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Citation for the published paper:

Abdgawad M, Hellmark T, Gunnarsson L, Westman KW,
Segelmark M.

"Increased neutrophil membrane expression and plasma level of proteinase 3 in systemic vasculitis are not a consequence of the -564 A/G promotor polymorphism."

Clinical and Experimental Immunology, 2006, Vol: 145, Issue: 1, pp. 63-70.

<http://dx.doi.org/10.1111/j.1365-2249.2006.03119.x>

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Increased neutrophil membrane expression and plasma level of proteinase 3 in systemic vasculitis are not a consequence of the -564 A/G promotor polymorphism

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Summary

Several findings link Proteinase 3 (PR3) to small vessel vasculitis. Besides being a major target of ANCA (Anti Neutrophil Cytoplasm Antibodies), previous findings have shown increased circulating levels of PR3 in vasculitis patients, increased levels of neutrophil membrane-PR3 (mPR3) expression and a skewed distribution of the -564 A/G polymorphism in the promotor region of the PR3 gene. In this study we elucidate how these three findings relate to each other.

The plasma concentration of PR3 was measured by ELISA, mPR3 expression by FACS and the gene polymorphism by real-time PCR. We compared results from 63 patients with ANCA Associated Systemic Vasculitis (AASV) with 107 healthy blood donors.

In accordance with previous reports, AASV patients had increased plasma concentrations of PR3 compared to healthy controls (mean 224 $\mu\text{g/l}$ versus 155 $\mu\text{g/l}$, $p < 0.0001$). They also showed an increased number of mPR3 positive neutrophils (60% versus 42%, $p < 0.001$). However, contrary to a previous report, we found no skewed distribution of the polymorphism in PR3 gene. There was a weak correlation between mPR3-Mean Fluorescence Intensity (MFI) and plasma PR3 among healthy controls and Myeloperoxidase-ANCA (MPO-ANCA) positive patients ($r = 0.24$, $p = 0.015$ and $r = 0.52$, $p = 0.011$ respectively).

In conclusion, increased plasma PR3 and high expression of mPR3 are associated with small vessel vasculitis, but neither of them is a consequence of the -564 A/G polymorphism of the PR3 gene promotor.

Introduction

The most common forms of small vessel vasculitis are Wegener's Granulomatosis (WG) and Microscopic Polyangiitis (MPA) and these diseases are strongly associated with ANCA. The main target of ANCA in WG is PR3. PR3 is an intracellular serine protease produced during the development of neutrophils and monocytes. It was originally described as an enzyme which degrades elastin and belongs to the family of neutrophil serine proteases[1-4].

Intriguingly, there are also several observations linking PR3 to AASV; (i) AASV patients have increased levels of circulating PR3 in the plasma[5], (ii) the proportion of neutrophils expressing PR3 on their plasma membrane is increased among AASV patients[6], (iii) circulating leukocytes from patients with AASV display up-regulated transcription of the PR3 gene[7, 8], (iv) deficiency of alpha1-Antitrypsin, the main inhibitor of PR3, seem to predispose for PR3-AASV[9, 10] and (v) a single nucleotide polymorphism in the promotor region of the PR3 gene have been found associated with WG[11].

Increased circulating levels of PR3 was first described by Henshaw et al.[12] and was later explored in our group by Baslund et al.[13] and in more detail by Ohlsson and co-workers[5]. It was shown that AASV patients had significantly higher levels of plasma PR3 compared to healthy blood donors as well as disease controls, this difference was independent of disease activity or ANCA type. The high plasma PR3 levels could not be explained by increased general inflammatory activity or decreased renal function and could not be connected to neutrophil degranulation[5].

In addition to azurophilic granules, PR3 is localized in specific granules and secretory vesicles[14]. It has also been found on the membrane of resting neutrophils[15]. Intriguingly, PR3 can be detected on the membrane of either the total neutrophil population or on a subset of neutrophils (Bimodal expression)[16]. The proportion of mPR3 positive (mPR3⁺)

neutrophils varies between individuals, but is highly stable over prolonged periods of time in a given individual suggesting a genetic background for this phenomenon[16]. This suggestion was reinforced by observations in two families and twin studies[6, 17].

Witko-Sarsat et al. reported that the vasculitis patients were skewed toward the mPR3^{high} phenotype. Considering the stability over time and the absence of relation to disease activity, they proposed the mPR3^{high} phenotype to be a genetic risk factor for vasculitis[6]. In contrast to these findings, Harper et al, reported that the proportion of mPR3⁺ neutrophils is higher in patients with acute vasculitis and sepsis and lower in patients with vasculitis in remission and healthy controls[18]. Results in the same direction were presented by Muller Kobold et al, who observed that the total level of mPR3 expression on neutrophils, measured as an expression index, was increased during active WG while during remission the values decrease to levels comparable to those of healthy controls[19]. Supporting the French results, Rarok et al showed that the mPR3⁺ proportion indeed is a stable feature in a given individual but, on the other hand, they reported that the total level of mPR3 expression is significantly associated with the incidence and rate of relapse in WG[20]. Taken together, mPR3 seems to be associated with AASV, although the mechanisms underlying this association have not been completely elucidated yet.

