



PR3-ANCA: A promising biomarker for ulcerative colitis with extensive disease



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ABSTRACT

Background: We determined if PR3-ANCA is a biomarker that differentiates ulcerative colitis (UC) from Crohn's disease (CrD).

Methods: A total of 946 sera were tested, including 86 granulomatosis with polyangiitis (GPA) and 491 inflammatory bowel disease (IBD) patients (283 UC and 208 CrD), 264 pathological controls (various diseases) and 105 healthy individuals. All samples were tested for PR3-ANCA by ELISA (QUANTA Flash Lite®, INOVA Diagnostics) and chemiluminescent immunoassays (CIA QUANTA Flash PR3). Conventional anti-neutrophil cytoplasmic antibody (ANCA) indirect immunofluorescence assays (IIF) was performed with NOVA Lite™ (INOVA Diagnostics).

Results: PR3-ANCA by CIA were detected in 31.1% UC vs. 1.9% CrD sera ($p = 2.2E - 16$), and by ELISA in 6% UC and 0% CrD ($p = 0.0003$). In GPA patients, PR3-ANCA were detected in 75.6% by CIA and 61.6% by ELISA ($p < 0.05$). PR3-ANCA by CIA were more prevalent in E3-UC compared to E1/2-UC ($p < 0.05$), and in patients with shorter disease duration ($p < 0.0001$). PR3-ANCA showed similar sensitivity, but significantly higher specificity ($p < 0.05$), compared to atypical pANCA by IIF.

Conclusion: The novel PR3 CIA may prove helpful in the differentiation of CrD from UC, as well as in the identification of UC patients with more extensive disease.

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1. Introduction

The diagnosis of ulcerative colitis (UC) and Crohn's disease (CrD) is largely based on endoscopic and histologic assessment of the inflamed tissue [1]. The terms "indeterminate colitis" or "inflammatory bowel disease unclassified" (IBD-U) describe patients where the diagnosis of UC or CrD is not clear [2]. The differential diagnosis may be complicated in patients with irritable bowel syndrome and celiac disease or other colorectal diseases, with features indistinguishable from those seen in patients with IBD [3].

Non-invasive, economical tests that could accurately rule in or rule out IBD, as well as differentiate CrD from UC would provide a valuable

clinical resource [2,4]. Autoantibody testing for anti-neutrophil cytoplasmic antibodies (ANCA) giving an atypical perinuclear (pANCA) pattern sometimes referred to as xANCA [5], and anti-*Saccharomyces cerevisiae* antibodies (ASCA), has been recommended as a way to help distinguish UC from CrD [3,6–9]. ASCA sero-positivity is a predominant feature of CrD, while atypical pANCA is a marker of UC [6,8]. Both ASCA and ANCA have been reported to predict the development of IBD [10]. ANCA directed against proteinase 3 (PR3-ANCA), or myeloperoxidase (MPO) are used in the diagnostic workup of vasculitis such as granulomatosis with polyangiitis (GPA), until recently known as Wegener's granulomatosis (WG) [11], and microscopic polyangiitis (MPA). Conventional screening for PR3-ANCA includes the indirect immunofluorescence assay (IIF) on ethanol-fixed human neutrophils followed by confirmatory ELISA using immobilized PR3 [11]. PR3-ANCA generate a cytoplasmic staining pattern (cANCA) on ethanol-fixed human neutrophils [11]. Several laboratories also evaluate specimens on formalin-fixed human neutrophils [6]. Despite several studies, the specificity of ANCA in IBD, however, is poorly defined [3,12].

Over the last decade, a variety of different methodologies have been developed and commercialized for the detection of PR3-ANCA,

Abbreviations: ASCA, anti-*Saccharomyces cerevisiae* antibody; cANCA, cytoplasmic anti-neutrophil cytoplasmic antibodies; CIA, chemiluminescence assay; CrD, Crohn's disease; CU, calculated units; GPA, granulomatosis with polyangiitis; IBD, inflammatory bowel disease; IIF, indirect immunofluorescence assay; LIA, line immunoassay; pANCA, perinuclear anti-neutrophil cytoplasmic antibodies; UC, ulcerative colitis; WG, Wegener's granulomatosis.

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including IIF on human neutrophils, enzyme-linked immunosorbent assays (ELISA) [13], capture [14,15] and anchor assays [16,17], line immunoassays (LIA) [18], and multiplex assays [19,20] using native purified PR3 and, more recently, recombinant PR3 antigen [21]. Despite several comparative studies, it remains debatable as to which methodology for PR3-ANCA detection provides the highest clinical accuracy for the diagnosis of GPA [13,22]. Several studies published over the last decade suggested that the sensitivity of both capture as well as novel anchor assays were superior to classical ELISA and even to IIF [13,17,23]. Whether this also holds true for the detection of PR3-ANCA in patients with IBD remains unclear. Moreover, the relevance of PR3-ANCA in the routine investigation of IBD has not been established.

