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IgA- and SIgA anti-PR3 antibodies in serum *versus* organ involvement and disease activity in PR3-ANCA-associated vasculitis

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Summary

Circulating immunoglobulin (Ig)A class anti-neutrophil cytoplasm antibodies (ANCA) directed against proteinase 3 (PR3) have been reported in ANCA-associated vasculitis (AAV) with mucosal involvement. However, secretory IgA (SIgA) PR3-ANCA has not been reported previously. In this study we compared serum levels of SIgA PR3-ANCA and IgA PR3-ANCA with IgG PR3-ANCA in relation to disease characteristics. Among 73 patients with AAV and PR3-ANCA at diagnosis, 84% tested positive for IgG PR3-ANCA, 47% for IgA-ANCA and 36% for SIgA PR3-ANCA at the time of sampling for the present study. IgA and IgG PR3-ANCA were represented similarly among patients with different organ manifestations, i.e. upper airway, lung or kidney at time of sampling. However, SIgA PR3-ANCA was significantly less represented among patients with upper airway involvement. During active disease, the proportions of IgA PR3-ANCA and SIgA PR3-ANCA-positive patients were significantly higher compared to inactive disease. Eight patients were sampled prospectively during 24 months from onset of active disease. In these patients, IgA PR3-ANCA and SIgA PR3-ANCA turned negative more often after remission induction compared to IgG PR3-ANCA. Our findings suggest that serum IgA PR3-ANCA and SIgA PR3-ANCA are related more closely to disease activity in AAV compared to IgG PR3-ANCA. Further studies are required to reveal if this has implications for disease activity monitoring. The mean number of PR3-ANCA isotypes increased along with disease activity, suggesting a global B cell activation during active disease.

Keywords: ANCA-associated vasculitis, disease, IgA PR3-ANCA, mucosal immunity activity

Introduction

Granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) are the two major forms of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). AAV predominantly affects small and medium sized arteries, and can thus engage multiple organs, mainly the upper and lower respiratory tract and kidneys, but less frequently skin, joints and peripheral nerves [1,2]. Experimental animal models and *in-vitro* set-ups support the concept that ANCA is of pathogenic importance in AAV by targeting surface-exposed myeloperoxidase (MPO) or proteinase 3 (PR3) either on cytokine-primed neutrophils, vascular endothelial cells [3–6] or on epithelial cells in glomeruli or lungs [7,8]. In experimental murine models, it has been demonstrated that ANCA-stimulated neutrophils reacted by forming 'neutrophil extracellular traps' (NET) exposing PR3 and MPO [9], which may induce ANCA and subsequent autoimmunity [10].

Immunoglobulin (Ig)G-class PR3-ANCA as well as MPO-ANCA can bind their target antigens exposed on the neutrophil surface (for instance, after cytokine-priming), resulting in cross-linking of Fc-receptors, complement activation and neutrophil oxidative burst [3,11–18]. ANCA of different isotypes have been described previously, including IgG, IgA and IgM-ANCA, where IgG-ANCA is the predominating circulating isotype in AAV, and is monitored frequently in GPA as a means to assess disease activity [19–21], although the clinical utility remains controversial [22–24]. With regard to mucosal manifestations in GPA, and as secretory IgA (SIgA) is the dominating isotype at mucosal sites, it is of interest to study IgA- and SIgA-class PR3-ANCA in

relation to organ manifestations and disease activity in AAV. Circulating IgA-class PR3-ANCA has been described previously in GPA [25], and IgA-ANCAs have been observed in IgA vasculitis (formerly known as Henoch–Schönlein purpura) [26], IgA-nephropathy [27], cutaneous vasculitis [28], liver cirrhosis [29] and inflammatory bowel diseases [30,31]. SIgA PR3-ANCA, however, has not been described previously in AAV. The present study was undertaken to analyse the occurrence, levels and clinical correlates of circulating IgA and SIgA PR3-ANCA in patients with IgG PR3-AAV based on the hypothesis that IgA/SIgA PR3-ANCAs correlate with mucosal disease manifestations (i.e. upper and/or lower respiratory tract) and disease activity.