Studies by Gencik et al. revealed 10 polymorphisms in the PR3 gene but only one of them was found to be associated with WG. This was a Single Nucleotide Polymorphism (SNP) in the promotor region of the PR3 gene (-564 A/G). Allele frequency revealed a strong preponderance for the G allele in WG patients (49.2%) compared with the control group (34.4%, $P < 0.01$, Odds ratio= 0.5)[11]. The -564 G allele of this polymorphism results in a GC-box element, which is the potential binding site for the transcription factor SP1 that could result in increased transcription of the gene and subsequently increased expression of the

protein. Recent in vitro experiments did not exhibit any differences in promotor activity between G and A allele-containing reporter constructs[21].

In this study, we have investigated whether the level of mPR3 on isolated neutrophils and the level of PR3 in the plasma have a common origin and if that is the described polymorphism in the promotor region of the PR3 gene. This has been done by measuring the mPR3, plasma PR3 levels and ANCA in the same sample and correlating these parameters to the genotype as well as to each other.

Materials and Methods

Patients

Patients with systemic vasculitis were recruited from the departments of Nephrology, Lund University Hospital and Malmö University Hospital. The distinction between WG and MPA was made according to Chapel Hill Consensus Conference nomenclature [22]. Samples for DNA analysis were collected between 1996 and 2004, while all samples for surface expression and plasma concentrations were drawn during 2004. Most patients were in stable remission at time of sampling. Healthy blood donors from Blood Center Skåne were included as a control group. The study was approved by the Local Ethical Committee at the Faculty of Medicine, Lund University and informed consent was obtained from all subjects participated in the study.

Blood Sampling and separation

About 6 ml of peripheral blood were collected from each patient or donor in EDTA-anticoagulated tubes and then leukocytes were freshly isolated from whole blood by centrifugation on Polymorphprep™ according to the manufacturer's instructions (Axis-Shield, Oslo, Norway). After centrifugation, two bands were formed; the upper band contains

mononuclear cells and the lower band contains polymorphonuclear cells. The contaminating red blood cells among polymorphonuclear band were lysed using 0.83% NH₄ Cl 10 mM Hepes pH 7.0 for 7 min. at 37°C. The plasma layer, on top of the mononuclear band, was used to measure PR3 level in the plasma and ANCA level. The mononuclear band was used to extract DNA for studies on PR3 gene and the polymorphonuclear band was used to study membrane PR3.

DNA isolation

DNA was extracted as described by Miller et al.[23]. The DNA was quantified by spectrophotometer; the 260/280 ratios were consistently 1.8-2.0.

Gene typing

The SNP-specific PCR primers and fluorogenic probes were designed using Primer Express (Version 1.5; Applied Biosystems, Foster City, CA) following Applied Biosystems' guidelines as the following; -564-Forward Primer: 5'- GGCCTCCACCCACTCCAT -3', -564-Reverse Primer: 5'- AGGATTCTCAATCAAGAGGTGATTCT -3'. The fluorogenic probes were labelled with a reporter dye (either FAM or VIC) and are specific for one of the two possible bases (-564 G or A) in the PR3 promoter region; -564A-Taqman Probe: FAM-AGACCTCACCCAGGGT-MGB, -564G-Taqman Probe: VIC- ACCTCGCCCAGGGT-MGB. A MGB quencher probe was utilized on the 3' end by a linker arm. TaqMan Universal PCR Master Mix (Applied Biosystems) was used to prepare the PCR. Primers, probes and genomic DNA were added to final concentrations of 300 nM, 100 nM, and 0.5–2.5 ng/μl respectively. Controls (no DNA template) and reference control DNA were run in each 96 well plate to ensure there was no amplification of contaminating DNA and that signals from both probes was achieved. All PCR reactions were made in triplicates (25 μl per reaction) in optical 96 well plates using the ABI Prism 7000 Sequence Detection System (Applied

Biosystems) with two initial hold steps (50°C for 2 min., followed by 95°C for 10 min.) and 40 cycles of a two step PCR (95°C for 15 sec., 60°C for 1 min.). The -564 nucleotide was determined by the fluorescence ratio of the two SNP-specific fluorogenic probes.

