukocytes, direct ELISA, capture ELISA and immunoblotting for PR3-ANCA were performed as previously described by our laboratory (5, 6, 13). ANCA detection by IFT and direct ELISA were performed according to standardised guidelines (3).

*Anchor ELISA.*

Conventional 96-well microtiter plates (Nunc, GmbH & Co. KG, Wiesbaden, Germany) were sequentially coated with a bridging molecule and human PR3 from neutrophile granulocytes (ORGENTEC GmbH, Mainz, Germany) at a concentration of 1 µg/ml. Sera were diluted at 1:200 in a buffer solution (Tris $NaN_3$ < 0.1%) and the coated microtiter plates were incubated with 100µL of serum per well in duplicate for 30 minutes at room temperature (RT). After washing, ready to use peroxidase-conjugated anti-human IgG was added and the microtiter plates were incubated for 15 minutes at RT. After 3 washing-cycles, tetramethylbenzidine-containing substrate solution was added to each well and incubation continued for 15 minutes. The enzyme reaction was stopped by adding 100µL hydrochloric acid (1M). The plates were analysed at 450/620nm using a microtiter plate reader (sunrise, TECAN, Trading AG, Switzerland ).

*Statistical analysis*

Performance characteristics were compared by receiver operating characteristic (ROC) curve analysis according to the method described by Hanley (14). A difference of $P < 0.05$ was considered to be statistically significant. Sensitivity and Specificity were calculated by 2x2 tables. The software MedCalc® (MedCalc®, Mariakerke, Belgium) was used for statistical analysis.

## Results

*Prospective evaluation of diagnostic performance*

Anchor ELISA recognized PR3-ANCA in 27 of 28 patients with WG and showed the highest sensitivity for a diagnosis of WG compared to disease controls of 96.0% (95% CI: 79.6-99.3), followed by IFT, capture ELISA and direct ELISA (Table II). Using the anchor ELISA, PR3-ANCA were found in none of the healthy control sera (median titre 1.3 U/ml [1.1-1.5]). Specificity was high for all methods and ranged from 98.5 (97.0 -99.4) to 99.5 % (97.9-99.8) (Table II). The overall diagnostic performance, assessed as the area under the ROC curve (AUC), of the anchor ELISA (0.96 [0.94 -0.98]) was significantly superior compared to the direct ELISA (0.80 [0.76 -0.83]; $P = 0.002$) and the capture ELISA (0.86 [0.82 -0.89]; $P = 0.032$) (Fig. 1, panel A). The AUC of the IFT was similar to the anchor ELISA and was also significantly greater compared to the direct ELISA and the capture ELISA (Table II).

**Table I.** Clinical characteristics of the four different cohorts of patients with WG.

| | Generalised prospective | Generalised* IFT +/ ELISA − | Generalised* IFT−/ELISA− | Localised* |
|---|---|---|---|---|
| Number | 25 | 14 | 19 | 28 |
| Sex (male/female) | 13/12 | 9/5 | 7/12 | 8/20 |
| Age (years) | 49.8 ± 14.8 | 51.2 ± 14.2 | 53.3 ± 17.7 | 50.5 ± 15.8 |
| Active/remission | 9/16 | 8/6 | 19/0 | 13/15 |
| DEI score | 3.2 ± 2.5 | 3.5 ± 1.9 | 3.9 ± 1.9 | 2.0 ± 0.8 |

ANCA: Antineutrophil Cytoplasm Antibodies; DEI: Disease Extent Index; ELISA: Enzyme-Linked Immunosorbent Assay; iIFT: indirect Immunofluorescence Test; *retrospectively collected sera.

*Ability to detect PR-3 ANCA in sera tested positive for C-ANCA, but negative for PR3- ANCA by conventional direct ELISA*

PR3-ANCA were detected by anchor ELISA in sera of 10 of 14 patients with WG who had tested positive for C-ANCA, but negative for PR3-ANCA by conventional direct ELISA. Binding to PR3 was confirmed by immunoblotting in all 10 cases. One of 4 the patients tested negative by direct-ELISA had BPI-ANCA while the remaining three had no antibodies against other tested ANCA-target antigens. Thus, the sensitivity of the anchor ELISA was 71.4% (41.9-91.4) for both assays with a specificity of 98.4% (96.8-99.4) for the anchor ELISA and 97.9% (96.0 -99.1) for the immunoblot. In contrast, PR3-ANCA were detected by capture ELISA in only one of the 14 patients (sensitivity 7.1% [1.2-33.9]). Thus in this group, the overall diagnostic performance of the anchor ELISA (AUC 0.89 [0.86-0.92]; $P < 0.001$) and Immunoblot (AUC 0.85 [0.81-0.99]; $P < 0.001$) was significantly higher compared to the capture ELISA (AUC 0.53 [0.48-0.58]).