In contrast to previously reported data [24], more recent findings indicate that PR3-ANCA can also be detected in a significant percentage of patients with IBD [20]. This raises the possibility that akin to GPA, PR3-ANCA may be a diagnostic marker for IBD. While several antibody tests can assist in the diagnosis of CrD (including ASCA and GP2) [25,26], the only reliable test for UC is atypical pANCA detected by IIF [6]. However, IIF is time consuming, observer-dependent, has low throughput requiring highly-trained personnel and can generate significant variation [27]. Equally important, ANCA testing as part of IBD serology is unspecific since a significant portion of patients with chronic inflammatory diseases, not directly related to IBD, can generate a positive test result [2,7]. IIF is also unable to provide information about specific reactivity to individual ANCA antigens [3,7]. During the development phase of a novel chemiluminescence PR3-ANCA immunoassay (CIA) on a random access auto-analyzer (Bio-Flash®), we confirmed that a significant percentage of IBD patients tested positive for PR3-ANCA. This observation prompted us to investigate the potential clinical utility of the novel PR3 CIA using covalently bound native PR3 antigen for the detection of PR3-ANCA in IBD.

2. Materials and methods

2.1. Subjects

Tests were performed in a total of 946 serum samples. The main study cohort included 491 consecutive IBD patients who were followed by a single physician (AF) in the outpatient clinics of 2 Tertiary Referral Centers in England (St. Mark's Hospital and University College Hospital, London). These included 283 samples from UC patients (130 males, 46%) with a mean \pm SD age of 49 ± 16.06 years and a disease duration of 14 ± 13.02 years; and, 208 CrD patients (89 males, 43%) with a mean \pm SD age of 35.5 ± 14.04 years and a disease duration of 12 ± 9.91 years. The diagnoses of CrD and UC were made based on current standard clinical, radiological, endoscopic and histological criteria (Lennard-Jones criteria) [28], and the Montreal Classification [29] was used to describe the extent of colitis (E1: 9%, E2: 25%, E3: 62%, 4% of patients had colon resection) and 25% of them had at least one episode of severe colitis (Montreal Classification: S3). Additionally, 4 samples from patients with indeterminate colitis (IC) were tested from the same study cohort. A written consent was obtained from patients and the protocol was approved by the Local Ethics Committee (UK). Patient data were anonymously used under consideration of the latest version of the Helsinki Declaration of human research ethics.

Tests were also performed on serum samples collected from 86 GPA patients originating from 2 clinical centers (Center I: Maastricht, The Netherlands, $n = 26$; Center II: Bad Bramstedt, Germany, $n = 60$). Two hundred sixty-four serum samples from patients with various diseases were also included as pathological controls (Table 1). Celiac disease (CD) was diagnosed using CD-specific serology and confirmed by intestinal biopsy. In addition, 105 serum samples from apparently healthy donors, obtained from a commercial source (ProMedDx,

Table 1

PR3-ANCA in granulomatosis with polyangiitis, inflammatory bowel disease and various controls.

Group	QUANTA Flash PR3		QUANTA Lite PR-3		Fisher test <i>p</i> value
	<i>n</i> =	No. (%) pos	<i>n</i> =	No. (%) pos	
Granulomatosis with polyangiitis	86	65 (75.6%)	86	53 (61.6%)	< 0.001
Microscopic polyangiitis	10	1 (10.0%)	10	1 (10.0%)	NS
Churg–Strauss syndrome	10	0 (0.0%)	10	0 (0.0%)	NS
Ulcerative colitis	283	88 (31.1%)	283	17 (6.0%)	< 0.001
Crohn's disease	208	4 (1.9%)	208	0 (0.0%)	NS
Celiac disease	50	1 (2.0%)	ND	ND	ND
Healthy individuals	105	2 (1.9%)	105	0 (0.0%)	NS
Systemic lupus erythematosus	52	0 (0.0%)	53	0 (0.0%)	NS
Rheumatoid arthritis	14	1 (7.1%)	14	0 (0.0%)	NS
Systemic sclerosis	50	0 (0.0%)	50	0 (0.0%)	NS
Hepatitis C virus	6	0 (0.0%)	6	0 (0.0%)	NS
Multiple sclerosis	17	0 (0.0%)	17	0 (0.0%)	NS

ND = not determined.

NS = not significant.

^a Cut-off that yields 99% specificity (11 CU for QUANTA Lite PR-3 ELISA).

Norton, MA), were tested as normal controls. All tests were performed blinded to the operator.

2.2. PR3-ANCA assays

The QUANTA Lite® PR-3 (INOVA Diagnostics, Inc., San Diego, CA) is an ELISA based on native PR3 purified from human neutrophils immobilized on the surface of ELISA plates by direct adsorption. The QUANTA Flash® PR3 assay is a novel CIA performed on the Bio-Flash® instrument (Biokit S.A., Barcelona, Spain) using the same native antigen as used in the ELISA version.

The PR3 CIA is designed around the Bio-Flash® instrument, containing a luminometer, as well as all the hardware and liquid handling accessories necessary to perform the assay. Native purified human PR3 is coated onto paramagnetic beads and assayed on the Bio-Flash® system as previously described [30]. The PR3 CIA utilizes a predefined lot specific Master Curve that is uploaded into the instrument through the reagent pack barcode. Based on the results of running two calibrators, an instrument specific working curve is created, which is used to calculate chemiluminescent units (CUs) for each serum.

For the PR3 CIA IgA version, the isoluminol conjugated anti-human IgG was replaced by isoluminol conjugated anti-human IgA. All samples were tested by PR-3 ELISA and by the PR3 CIA.