Materials and methods

Patients and controls

Seventy-three patients diagnosed previously with AAV (GPA, $n = 61$ or MPA, $n = 12$), according to the 2012 revised international Chapel Hill consensus nomenclature of vasculitis [32], participated in the study. Baseline patient characteristics are presented in Table 1. The majority were Swedish by ancestry (70 of 73; 96%). Disease activity was assessed according to the Birmingham Vasculitis Activity Score (BVAS) 3·.0 [33]. At the time of diagnosis, all patients tested positive for IgG PR3-ANCA (performed at the accredited Clinical Immunology Laboratory at Linköping University Hospital, Linköping, Sweden). At the time of sampling, 22 of the 73 patients (30%) had active disease (BVAS > 0), and 51 were in remission (Table 1). In eight cases, we had access to serum samples collected longitudinally (every third month during 24 months). Plasma samples from 31 patients diagnosed previously with IgA nephropathy ($n = 17$) or IgA vasculitis ($n = 14$) and serum samples from 101 blood donors were used as controls. All samples were obtained with informed consent and the study was approved by the regional ethical review board in Linköping, Sweden. Serum samples were stored at −80°C until analysed.

Table 1. Baseline characteristics of patients diagnosed previously with ANCA-associated vasculitis (AAV) at the time of sampling.

	Cohort $n = 73$
Men/women	42/31
Mean age (years) (s.d.)	62·41 (15·88)
GPA/MPA* diagnosis	61/12
Mean disease duration (years) (range)	6·96 (0–21·3)
Active disease (%)	30
Mean prednisone dose (mg) (range)	15·2 (0–80)
Other immune suppressive therapy (%)†	63

*Granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA); s.d. = standard deviation.

†Other immune suppressive therapy besides prednisone were: methotrexate, mycophenolate mofetil, azathioprine, leflunomide, chlorambucil, and rituximab.

PR3-ANCA analyses

Detection of circulating PR3-ANCA was performed by high-sensitivity anti-PR3 enzyme-linked immunosorbent assays (ELISAs) (Orgentec Diagnostika GmbH, Mainz, Germany). For IgG PR3-ANCA antibodies, analyses and cut-off procedures were performed according to the manufacturer's instructions. Regarding IgA PR3-ANCA antibodies, samples were diluted 1 : 100 and the detection antibody was replaced by a horseradish peroxidase (HRP)-conjugated rabbit anti-human α-chain antibody (Dako A/S, Glostrup, Denmark) diluted 1 : 3000. For the detection of serum SIgA PR3-ANCA antibodies, samples were diluted 1 : 25, and an HRP-conjugated goat detection antibody towards human secretory component (Nordic BioSite AB, Täby, Sweden) was applied. Incubation conditions for all assays were in accordance with the manufacturer's recommendations for the IgG assay. A serially diluted strongly positive reference serum served as standard curve in each IgA and SIgA experiment (Supporting information, Fig. S1). Cut-off levels for the IgA and SIgA PR3-ANCA antibody assays were set to $>$ 99th percentile among 101 blood donors (IgA PR3-ANCA cut-off; 12·9 AU/ml, and SIgA PR3-ANCA cut-off; 18·0 AU/ml). Regarding IgG PR3-ANCA, we used the cut-off level suggested by the manufacturer (10 U/ml). Intra- and interassay variations were assessed by testing a medium-level sample (mean SIgA PR3-ANCA 119 AU/ml and mean IgA PR3-ANCA 96 AU/ml) 10 and five times, respectively. SIgA PR3-ANCA intra- and interassay variation was 7·2 and 11·1%, respectively. Regarding IgA PR3-ANCA, the intra- and interassay variation was 10·0 and 10·3%, respectively.