Determination of surface PR3 expression by FACS analysis

Membrane PR3 expression on neutrophils was measured using flow cytometry as described previously[6]. Shortly, isolated cells were treated with 0.5 mg/ml heat-aggregated rabbit immunoglobulin (IgG; Sigma, St. Louis, MO) for 30 min. After washing, cells were incubated with murine monoclonal anti-human PR3 antibodies (4A5, Wieslab, Lund, Sweden), or with an isotype matched murine control antibody (IgG1, Sigma Biosciences, St. Louis, MO) for 30 min. Followed by a 30 min. incubation with FITC-conjugated (Fab')² fragments of rabbit anti-mouse antibodies (Dako Cytomation, Denmark). Labeled neutrophils were fixed with 1% paraformaldehyde for 10 min.

Fluorescence was analyzed on an EPICS XL-MCL flow cytometer (Beckman Coulter Inc., Miami, FL), and fluorescence intensity was standardized using Flow-Set Fluorospheres (Beckman Coulter Inc., Fullerton, CA). The cytometer can show the % of mPR3⁺ neutrophils as well as MFI.

The expression index of mPR3 was calculated as previously described by Rarok et al., the MFI was corrected for the Non-Specific Binding of an isotype matched irrelevant antibody (NSB) and multiplied by the percentage of mPR3⁺ neutrophils; Expression index = (MFI- NSB) × % mPR3⁺ neutrophils, and expressed in Arbitrary Units (AU)[20]. The individuals were divided into three mPR3 phenotypes, as previously described by Witko-Sarsat et al, according to their percentage of mPR3⁺ neutrophils corresponding respectively to 0 to 20% of

mPR3⁺ neutrophils (mPR3^{low} phenotype), 21 to 58% of mPR3⁺ neutrophils (mPR3^{intermediate} phenotype), and 59 to 100% of mPR3⁺ neutrophils (mPR3^{high} phenotype)[6].

ELISA assays of plasma PR3 and ANCA

Plasma PR3 level was detected as previously described[5]. Briefly, a microtitre plate was coated overnight with a purified mixture of two monoclonal PR3 antibodies (4A3 and 4A5, 3µg/ml each). Plasma samples diluted to 1/20 and 1/40 in sample buffer were added and the plates were incubated for 2 hours. After washing, bound PR3 was detected by incubation for 2 hours with affinity-purified rabbit anti-PR3 diluted to 1/300 in sample buffer. Washing was followed by the addition of the conjugate (alkaline phosphatase-labelled swine anti-rabbit IgG from Dako Cytomation, Glostrup, Denmark), diluted to 1/1000 in sample buffer and then incubated for 1 hour. ANCA was detected by standard direct ELISA using Wielisa[®] PR3-ANCA kit.

Statistical Analyses

Differences in continuous variables between two groups were analyzed using the Mann-Whitney U-test, some data sets that did not follow Gaussian distribution were transformed and then analyzed by unpaired t-test. For data sets that follow Gaussian distribution, t-test was used and results are given as Mean ± SD, All other results are given as Median, Inter Quartile range (IQR).

One-way ANOVA with Bonferroni's post-test was used for comparisons between more than two groups. Correlations were analyzed using Pearson rank test. Proportions between groups

were compared with the χ^2 test (Chi-squared test). A two-sided $P < 0.05$ was considered to be statistically significant.

Results

Genotype

DNA purified from peripheral blood of 189 blood donors and 132 patients with AASV (76 with WG and 56 with MPA) was subjected to genotyping using Allelic Discrimination-Real Time PCR. No significant differences were seen between any group of patients regarding gene or allele frequencies. Figure 1 shows the gene frequencies of patients and controls of the -564 A/G polymorphism in the promoter region of the PR3 gene. The allele frequencies in the patients were 36% for the A allele and 64% for the G allele compared to 31% and 69% for the healthy controls. When limiting the analysis to the patients with a clinical diagnosis of WG, we found an allele frequency of 40% for the A allele and 60% for the G allele, the corresponding figures for MPA patients were 30% and 70%. Thus, we could not verify the previous findings of an increased frequency of the G allele among WG patients.

Membrane PR3

Flow cytometry analysis of mPR3 on isolated neutrophils was performed on 107 healthy blood donors and 58 patients with AASV, 31 of these patients were men and 27 were women. Their mean age (at sampling) was 63.2 ± 16.4 years ranging from 17.8 to 86.5 years. A clinical diagnosis of WG was made for 35 patients (31 were PR3-ANCA positive, 3 MPO-ANCA positive and 1 ANCA-negative) and 23 patients were diagnosed as having MPA (20 MPO-ANCA positive, 3 PR3-ANCA positive). In total, 34 patients were PR3-ANCA positive, 23 patients were MPO-ANCA positive at diagnosis and one patient never exhibited any positive ANCA test.