2.3. 2-D electrophoresis and mass spectrometry

The purity of the PR3 protein was confirmed by 2-dimensional electrophoresis using a Zoom® IPGRunner™ System and XCell SureLock™ Mini-Cell (Invitrogen™ Life Technologies: Carlsbad, CA). The PR3 sample was solubilized in the sample rehydration buffer (8 M urea, 2% CHAPS, 0.5% (v/v) Zoom® Carrier Ampholytes, 0.0002% Bromophenol Blue, 20 mmol/l dithiothreitol (DTT)) and applied to the ZOOM® IEF Strips for 8–16 h. Isoelectric focusing (IEF) of the strip was sequentially performed: 200 V for 15 min, 450 V for 15 min; and then 750 V for 15 min, 2000 V for 30 min. The IEF Strip was equilibrated in NuPAGE® LDS Sample Buffer and NuPAGE® Sample Reducing Agent for 15 min. SDS/PAGE was performed at 200 V using NuPAGE Novex 4–12% Bis-Tris Zoom® Gels and NuPAGE® MOPS Running Buffer. The gel was stained with SilverQuest™ Silver Staining (Invitrogen™ Life Technologies) and the spot excised and submitted for mass spectrometry analysis at Southern Alberta Mass Spectrometry Centre (SAMS, University of Calgary, AB) for matrix-assisted laser desorption-ionization mass spectrometry (MALD-MS) using a trypsin digestion protocol. MS data was analyzed using ProFound (<http://prowl.rockefeller.edu/>)

cgi-bin/ProFound) and MoverZ software to observe the spectra (<http://prowl.rockefeller.edu/software/mz.htm>). Sequence alignment was confirmed using amino acid sequences from the national resource for molecular biology information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) and LALIGN tool on Expert Protein Analysis System (ExpASY) (<http://www.expasy.org/>).

2.4. Immunoblotting

The PR3 antigen was separated by 12% SDS-PAGE and the proteins then electrotransferred to nitrocellulose membranes. Serum samples positive for PR3 ANCA antibodies, diluted 1:100 in Sample Diluent (INOVA Diagnostics) were incubated for 1 h at room temperature, with rocking. After four 5-min washing steps in ELISA HRP Wash (INOVA), the nitrocellulose strips were incubated with alkaline phosphatase conjugated to goat anti-human IgG antibodies (Jackson ImmunoResearch, West Grove, PA: Fc γ Specific) diluted 1:3000 in Sample Diluent for 1 h at room temperature, with rocking. After four 5-minute washing steps in HRP Wash, the strips were incubated with BCIP/NBT alkaline phosphatase (Moss, Inc., Pasadena, Maryland). The reaction was stopped with deionized water and the results were digitized using the Biospectrum AC Imaging System (UVP, Inc., Upland, CA) and the software package VisionWorks LS Image Acquisition and Analysis Software (Ver 5.5.4, UVP, Inc.).

2.5. Other assays for autoantibody detection

Anti-*S. cerevisiae* antibodies (ASCA, IgA and IgG) were detected by QUANTA Lite® ASCA IgA and IgG (INOVA Diagnostics). Samples were also evaluated by IIF on ethanol and formalin fixed human neutrophils (NOVA Lite™, INOVA Diagnostics) as recommended [6,31]. ANCA with a perinuclear pattern on ethanol fixed neutrophils and a negative result on formalin fixed cells were considered as atypical pANCA [5,6]. Results of IIF were read by trained operators with at least 3 years of experience in reading ANCA IIF slides.

2.6. Inhibition experiments

Four samples were selected for inhibition experiments, one GPA patient sample with high titers of PR3-ANCA (by CIA), the UC patient sample with high titers of PR3-ANCA (by CIA), 1 sample from a UC patient negative for PR3-ANCA (by CIA) and 1 normal control were used in this experiment. Each sample was then diluted in PBS according to the instructions of NOVA Lite ANCA and incubated with PR3 coated magnetic beads (MPO beads as control) for 30 min. After incubation, the samples were magnetized and the supernatant was collected in a new tube for testing. The 4 immunoadsorbed samples were tested on the PR3-ANCA CIA and IIF (ethanol and formalin fixed cells) along with the same four samples which had not been treated by immunoadsorption. In addition, liquid phase titration studies with non-immobilized PR3 antigen were performed using one GPA and one UC patient sample that were previously found to have anti-PR3 activity. The PR3 antigen was serially diluted in an assay buffer starting with a concentration of 500 μ g/ml and dilutions were mixed with the PR3-ANCA positive sera and then residual PR3-ANCA reactivity was determined by CIA.

2.7. Statistical evaluation

The data was statistically evaluated using the Analyse-it software (Ver 2.03; Analyse-it Software, Ltd., Leeds, UK). Mann-Whitney U-test was used to analyze the difference between groups and Fisher exact test to qualitatively compare groups. For both evaluations, $p < 0.05$ were considered as significant. Correlations between different assays were analyzed using the Spearman equation. Receiver-operating characteristic (ROC) analysis was carried out to analyze the discrimination

between patients and controls. Where appropriate, 95% confidence intervals were calculated. No outlier was excluded from the study.

3. Results

3.1. Prevalence of PR3-ANCA in patients with GPA and non-IBD controls

The PR3-ANCA results obtained from testing of 86 GPA and 264 controls were used to generate a comparative descriptive analysis (Fig. 1a, Table 1) and to perform a comparative ROC analysis. The area under the curve (AUC) values were 0.93 (95% CI 0.89–0.97) for the CIA and 0.96 (95% CI 0.93–0.98) for the ELISA ($p = 0.1165$, Fig. 2a). The calculated quantitative correlation between the ELISA and the CIA utilizing the Spearman equation was $\rho = 0.54$ (95% CI 0.46–0.61).