Antibody affinity purification

IgA anti-PR3 and IgG anti-PR3 were purified from a highly double-positive serum sample by affinity chromatography. A 3-ml serum sample was added to a Protein G column (Pierce Biotechnology, Woburn, MA, USA) and IgG antibodies were eluted using IgG Elution buffer (Pierce Biotechnology). The flow-through containing IgA-class antibodies was applied to a peptide M column (Invivogen Inc., San Diego, CA, USA). IgA-antibodies were eluted using 0·1 M sodium acetate (pH 4·0). Immediately after elution, both IgG and IgA class antibodies were neutralized with 1 M Tris-HCl (pH 8·3). The IgG and IgA class antibodies were applied to an in-house PR3 column (Euro Diagnostica AB, Malmö, Sweden). Bound antibodies were eluted with 0·1 M glycine (pH 2·7) and neutralized immediately with 1 M Tris-HCl (pH 8·3). The purified antibodies were stored at −20°C until further use.

Western blot

Purified antibodies were mixed with Laemmli buffer 1 : 1 (BioRad, Hercules, CA, USA) without 2-beta

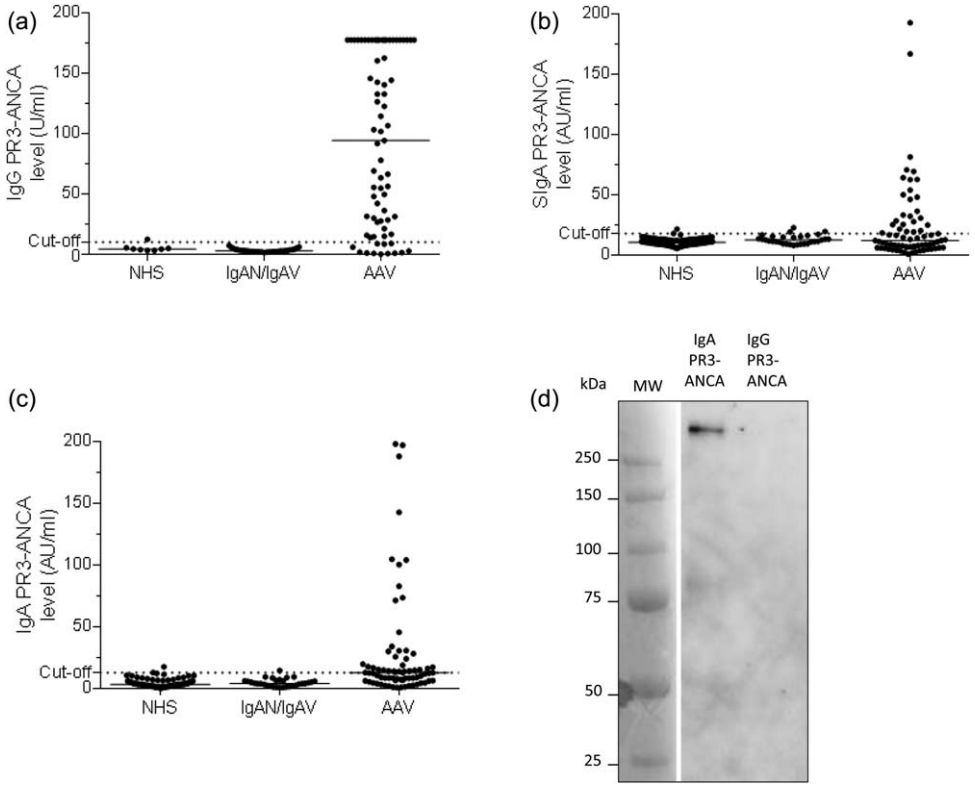

Fig. 1. Occurrence and levels of immunoglobulin (Ig)G proteinase 3- anti-neutrophil cytoplasm antibodies (PR3-ANCA) (a), secretory IgA (SIgA) PR3-ANCA (b), and IgA PR3-ANCA (c) in sera from patients diagnosed with ANCA-associated vasculitis (AAV). Western blot for secretory component (d). IgA and IgG were purified from a serum sample taken from a double-positive AAV patient and were then subjected separately to affinity purification on a PR3-column. Anti-secretory component reactivity was detected only in the IgA fraction.
NHS = normal healthy subjects; IgAN = IgA nephropathy; IgAV = IgA vasculitis.