The percentage of mPR3⁺ neutrophils as shown in Figure 2A was significantly higher in vasculitis patients 60 ± 27 percent compared to healthy blood donors 42 ± 22 , ($P < 0.0001$). This difference was independent of diagnosis as the MPA group had 62 ± 24 percent PR3 positive cells and the WG group had 58 ± 29 percent ($P = 0.0002$ and $P = 0.0007$ respectively). Similar results were observed when the patients were divided according to ANCA serology. When the patients were divided into the three predefined mPR3 phenotypes[6], the distribution of the vasculitis patients were skewed toward the mPR3^{high} phenotype, as shown in Figure 3. This stands in contrast to the control group that exhibited an equal distribution between the high and low phenotype groups (27% high, 50% intermediate, and 23% low). This difference between the two distributions was significant ($\chi^2 = 13.47$, $P = 0.0012$).

Also when comparing the MFI of mPR3, we found higher values for the vasculitis patients (median 1.43, IQR 1.03-2.12) compared to healthy blood donors (1.19, 0.915-1.58). However the differences were relatively small and only statistically significant, according to the Mann-Whitney U-test, when comparing all patients or the PR3-ANCA positive subgroup with the controls ($p = 0.04$ and 0.03 respectively).

Consequently when comparing the Expression Index of mPR3, we found higher values for the AASV patients (median 89.3, IQR 43.6-163 Units) compared to the group of controls (53.1, 25.2-87.8 Units, $P = 0.001$, Figure 2B). The median expression index was significantly higher for all subgroups compared to healthy blood donors. There were no significant differences between the subgroups neither based on diagnosis nor on serology.

Plasma PR3 and ANCA

Plasma PR3 was significantly elevated in the patients (mean \pm SD 224 ± 128 $\mu\text{g/l}$, $N = 63$) compared to healthy blood donors (155 ± 52 $\mu\text{g/l}$, $N = 130$), (unpaired t-test, $P < 0.0001$). When dividing the AASV patients based on their clinical diagnosis, we found that the MPA patients

had significantly higher PR3 concentrations ($256 \pm 162 \mu\text{g/l}$, N=26) than healthy blood donors ($P < 0.0001$). Similarly but to a lesser extent, WG patients had higher PR3 concentrations ($202 \pm 95 \mu\text{g/l}$, N=37) compared to healthy blood donors ($P = 0.0029$), as shown in Figure 4. Similar results were found when we divided the AASV patients into two subgroups based on their ANCA serology.

ANCA level in the patient plasma was also tested, 13 patients were PR3-ANCA positive at the time of sampling, out of the 35 who were PR3-ANCA positive at the time of diagnosis. Regarding MPO-ANCA, 10 patients were MPO-ANCA positive at the time of sampling out of the 26 who were MPO-ANCA positive at the time of diagnosis.

Genotype-Phenotype Correlations

There was no significant difference between the three promotor genotypes regarding any of the three mPR3 parameters (% of mPR3⁺ cells, MFI and E.I.) in the healthy blood donors (Chi-square test). Similarly, there was no difference between the three promotor genotypes regarding their mPR3 expression in the patients, Table 1.

When correlating the three promotor genotypes with the plasma PR3 in the healthy blood donors, there was a significant difference between the three genotypes, ($P = 0.0002$). The GG genotype had lower levels, which is opposite to the expected. A similar difference was observed in the patient group, but it was not significant ($P = 0.08$), Table 1.

Phenotype-Phenotype Correlations

There was no correlation between plasma PR3 and percentage of mPR3⁺ neutrophils in either controls or AASV patients. There was, however, a weak but significant correlation between plasma PR3 and MFI in healthy controls ($r = 0.24$, $P = 0.015$). A similar correlation was found in the MPO-ANCA positive subgroup of AASV patients ($r = 0.52$, $P = 0.011$). While no

correlation was found in the PR3-ANCA subgroup (Figure 5). On the other hand, we did observe a significant negative correlation between plasma ANCA levels and Plasma PR3 levels in the subgroup of PR3-ANCA patients ($r = -0.486$, $P = 0.0031$), while MPO-ANCA levels did not correlate with plasma concentrations of PR3.

Discussion

The aim of this study was to shed light on the possible relationship between different features of the PR3 protein previously linked to AASV. To this end, we have analyzed all these parameters in the same sample from a new cohort of AASV patients. In this study, we do confirm that AASV patients have more PR3 on the surface of isolated neutrophils compared to healthy blood donors. This was primarily due to an increased number of mPR3⁺ cells, which is in accordance with studies suggesting that a high percentage of mPR3⁺ cells is a risk factor for vasculitis[6]. In addition to high percentage, we also observed that our AASV patients had slightly increased mPR3 expression per cell (MFI) compared to healthy controls. This observation is compatible with the notion that MFI is a variable feature that correlates with disease activity and a possible marker of disease activity[19]. The slightly elevated MFI levels could be caused by low-grade inflammation, and our earlier studies have shown that AASV patients have increased levels of circulating cytokines also during remission[24]. Moreover, we found no significant differences regarding mPR3 expression (mPR3⁺ proportion or MFI) in vasculitis patients according to ANCA status.