*Ability to detect PR3-ANCA in sera tested negative for C-ANCA and PR3-ANCA by conventional direct ELISA*

PR3-ANCA were detected at low level (13.7 AU/ml) by anchor ELISA in serum from one of 19 patients with biopsy-proven WG which all had been tested negative for C-ANCA and PR3-ANCA by conventional direct ELISA. Immunoblots for PR3-ANCA were negative for all 19 patients and no other flourescence patterns or ANCA target antigens were detected by IFT and direct ELISA.

*Ability to detect PR-3 ANCA in sera from patients with localised WG*

Using a cut off level of 10 AU/ml as above, PR3-ANCA or C-ANCA were detected in 4 of 28 patients with localised WG by anchor ELISA, IFT and immunoblotting (sensitivity 14.3% [4.1-32.7], respectively), in 3 patients by direct ELISA (sensitivity 10.7% [2.4-28.3]) and one patient by capture ELISA (sensitivity 3.6% (0.6-18.4). Specificity was high for all tests and ranged from 98.4 to 99.2%. However, ROC curve analysis (Fig. 1, panel B) demonstrated that in these patients with localised WG the overall diagnostic performance of the anchor ELISA (AUC 0.80 [0.76-084]) was significantly greater as compared to indirect IFT (AUC 0.57 [0.52-0.62)]; $P = 0.003$), direct ELISA (AUC 0.55 [0.50-0.60]; $P < 0.001$), capture ELISA (AUC 0.51 [0.46 - 0.56)]; $P < 0.001$), and immunoblotting (AUC 0.56 [0.51-0.61)]; $P < 0.001$). Thus, if the cut-off level for the anchor ELISA of 10 AU/ml was lowered to 2.7 AU/ml PR3-ANCA were detected in 50% of patients with localised WG (sensitivity 50.0% (30.7-69.3) with a still acceptably high specificity of 84.8% (81.1-88.0). The "optimum cut-off" by ROC analysis was determined to be at 1.8 AU/ml and resulted in a sensitivity of 82.1% (63.1-93.9) and a specificity of 68.4 (63.8-72.7). Sera from the 450 disease controls that showed positive binding in the borderline range from 1.8 to 10 AU/ml were equally distributed between the different diagnoses. In contrast, lowering the cut-off levels for the direct ELISA and capture ELISA did not result in a significant increase of sensitivity.

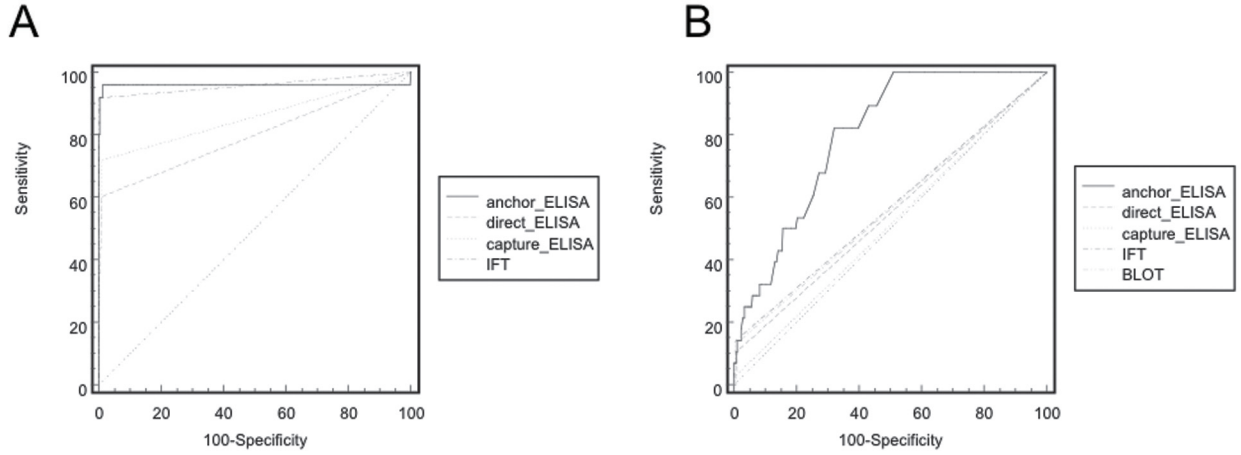


**Fig. 1.** Receiver operating characteristics curves of anchor ELISA, direct ELISA, capture ELISA, indirect immunoflourescence (IFT) and immunoblotting (BLOT) for detection of PR3-ANCA in 25 prospectively collected patients with early systemic or generalised WG (**panel A**) and 28 retrospectively studied patients with localised WG (**panel B**).