Using the cut-off values recommended by the manufacturers, the sensitivities were 61.6% (95% CI 50.5–71.9%) for ELISA and 75.6% (95% CI 65.1–84.2%) for CIA. Specificities were 99.6% (95% CI 97.9–1.00%) for ELISA and 98.9% (95% CI 96.7–99.8%) for CIA. When cut-off values were selected resulting in 99% specificity for both assays, the sensitivities for ELISA (73.3%, 95% CI 62.6–82.2%) and CIA (75.6%, 95% CI 65.1–84.2%) were comparable.

3.2. PR3-ANCA in ulcerative colitis and Crohn's disease

To analyze the presence and clinical utility of PR3-ANCA in IBD, samples from 283 UC and 208 CrD were tested by PR3 CIA and ELISA. Using the CIA, 88/283 (31.1%) of patients with UC were positive for PR3-ANCA compared to 4/208 (1.9%) of CrD patients ($p = 2.22E - 16$). By ELISA, only 17/283 (6.0%) UC patients and none of the CrD patients were positive for PR3-ANCA ($p = 0.0003$). ROC analysis demonstrated a significant difference between ELISA and CIA in terms of discrimination between UC and CrD (AUC 0.76 vs. 0.60, $p < 0.0001$; Fig. 2 b). To assess the prevalence of PR3-ANCA where the diagnosis of UC or CrD was unclear, four IC patients were tested by PR3 CIA and 0/4 (0.0%) were positive for PR3-ANCA.

3.3. Comparison of IIF and CIA PR3-ANCA assays

Since ANCA determined by IIF is a commonly used serological test for UC we compared the diagnostic accuracy of ANCA by IIF and PR3-ANCA by CIA. The sensitivity of ANCA (atypical pANCA being positive) and PR3-ANCA was 34.9% and 31.1%, respectively. In terms of specificity, PR3-ANCA was superior to ANCA by IIF (98.1% vs. 90.4%, $p < 0.05$). When the assays were compared at the same specificity (90.4%), the sensitivity of the PR3-ANCA was significantly higher (52.7 vs. 34.9%). The likelihood ratio (LR +) was 16.17 and the LR (–) was 0.70 for PR3 CIA in UC vs. CrD. In contrast, the LR (+) and LR (–) values for atypical pANCA were 3.64 and 0.72, respectively (see Table 2). All 4 PR3-ANCA negative samples from IC patients were also negative by IIF.

3.4. Clinical associations of PR3-ANCA in UC

PR3-ANCA (CIA) positive UC patients had shorter disease duration (12.5 ± 12.1 years) compared to the PR3-ANCA negative patients (18.5 ± 13.0 years; $p < 0.0001$). Next patients were divided into seven groups according to their disease duration (0–2, 3–4, 5–7, 8–11, 12–16, 17–20 and >20 years), and analyzed in relation to their PR3-ANCA positivity. The highest prevalence of PR3-ANCA was found in patients with a disease duration of 0–2 years (16/33, 48.5%) and the lowest prevalence was found in patients with a disease duration of 17–20 years (16.7%, $p = 0.0172$). As shown in Fig. 3, longer disease duration was associated with a lower PR3-ANCA positive rate.

PR3-ANCA positive and PR3-ANCA negative UC patients did not differ significantly in terms of sex (34/88, 38.6% female vs. 97/195, 49.7%, respectively, $p = 0.1076$). PR3-ANCA positive patients were significantly