In contrast to the mPR3 case, we were not able to confirm the findings from Gencik et al regarding the -564 A/G polymorphism. In our cohort, there was no skewing of this polymorphism towards the G allele (60% for WG patients versus 69% for healthy controls). The main discrepancy between our results and theirs was a lower percentage among their healthy controls (34%); a difference that could be explained by a genetic heterogeneity in the

background populations[11]. As no skewing was observed, it is not surprising that the mPR3⁺ high phenotype did not correlate to the GG genotype. This together with our previous finding that the -564 A/G SNP in the promoter region of proteinase 3 does not affect the promoter activity in vitro, we conclude that this SNP is not associated with increased mPR3 expression[21].

In this study, we confirm our previous finding that vasculitis patients have elevated plasma levels of PR3 compared to healthy controls (224 versus 155, $P < 0.0001$)[5]. We also found a significant correlation between plasma PR3 and the -564 A/G polymorphism in the healthy controls ($P < 0.0002$). This argues for a genetic component of plasma PR3 levels. However, the high plasma PR3 can not be explained by the binding of the SP1 transcription factor to the G allele, as the highest levels were found among those with the AA genotype (185 for AA versus 130 for GG). A more plausible explanation is that the A allele is in linkage disequilibrium with an allele at another locus that is actually associated with the high plasma phenotype. However, the existence of such gene locus could not explain the difference in plasma levels between patients and controls, as patients exhibited a similar difference plasma levels with regard to genotypes (223 for AA versus 154 for GG).

Our earlier studies did not show any correlation between plasma PR3 and NGAL, a marker of secondary granules, strongly arguing against that increased degranulation is the source for high plasma PR3 levels[5]. The present study shows a weak but significant correlation between plasma PR3 and mPR3-MFI among controls and among the MPO-ANCA positive patients. The existence of such correlation leads us to suggest that at least part of the plasma PR3 comes from shedding of PR3 from the membrane of neutrophils. The absence of correlation in PR3-ANCA patients could be explained by interference of the autoantibodies.

PR3-ANCA may enhance the clearance of plasma PR3 from the circulation or may act as a blocking agent for PR3, preventing its detection by ELISA. Supporting this notion, we found a significant negative correlation between plasma ANCA levels and Plasma PR3 levels in the subgroup of PR3-ANCA patients but not among those with MPO-ANCA.

This study shows increased mPR3 and plasma PR3 levels among AASV patients but fails to reveal which precedes the other. Nor can we answer if either of them is directly implicated in the pathogenesis of vasculitis as a risk factor or activity marker. As these questions might be fundamental for the understanding of the underlying mechanisms of the disease, they deserve further study. In order to resolve these questions, more knowledge is needed regarding the origin of the plasma PR3 and the nature of the association of PR3 with the plasma membrane.

In conclusion, this study demonstrates that the elevated mPR3 expression on the membrane of neutrophils and the elevated plasma PR3 are associated with vasculitis. In addition, our data shows that the elevated mPR3 expression and the elevated plasma PR3 are correlated to each other. According to our results, these elevated PR3 levels found in AASV patients are not a consequence of the -564 A/G polymorphism in the PR3 gene and further studies are needed to reveal their origin, as it may be a fundamental predisposing factor for the generation of PR3-ANCA.

Acknowledgements

This study was supported by grants from the Swedish Research Council (71X-15152 and 73X-09487), Crafoord Foundation and the Renal Foundation. We would like to thank Blood Center Skåne for providing blood samples, Wieslab AB for measuring plasma ANCA levels, Annika Andreasson for her assistance in FACS measurements.

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Tables

Table 1. Genotype-phenotype correlation. Comparison table showing the correlation between the three genotypes of the -564 A/G promotor polymorphism in the PR3 gene and the four PR3 parameters, % of mPR3 positive cells, MFI, E.I. and Plasma PR3 (median). **(A)** Shows the results of healthy blood donors. **(B)** Shows the results of patients with AASV.

A

Genotype	% of +ve cells	MFI	E.I.	Plasma PR3
AA	45	1.61	54.8	185
AG	40	1.13	53.3	167
GG	45	1.13	50.3	130
p	0.98	0.57	0.79	0.0002
N	83	83	83	110

B

Genotype	% of +ve cells	MFI	E.I.	Plasma PR3
AA	71	1.52	100	223
AG	65	1.42	109	199
GG	52	1.01	64.8	154
p	0.28	0.07	0.17	0.082
N	52	52	52	57

Figure legends

Figure 1. Distribution of the –564 A/G polymorphism in the promotor region of the PR3 gene. Black colour represents individuals homozygous for the A allele, white colour represents individuals homozygous for the G allele and grey colour represents heterozygotes. The results of 189 healthy blood donors are shown in (A) and 136 AASV patients are shown in (B).