**Table II.** Diagnostic performance of anchor ELISA, direct-ELISA, capture ELISA and indirect immunofluorescence for diagnosis of WG.

| | Sensitivity (% [95% CI]) | Specificity (% [95% CI]) | AUC/ROC [95% CI] | *P* (AUC) versus Anchor ELISA | Direct ELISA | Capture ELISA | IFT |
|---|---|---|---|---|---|---|---|
| Anchor ELISA | 96.0 [79.6 -99.3] | 98.5 [97.0 - 99.4] | 0.96 [0.94 -0.98] | - | 0.002 | 0.032 | 0.997 |
| Direct ELISA | 60.0 [38.7 - 78.8] | 99.0 [97.6 - 99.7] | 0.80 [0.76 -0.83] | 0.002 | - | 0.135 | 0.001 |
| Capture ELISA | 72.0 [50.6 - 87.9] | 99.3 [97.9 - 99.8] | 0.86 [0.82 -0.89] | 0.032 | 0.135 | - | 0.019 |
| Indirect IFT | 92.0 [73.9 - 98.8] | 99.0 [97.6 - 99.7] | 0.96 [0.94 -0.98] | 0.997 | 0.001 | 0.019 | - |

AUC/ROC: Area Under Receiver Operating Characteristics Curve; ELISA: Enzyme Linked Immunosorbent Assay.

## Discussion

Results of the present study show that anchor ELISA is a highly sensitive, but very specific method for detection of PR3-ANCA in patients with WG. In prospectively collected patients with WG, the overall diagnostic performance of the anchor ELISA as analysed by ROC curve analysis was significantly better compared to the direct ELISA and capture ELISA and was at least equivalent compared to IFT.

In retrospectively collected patients with WG who had tested positive for C-ANCA on IFT, but who were negative for PR3-ANCA on direct ELISA the anchor ELISA detected PR3-ANCA in 71.4% of patients with high specificity. The diagnostic performance in this cohort was again superior to capture ELISA, which is often used as a second confirmatory assay in patients with negative direct ELISA. The sensitivity and specificity of this novel method was equivalent to immunoblotting. Given the fact that a C-ANCA pattern on IFT is not necessarily due to PR3-antibodies, but may be caused by other target antigens such as BPI, a negative confirmatory ELISA can lead to diagnostic uncertainties, particularly in patients with negative biopsy.

One of the most surprising findings of this study is however, the superior sensitivity of anchor ELISA in patients with localised WG. ROC curve analysis revealed a significantly better overall diagnostic performance of anchor ELISA compared to all other methods, suggesting that low levels of PR3-ANCA were present. Using a lower cut off level of 2.7 AU/ml PR3-ANCA were detected by anchor ELISA in 50% of patients with localised WG with a still acceptably high specificity of 84.8 %. This supports *in vitro* observations from our laboratory indicating that affinity-maturation of autoreactive B cells towards PR3 occurs in localised WG (15, 16) which results in the production of low levels of PR3-ANCA, which can only be detected with a very sensitive method such as the anchor ELISA.

The superior sensitivity of the anchor ELISA used in this study compared to conventional direct ELISA and capture ELISA is most likely due to a novel method of antigen-immobilisation. In conventional ELISA systems, coating is accomplished by binding of hydrophobic regions of the antigen to the plastic surface of the microtiter plate. However, those parts of the molecule that bind to the plate are then hidden what restricts the presentation of epitopes for binding with ANCA. Furthermore, binding by adhesion can alter the conformation of the epitopes. and thus can be another cause of falsely negative results on conventional ELISA. Capture ELISA preserves the conformation of the PR3 molecule by using a monoclonal antibody for binding to the plastic surface and thus is often of higher sensitivity compared to conventional direct ELISA. However, ANCA directed against the same epitopes used by the adhesion antibody are hidden which can decrease sensitivity. The anchor ELISA tested inhere uses a bridging molecule for binding to the microtiter plate which prevents adhesion to the plastic surface, preserves the conformation of PR3 and allows presentation of the native PR3 molecule without any hidden epitopes what ultimately leads to a high sensitivity of the assay.

We believe that the strengths of this study are the evaluation of different clinical scenarios with well-characterised patients with WG and the analysis of a large number of disease controls with inflammatory and non-inflammatory disorders that may resemble certain disease manifestations of WG and thus reflect the situation in which ANCA testing is performed in clinical practice. We acknowledge that patients with MPA may have PR3-ANCA as well as patients with WG may have MPO-ANCA, which was both not the case in the present study. Thus the absolute results may differ in cohorts with a different disease spectrum, e.g. from nephrology centers, but is not expected to be different in terms of the relative diagnostic performance of the assays tested inhere. Furthermore, prospective longitudinal studies are needed to evaluate the value of this method for monitoring disease activity.

In summary, anchor ELISA is a novel highly sensitive and specific method for detection of PR3-ANCA in patients with WG. In case future studies should confirm the high sensitivity and specificity of this technique, anchor ELISA may replace the need to perform both IFT and ELISA in routine clinical testing.

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