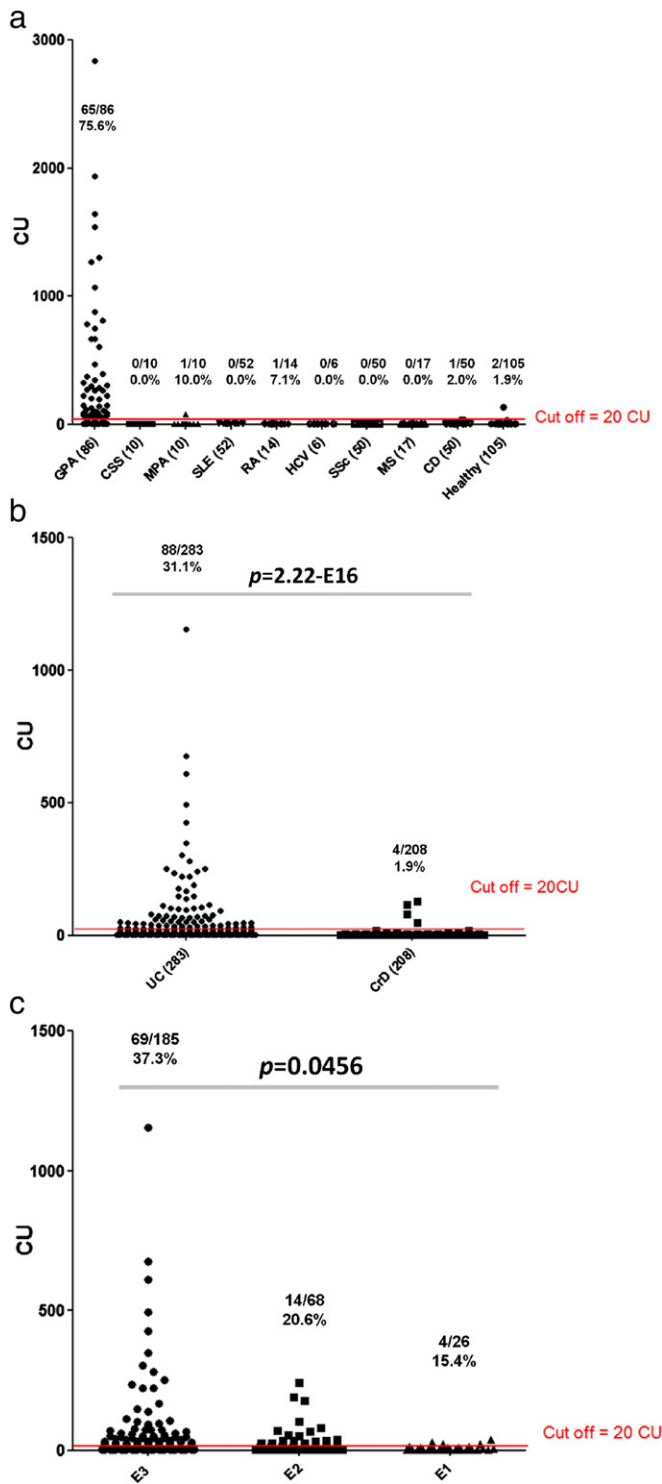


Fig. 1. Comparative descriptive analysis. a) PR3-ANCA in granulomatosis with polyangiitis (GPA) and various pathological and healthy controls. b) PR3-ANCA in ulcerative colitis (UC) and Crohn's disease (CrD). c.) Comparative descriptive diagram shows the PR3-ANCA titers in three different groups E1, E2 and E3. The titers in E3 were significantly higher compared to the other groups. No difference was found between E2 and E1. The recommended cut-off (20 CU) is indicated by the red lines. Groups are as follows: granulomatosis with polyangiitis (GPA); microscopic polyangiitis (MPA); Churg–Strauss syndrome (CSS); ulcerative colitis (UC); Crohn's disease (CrD); celiac disease (CD); systemic lupus erythematosus (SLE); systemic sclerosis (SSc); rheumatoid arthritis (RA); multiple sclerosis (MS); and chronic hepatitis C virus (HCV) infected patients.

younger at blood sampling (45.6 years vs. 49.9 years, $p = 0.0192$), but multi-variant analysis showed that this association was not independent on the disease duration.

In the PR3-ANCA positive and PR3-ANCA negative patients there was no apparent difference in the prevalence of either IgA or IgG ASCA (14.8% vs. 14.4%; Table 3). Of clinical relevance, PR3-ANCA was significantly more prevalent in E3 (37.3%) than in E2 (20.6%, $p = 0.0160$) and E1 (15.4%, $p = 0.0398$). Statistically significant differences were also observed between UC patients with E3 compared to E2/E1 combined (37.3% vs. 19.2%, $p = 0.0025$), but not between E2 and E1 (Fig. 1 c). When disease duration and classification (E3), i.e., the 2 parameters associated with increased PR3-ANCA prevalence, were simultaneously analyzed the highest prevalence of PR3-ANCA was found in patients with disease duration of 0–3 years (58.3%) and the lowest prevalence was found in patients with a disease duration of 17–20 years (22.7%; $p < 0.0001$; Fig. 3b).

Increased titers of IgA reactivity to PR3 by CIA were found in patients with GPA and in IBD patients, but neither IgA alone, or in combination with IgG enhanced discrimination of UC from CrD. The IgA and IgG PR3-ANCA reactivity was correlated (Spearman's rho = 0.47; $p < 0.0001$).

3.5. Immunoblotting and inhibition experiments

Immunoblotting and adsorption studies were performed to further assess the specificity of PR3-ANCA for the PR3 antigen preparation. Immunoblotting showed staining of a single band corresponding to the molecular mass of the PR3 antigen. This reactivity was specific for the PR3-ANCA positive serum samples from both GPA as well as UC patients, as PR3-ANCA negative control sera did not react (data not shown). 2-D electrophoresis and mass-spectrometry analyses confirmed that the single protein band was indeed human PR3.

Subsequently, reactivity to PR3-ANCA was tested before and after inhibition with PR3 coated beads (component of PR3-ANCA CIA) using serum samples of patients with UC and GPA. In the sample derived from the GPA patient, the PR3-ANCA reactivity as determined by CIA dropped to 1% (from 135,545 to 1880 RLU) and in the UC patient sample to 2% (from 57,498 to 1332 RLU). A significantly less pronounced decrease was observed with MPO coupled beads (control experiment). The sample from the GPA patient retained 85% (from 135,545 to 114,839 RLU) and the sample from the UC patient 81% (from 57,498 to 46,322 RLU) reactivity, respectively (Fig. 4 a.).

To further ascertain that the observed adsorption of PR3-ANCA is specifically due to PR3 (and not the bead surface), liquid phase inhibition studies were performed using PR3 antigen (not bound to paramagnetic particles). Results from both the GPA and UC patient samples showed a significant, concentration dependent decrease in reactivity (see Fig. 4 b.). The sample from the GPA patient decreased from 159,143 to 74,734 RLU (47%) and the sample from the UC patient from 68,580 to 39,015 RLU (57%).