Figure 2. Membrane expression of PR3 on neutrophils. Neutrophils stained with anti-PR3 murine monoclonal antibodies and followed by FITC-conjugated anti-mouse antibodies, were analyzed by Flow cytometry. (A) Shows the percentage of mPR3⁺ neutrophils in 107 healthy blood donors, 58 patients with AASV and two subsets of the same patients (23 having MPO-ANCA and 34 having PR3-ANCA). (B) Shows the expression index of mPR3 on neutrophils measured by arbitrary units (AU) for the same groups as in (A). All results are given as mean values \pm SD

Figure 3. Phenotype distribution of mPR3 on neutrophils. Individuals divided into three predefined groups, high (black)/intermediate (grey)/low (white), according to their percentage of mPR3⁺ neutrophils [6]. The distribution of these three groups is shown in 58 patients with AASV compared to 107 healthy blood donors.

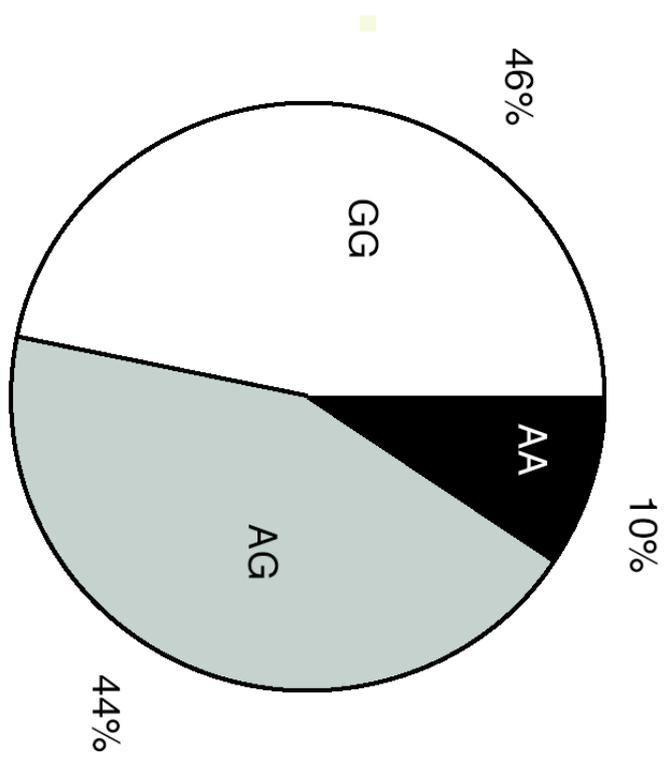
Figure 4. Concentration of PR3 in the plasma measured by ELISA. 130 healthy blood donors are compared with 63 patients with AASV and two subsets of the same patients (26 with MPA and 37 with WG).

Figure 5. Scatter diagrams showing the correlation between the mean fluorescence intensity of mPR3 on neutrophils and plasma PR3 concentrations. (A) Shows the results

of 102 healthy blood donors **(B)** Shows the results of 58 patients with AASV. Circles indicate 23 MPO-ANCA positive patients and triangles 34 PR3-ANCA positive patients.