mercaptoethanol for a native sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and applied to a precast Mini-Protean TGX gel (BioRad), which was run for 45 min at 180 V (Bio-Rad Power Pac HC). Precision plus protein all blue standards (BioRad) was used as a molecular weight marker and could be used for fluorescent blot detection at red wavelengths (635 nm laser). Bands on the SDS-PAGE gel were transferred to a polyvinylidine fluoride (PVDF) membrane using a PVDF transfer pack (Bio-Rad) and the Trans-Blot Turbo Transfer system (BioRad) for 7 min at 25 V. The membrane was then blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline-Tween 20 (TBST) with 0·1% Tween for 1 h. After blocking the membrane was incubated with a goat anti-human secretory component peroxidase antibody (GAHU/SC/PO; Nordic-Biosite, Täby, Sweden) 1 : 5000 in TBS with 0·1% Tween for 1 h at room temperature (RT). Thereafter, the membrane was washed in TBST with 0·1% Tween, mounted and incubated with enhanced chemiluminescence (ECL) prime Western blotting detection reagent (GE Healthcare, Little Chalfont, UK) for 1 min. Bands on the membrane were detected using the ChemiDoc MP system (BioRad) and the molecular weight marker was detected with fluorescent Western blotting (Qdots 625).

Statistical analyses

Correlations were analysed by Spearman's correlation, categorical data by Fisher's exact test and ordinal data by Mann–Whitney *U*-test. All the above-mentioned analyses were performed with IBM SPSS statistics version 22. Statistical analyses for distribution of anti-PR3 isotypes in organ involvement were performed using two-way analysis of variance (ANOVA) with GraphPad Prism version 6.

Results

PR3-ANCA isotypes

PR3-ANCA levels of different isotypes in patients and controls are shown in Fig. 1a–c. IgG PR3-ANCA occurred in 84% (61 of 73) (Fig. 1a), whereas SIgA PR3-ANCA occurred in 36% (26 of 73) of the patients (Fig. 1b), and IgA PR3-ANCA in 34 of 73 (47%) of AAV patients (Fig. 1c). IgG PR3-ANCA was significantly more common than the occurrence of IgA PR3-ANCA ($P < 0{\cdot}0001$) or SIgA anti-PR3 ($P < 0{\cdot}0001$). The occurrence of IgA PR3-ANCA did not differ significantly from SIgA PR3-ANCA ($P = 0{\cdot}24$). Among IgG PR3-ANCA- positive patients ever diagnosed with MPA, 17% (two of 12) were positive regarding IgA PR3-ANCA, whereas among the GPA patients, 53% (32 of 61) were IgA PR3-ANCA-positive. Among AAV patients, all anti-PR3 isotype levels correlated significantly; for IgG *versus* IgA PR3-ANCA the correlation coefficient was 0·56 ($P < 0{\cdot}001$), for IgG *versus* SIgA PR3-ANCA 0·51 ($P < 0{\cdot}001$), and for IgA *versus* SIgA PR3-ANCA 0·53 ($P < 0{\cdot}001$).

None of the 31 sera from patients with IgA-nephropathy or IgA vasculitis tested positive for IgG PR3-ANCA (Fig. 1a). IgA

PR3-ANCA occurred in one patient (7%) diagnosed previously with IgA vasculitis (Fig. 1c), while SIgA PR3-ANCA occurred at low levels in two cases diagnosed previously with IgA vasculitis (14%), and in one patient diagnosed previously with IgA-nephropathy (6%) (Fig. 1b).

A shown by Western blot in Fig. 1d, the anti-human secretory component antibody used in the high-sensitivity anti-PR3 ELISA detected a > 250 kDa band (compatible with 385 kDa SIgA) in the IgA PR3-ANCA eluate, but not in the IgG PR3-ANCA fraction.