Testing of the PR3-ANCA-positive GPA sample before and after inhibition with PR3 led to the loss of the strong granular cytoplasmic staining on both ethanol- and formalin fixed neutrophils. Before inhibition with PR3, the UC sample showed a perinuclear, faint granular rimming on the outer nuclear membrane of ethanol- and a weak granular cytoplasmic pattern formalin-fixed neutrophils. After inhibition, this serum showed a perinuclear pattern on ethanol fixed cells and no detectable staining on formalin-fixed cells.

4. Discussion

This study has found that the frequency of PR3-ANCA detected by CIA is >30% in sera from patients with UC, but <2% of patients with CrD and at least as rare in relevant pathological controls. We have also shown that the PR3-ANCA-based discrimination between UC and CrD is superior by CIA compared to IIF (atypical pANCA), as the CIA has a

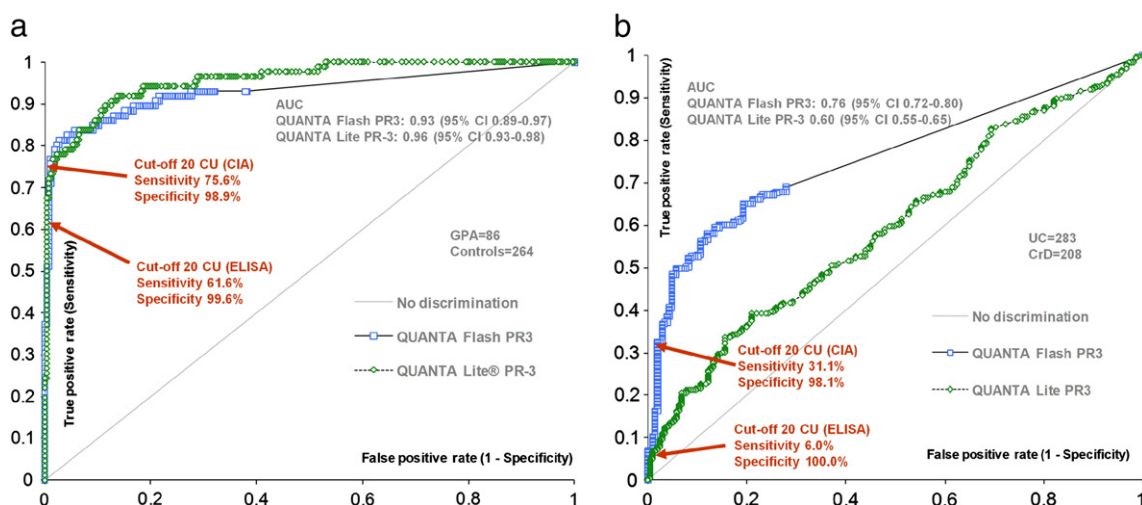


Fig. 2. Comparative receiver operating characteristic (ROC) analysis. Comparative ROC analysis was performed for PR-3 ELISA and PR-3 chemiluminescence immunoassay (CIA) using a) the results obtained from 86 granulomatosis with polyangiitis (GPA) samples and 264 controls (non-inflammatory bowel disease) and b) from 283 ulcerative colitis (UC) and 208 Crohn's disease (CrD) patients. The area under the curve values are shown for both comparisons.

much higher specificity. These data suggest that the application of CIA testing in routine practice can assist in the discrimination of UC from CrD. Since the diagnosis of UC and CrD is usually straight forward, and more challenging in patients with IC, we tested four patients with IC. None of our 4 IC samples in this study were positive for PR3-ANCA by PR3 CIA. Sample collection was limited and due to the lack of a unifying diagnostic criterion for IC, the utility of PR3-ANCA in IC remains unclear. However, a previous study using the same PR3-ANCA assay, reported PR3-ANCA in 2/19 (10.5%) of IC patients [32]. As expected, the prevalence of PR3-ANCA in IC was between the prevalence in UC and in CrD. Due to the small number of PR3-ANCA positive and negative patients, no comparison of the clinical phenotypes was possible. It is noteworthy that the prevalence of pANCA was lower compared to previous investigations. Further studies are mandatory to analyze the prevalence of PR3-ANCA and pANCA in large cohorts including patients with IC. Of clinical importance is the finding that the presence of PR3-ANCA in UC characterizes a subgroup of patients with more extensive colitis and shorter disease duration. Several studies aimed to identify the major target antigen of atypical pANCA in IBD, autoimmune hepatitis (AIH) and PSC [12,33], but the identity of major disease-specific target antigens remains poorly understood.

Previous investigation of patients with small vessel vasculitis [11] has shown that capture or anchor assays are more sensitive compared to conventional PR3-ANCA testing systems including IIF, likely because of the ability of the former to recognize conformational epitopes [13,16,34]. Based on these findings, several investigators have suggested that screening for PR3-ANCA should be done with a sensitive ELISA or other immunoassays and then confirmed by ANCA IIF, but a consensus has yet to be reached [21,35,36]. Although both assays utilize the identical native PR3 antigen preparation, the solid-phase and the antigen immobilization strategy used in the PR3 CIA differ significantly from ELISA technology in that the antigen is covalently attached to the

Table 2

Comparison of diagnostic performance of atypical pANCA and PR3-ANCA by QUANTA Flash PR3.