Figure 1

A



B

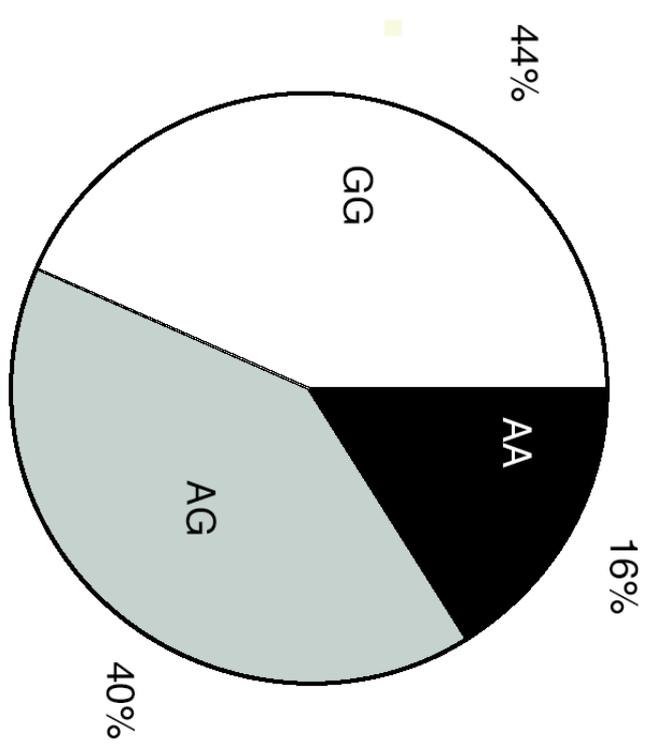


Figure 2

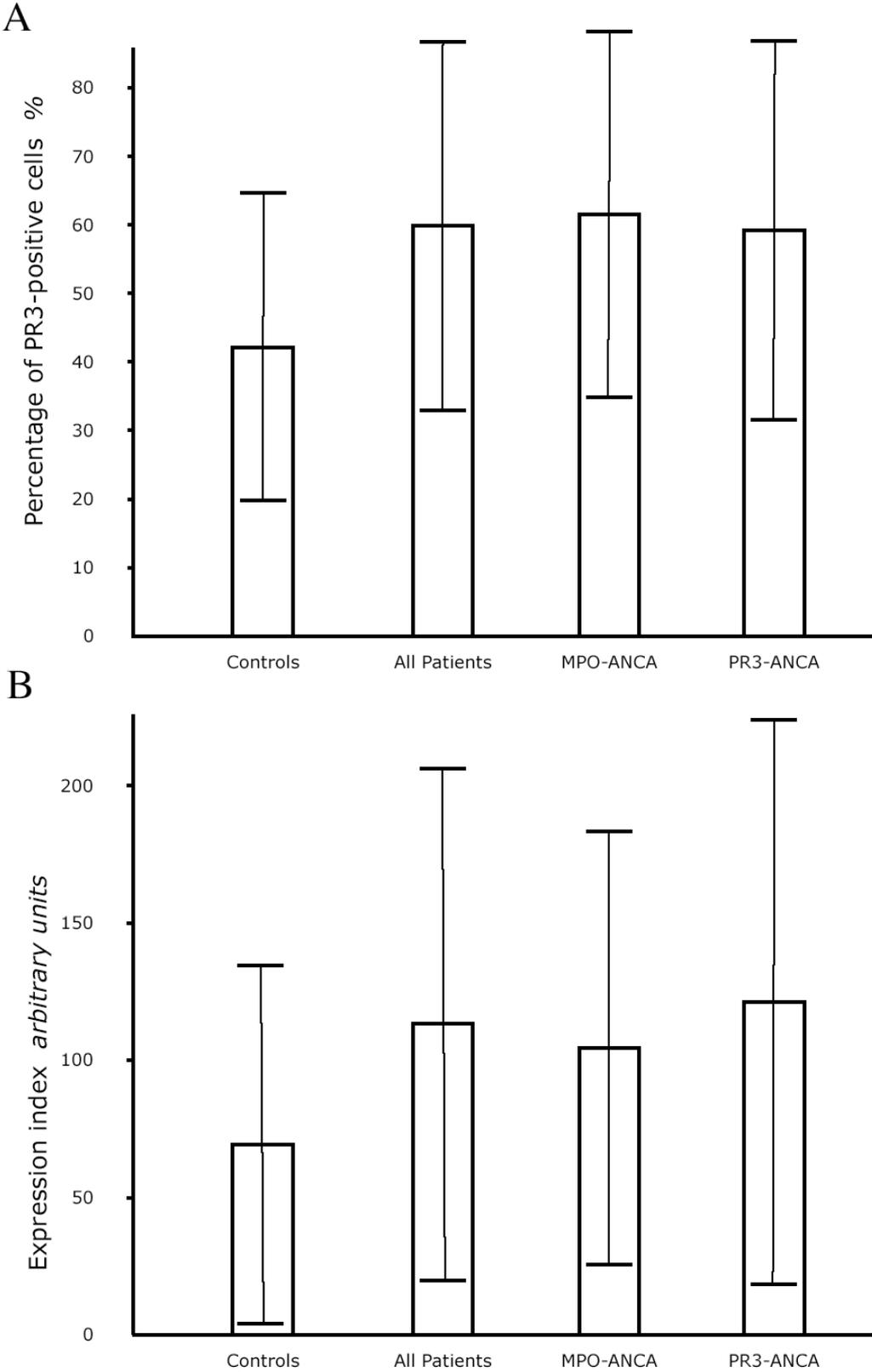