PR3-ANCA isotypes and disease activity

In patients with active disease (BVAS > 0) at the time of sampling ($n = 22$), the frequencies of IgA PR3-ANCA- and SIgA PR3-ANCA-positive patients were significantly higher ($P = 0{\cdot}0001$ and $P = 0{\cdot}035$, respectively) than in patients with inactive disease (BVAS = 0) (Fig. 2a). There was no significant difference regarding the occurrence of IgG PR3-ANCA ($P = 0{\cdot}092$). At the time of sampling, IgA and IgG PR3-ANCA were represented similarly among different types of organ involvement, i.e. upper airway, lung or kidney (Fig. 2b).The occurrence of serum SIgA PR3-ANCA, however, was significantly lower among patients with upper airway involvement ($P = 0{\cdot}013$). Three of 12 patients testing positive for SIgA PR3-ANCA antibodies had neither upper airway nor lung involvement. We found statistically significant, although moderate, correlations

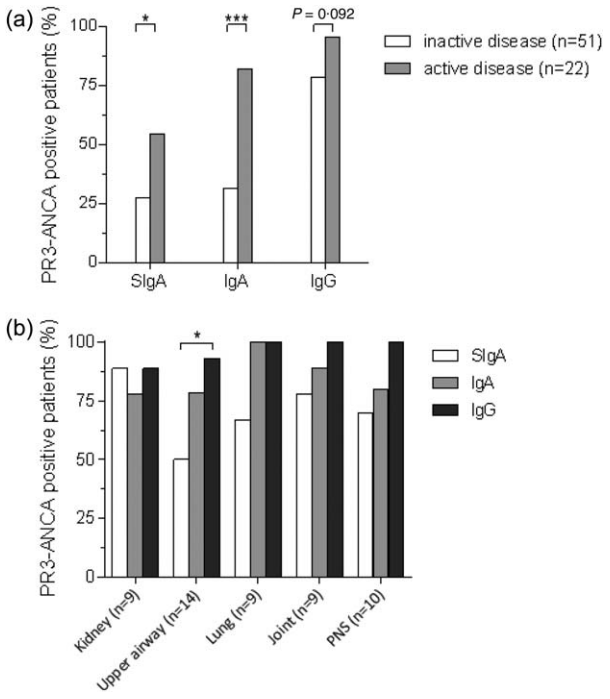

Fig. 2. Proteinase 3-anti-neutrophil cytoplasm antibodies (PR3-ANCA) levels in relation to disease activity (a) and in relation to organ involvement in patients with active disease at sampling ($n = 22$) (b). PNS = peripheral nervous system.

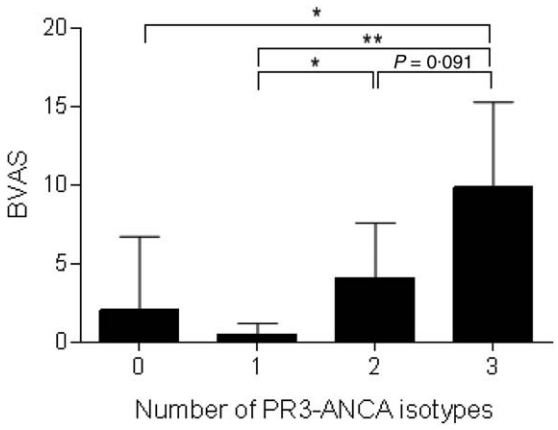

Fig. 3. Number of serum proteinase 3- anti-neutrophil cytoplasm antibodies (PR3-ANCA) isotypes in relation to disease activity in ANCA-associated vasculitis ($n = 73$). Bars represent mean Birmingham Vasculitis score with 95% confidence intervals depicted with error bars.

between PR3-ANCA isotype levels and BVAS: 0·43 for IgA PR3-ANCA ($P < 0{\cdot}001$), 0·37 for SIgA PR3-ANCA ($P = 0{\cdot}001$) and 0·33 for IgG PR3-ANCA ($P = 0{\cdot}005$). In the total cohort, mean BVAS was associated with the number of PR3-ANCA isotypes present in serum (Fig. 3).