	QUANTA Flash PR3	Atypical pANCA
Sensitivity	31.1% (25.7–36.8)	34.9% (29.4–40.9)
Specificity	98.1% (95.1–99.5)	90.4% (85.5–94.0)
Positive predictive value (PPV)	95.7% (89.2–98.8)	83.2% (75.2–89.4)
Negative predictive value (NPV)	51.1% (46.1–56.1)	50.5% (45.3–55.7)
Positive likelihood ratio (LR+)	16.17	3.64
Negative likelihood ratio (LR-)	0.70	0.72

surface of a particle, in contrast to the passive adsorption used in most ELISAs. Our observations suggest that this difference may confer enhanced performance characteristics to CIA compared to ELISA and/or IIF.

Studies of PR3-ANCA in IBD are limited and have been based on relatively small cohorts of UC patients [20,37,38]. When twelve PR3-ANCA assays were analyzed in a comparative study using 22 IBD sera, the

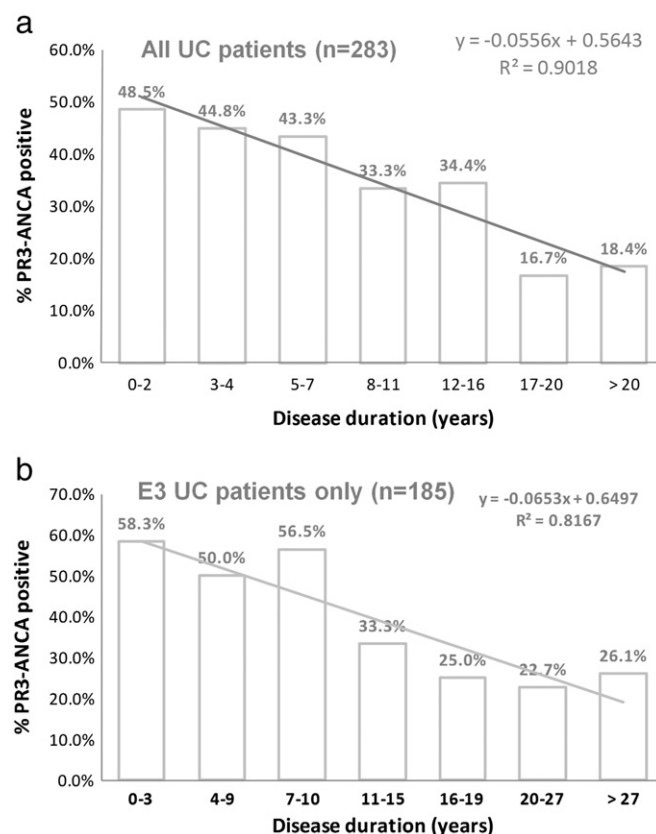


Fig. 3. PR3-ANCA and disease duration. The prevalence of PR3-ANCA in UC patients significantly decreases with increasing disease duration. a) In the entire UC cohort the highest prevalence of PR3-ANCA was found in patients with disease duration between 0 and 2 years (48.5%). The lowest prevalence was found in patients with disease duration of 17–20 years. b) In the UC classified as E3 according to the Montreal classification the highest prevalence of PR3-ANCA was found in patients with disease duration between 0 and 3 years (58.3%). The lowest prevalence was found in patients with disease duration of 17–20 years.

Table 3
Association of PR3-ANCA and demographic and serological data.

Autoantibody	PR3-ANCA neg (n = 195)	PR3-ANCA pos (n = 88)	p value
<i>Clinical features and symptoms</i>			
Mean age at diagnosis (SD) y	31.5 (13.3)	33.3 (15.4)	>0.1
Mean age at blood sampling (SD) y	49.9 (15.3)	45.6 (17.2)	0.0192
Mean disease duration (SD) y	18.5 (13.0)	12.5 (12.1)	0.0001
Females	97 (49.7%)	34 (38.6%)	>0.1
<i>Other autoantibodies</i>			
ASCA IgA positive	13 (6.7%)	10 (11.4%)	NS
ASCA IgG positive	28 (14.4%)	13 (14.8%)	>0.1

reported prevalence of PR3-ANCA ranged from 4 to 43%, raising concerns as to the reliability of the testing systems used in these reports [20,37,38]. The two assays with highest sensitivity (Bindzyme Anti-PR3 39% and Rainbow ELISA PR3 43%) also showed the lowest specificity (88%).

Different strategies to purify PR3 from human neutrophils have been established [39–41]. The production of recombinant PR3 (rPR3), which can be effectively used in PR3-ANCA immunoassays, has been difficult