Serial monitoring of PR3-ANCA isotype levels in patients with disease flare at baseline showed that, in four of eight patients, the IgG PR3-ANCA antibody levels remained stable during 24 months, while remission (BVAS = 0) was achieved from 3 months and onwards. By contrast, in six of eight patients IgA PR3-ANCA antibody levels declined below the cut-off level during the same period (Fig. 4). Regarding SIgA PR3-ANCA antibodies, only two patients tested positive for SIgA PR3-ANCA at baseline, and in both cases the SIgA antibody levels declined 3 months after disease flare onset (Fig. 4). None of the eight patients were in active disease flare during the 24-month follow-up or the following 6 months after the last samples were collected. Furthermore, these longitudinally collected samples displayed a significantly higher frequency of IgA PR3-ANCA and SIgA PR3-ANCA positive patients in active disease (at 0 months) compared to inactive disease (3 months to 24 months) ($P = 0{\cdot}022$, and $P = 0{\cdot}014$, respectively). This could not be demonstrated regarding IgG PR3-ANCA ($P = 0{\cdot}58$) (Fig. 5).

Discussion

In this study, based on IgG PR3-ANCA-positive AAV patients, we found that serum IgA PR3-ANCA correlated more clearly with disease activity than did IgG PR3-ANCA, regardless of clinically apparent mucosal involvement of the disease. To our knowledge, this is the first report on circulating SIgA-ANCA in vasculitis.

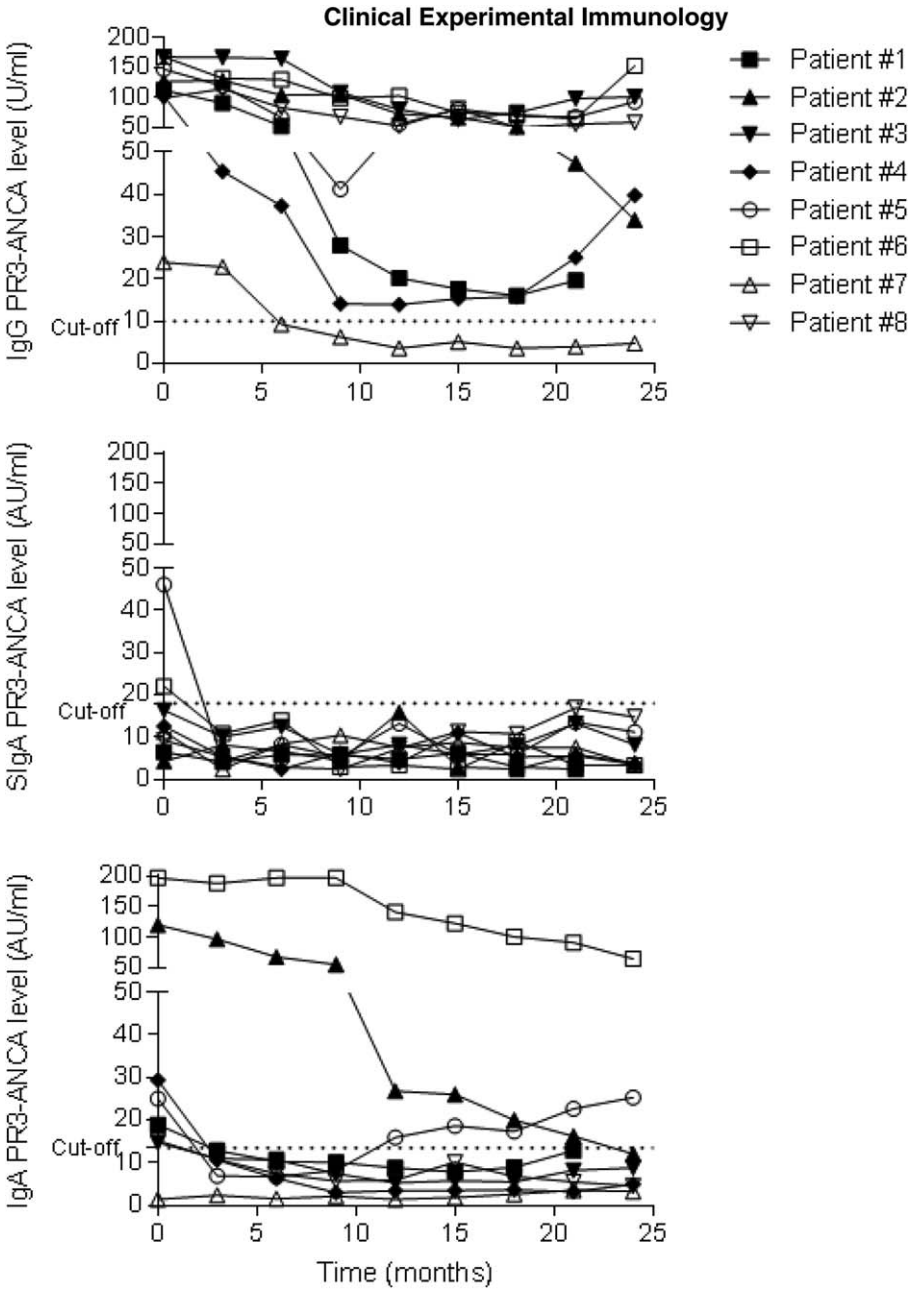

Fig. 4. Proteinase 3-anti-neutrophil cytoplasm antibodies (PR3-ANCA) levels in serum samples collected longitudinally from patients with disease flare at baseline and in remission from month 3 and onwards.

Kelley *et al.* have described previously that IgA PR3-ANCA antibodies in GPA occurred predominantly among patients with upper airway involvement and without renal disease [25]. With this in mind, and given the mucosal connection of IgA antibodies in general, we hypothesized that IgA PR3-ANCA, and SIgA in particular, would be better markers of 'mucosal disease activity' in AAV compared to IgG PR3-ANCA. Indeed, we found IgA PR3-ANCA to be less prevalent in inactive disease. However, in contrast to the study by Kelley *et al.*, we found no clear difference across different organ manifestations. Longitudinal sampling over 24 months revealed that IgG PR3-ANCA levels were high during disease flare and remained positive in remission. Conversely, IgA PR3-ANCA and SIgA PR3-ANCA tended to decline more obviously during remission. Accordingly, both the cross-sectional and longitudinal serum sample sets showed that the proportion of IgA- and SIgA PR3-ANCA-positive samples were significantly higher among patients with active disease compared to those in remission, while this was not seen regarding IgG PR3-ANCA. Moreover, among the tested isotypes, IgA PR3-ANCA had the highest correlation with BVAS. Taken together, this implies that analysis of IgA PR3-ANCA may be superior in monitoring disease activity.

To our surprise, serum SIgA PR3-ANCA was not more common among patients with upper or lower airway

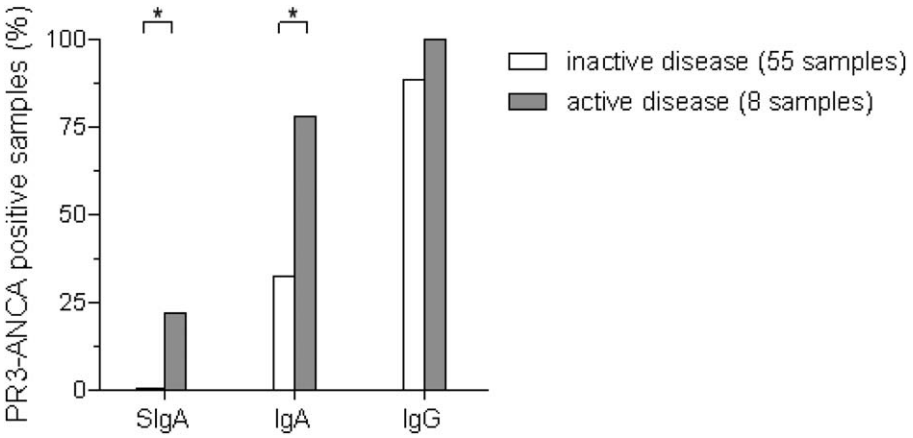

Fig. 5. Proteinase 3-anti-neutrophil cytoplasm antibodies (PR3-ANCA) isotype occurrence according to disease activity in longitudinally collected serum samples from eight patients.