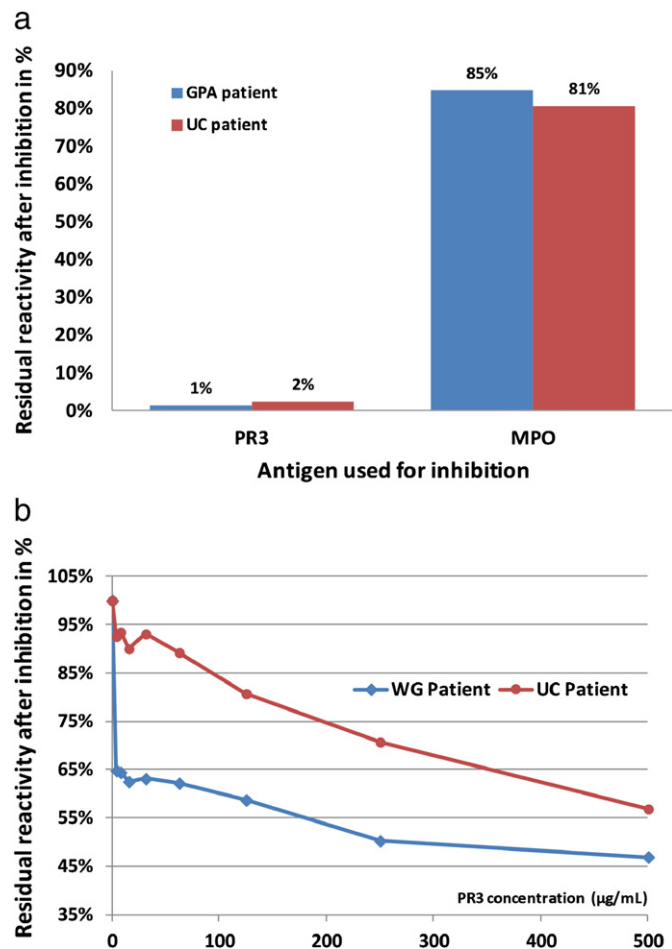


Fig. 4. Immunoadsorption of PR3-ANCA in samples from ulcerative colitis (UC) and granulomatosis with polyangiitis (GPA) patients. a.) Solid phase inhibition was performed using PR3- and control antigen (MPO) coupled to paramagnetic beads. Significant inhibition was observed with PR3, but not with MPO antigen. b.) Serum samples from a patient with UC and a patient with GPA were tested by CIA for PR3-ANCA with different concentrations of PR3 antigen in liquid phase inhibition test. Although PR3-ANCA reactivity was inhibited in both samples, stronger inhibition was observed in the patient sample from the GPA patient.

so far. It also appears that the majority of PR3-ANCA recognizes conformational epitopes on PR3 [11,41,42]. Several attempts have been made in different cell expression systems to produce immunoreactive recombinant PR3, but most of them have resulted in low sensitivity, possibly due to the instability of intact conformational epitopes [41]. However, more recent studies have obtained promising results, demonstrating good immunoreactivity of recombinant PR3 [21,41–43].

In the present study, we found PR3-ANCA in sera of IBD patients that reacted in two PR3 immunoassays, namely CIA and ELISA. Immunoblotting of purified native human PR3 used by the commercial assays revealed reactivity to a single band corresponding to the molecular mass of PR3. The identity of the protein was further confirmed by 2D gel electrophoresis and mass spectroscopy. These findings support the notion that the enhanced performance of the PR3 assays used in the present study is not likely due to cross-contamination of unrelated proteins. This was further supported by inhibition studies based on the purified PR3 antigen, which completely blocked the cANCA pattern by IFA and significantly reduced the PR3-ANCA reactivity by CIA using solid phase inhibition. Although the liquid phase inhibition was less pronounced, a significant and dose dependent inhibition of PR3-ANCA reactivity was observed both with a sample from a GPA and a UC patient. These results clearly demonstrate that the observed reactivity is due to antibody binding to PR3 antigen.

An association between PR3-ANCA by CIA and atypical pANCA in UC patients was found, but this was not strong ($p = 0.07$), supporting the existence of at least two independent ANCA specificities in a significant portion of UC patients. This is further supported by the observation that the majority of PR3-ANCA UC patients show a strong atypical pANCA but not a cANCA pattern in IIF, indicating the presence of several different autoantibody specificities in the serum of those patients.

IgA ANCA have been described in patients with vasculitis [44] and autoimmune liver diseases [45]. This prompted us to test for IgA PR3-ANCA using the PR3 CIA. We found that IgA PR3-ANCA are present in a significant portion of patients with IBD, and that the IgA reactivity with CIA was associated with IgG PR3-ANCA. However, in contrast to IgG PR3-ANCA, IgA PR3 ANCA was unable to discriminate UC from CrD. Therefore, IgA PR3-ANCA may be of limited diagnostic value in IBD.

PR3-ANCA is a marker autoantibody for the ANCA-associated vasculitides and in particular for GPA, and recent studies have described patients with overlapping features of UC and GPA [46–48]. The extent to which PR3-ANCA positive patients with UC will develop full-blown vasculitis over the course of their disease, needs to be assessed in large longitudinal studies.

Additional studies are also required to determine whether there is an overlap between the two chronic inflammatory diseases. While GPA typically affects the upper respiratory tract and the kidneys, UC typically is an inflammatory disease limited to the colon. Although 10% of patients with vasculitides can present with ulcerations of the colon [49], isolated gastro-intestinal tract involvement is infrequently seen in ANCA-positive patients with vasculitis [50]. In these cases, endoscopic assessment may be required in patients that are a diagnostic dilemma. It should be taken into account that the PR3-ANCA in patients with GPA is often associated with a cANCA pattern on ethanol-fixed neutrophils, while in UC most often an atypical ANCA is observed. The latter is most likely explained by reactivity to other antigens that have been reported in the past to be associated with UC [51].

5. Conclusion

Our data suggest that PR3-ANCA tested by highly sensitive assays, such as the novel CIA, are preferentially detected in patients with UC, as opposed to CrD, and may have clinical diagnostic and prognostic significance for IBD patients. Large multi-center studies are clearly warranted to validate this observation.

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