involvement. In fact, upper airway involvement was the only manifestation where SIgA PR3-ANCA was significantly less prevalent compared with IgA and IgG PR3-ANCA (Fig. 2b). The fact that 25% of the patients testing positive for SIgA PR3-ANCA had neither upper airway nor lung involvement shows that circulating SIgA PR3-ANCA can be present without clinically apparent AAV-related mucosal manifestations. It remains to be elucidated whether this reflects subclinical upper airway or lung involvement or, instead, originates from other mucosal compartments, e.g. gastrointestinal or urogenital mucosa. The mechanism by which SIgA PR3-ANCA may be redirected to the circulation in AAV remains to be determined. Leakage across epithelial surfaces disrupted by inflammation is one obvious possibility, but there are also reports on active apical-to-basal retransportation mechanisms of SIgA from the gut lumen to submucosa [34–36].

The PR3-ANCA isotype repertoire appears to increase with global disease activity, resembling the situation reported for IgG subclasses of ANCA, where all patients with active disease had more than one IgG ANCA subclass while a single subclass was common in patients in remission [37]. Taken together, this indicates that active vasculitis is associated with a generalized B cell activation, promoted by, for instance, elevated B cell activating factor/B lymphocyte stimulator (BAFF/BLyS) levels [38], decreased numbers of peripheral blood regulatory B cells [39] and defective regulatory T cell (T_{reg}) function [40].

The generalized B cell activation in active vasculitis most probably involves B1 cells (shown to produce IgA in lungs and intestinal lamina propria [41]) and marginal zone B lymphocytes (MZ cells), as well as B2 cells activated in germinal centres. The possibility that MZ cells and B1 cells are more prone to give rise to short-lived IgA-producing plasma cells, while B2 cells are more prone to give rise to long-lived IgG producing plasma cells, may provide an explanation to why IgA/SIgA PR3-ANCA diminishes quicker after onset of treatment and correlates more effectively with disease activity. Upon successful treatment, the resolution of inflammation would then lead hypothetically to degeneration of short-lived plasma cell survival niches, and thereby decreased IgA production. It is also possible that the shorter half-life of IgA compared with IgG may contribute to a faster decline in IgA autoantibody levels. Although any type of activated B cell can become a plasma cell precursor (plasmablast), not all plasma cells have the capacity to become long-lived plasma cells. This may explain transient peak levels of circulating immunoglobins. For instance, this was illustrated in systemic lupus erythematosus (SLE), where large numbers of plasmablasts were also found during disease flare [42]. Moreover, following acute viral infections, the number of short-lived class-switched IgA-producing plasmablasts was found to be increased [43].

A weakness of the current study is the relatively small number of patients with active disease, especially in subgroups according to organ involvement. However, the patients are well-characterized, and we believe that they constitute a representative 'real-world' PR3-ANCA-positive AAV patient sample.

In conclusion, this study describes the occurrence of circulating SIgA PR3-ANCA in AAV, and provides evidence that IgA PR3-ANCA antibody analyses may improve serological monitoring of AAV patients.

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Disclosure

The authors declare no financial disclosures.

Author contributions

A. K. conceived the study and C. S. performed the experiments. M. S. and P. E. were responsible for patient recruitment and characterization. C. S. and A. K. analysed the data. C. S., P. E., M. S., T. S. and A. K. were involved in the design of the study and manuscript preparation.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Representative standard curves for the detection of secretory immunoglobulin (Ig)A (SIgA) proteinase 3-anti-neutrophil cytoplasm antibodies (PR3-ANCA) (a) and IgA PR3-ANCA (b).