Figure 3

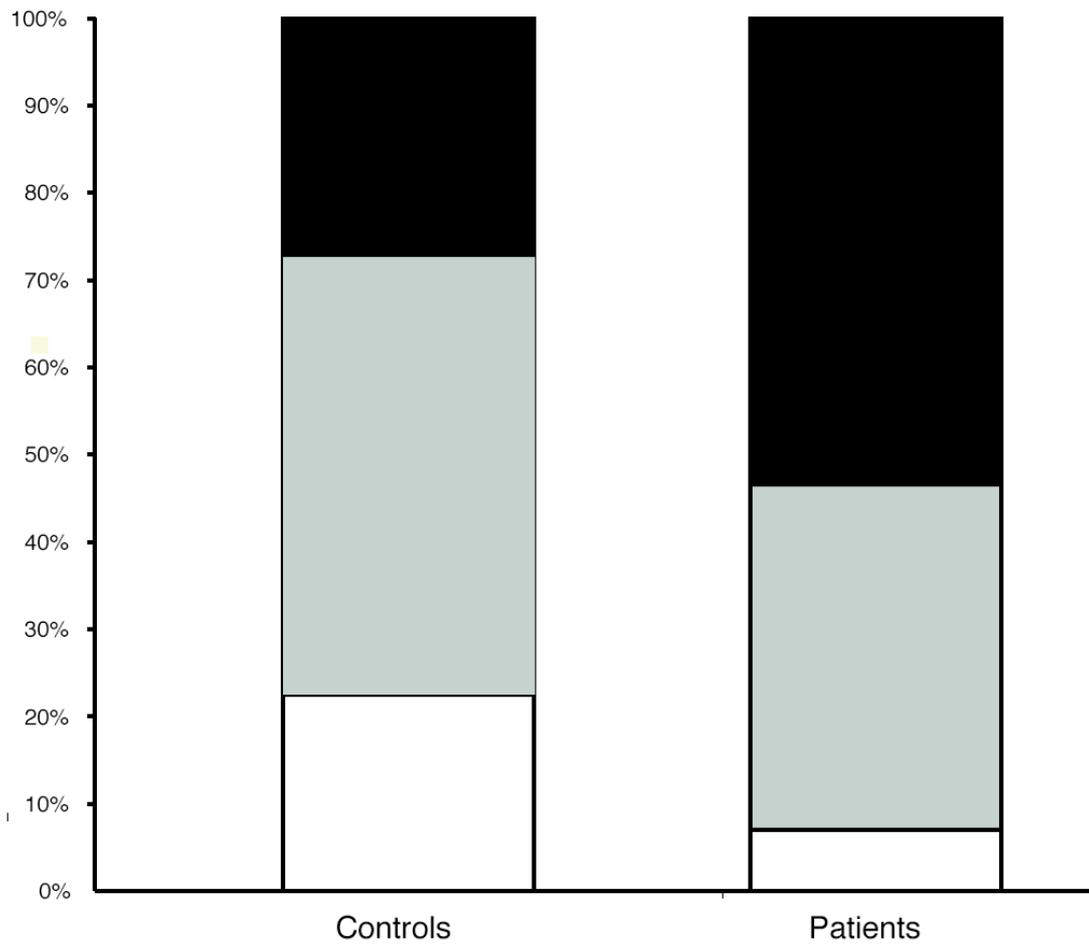


Figure 4

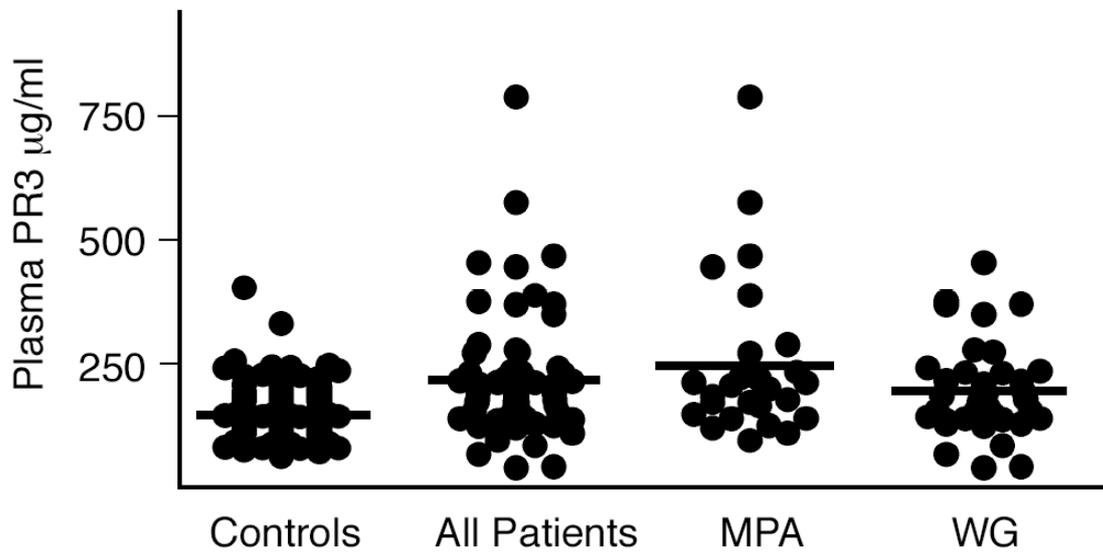
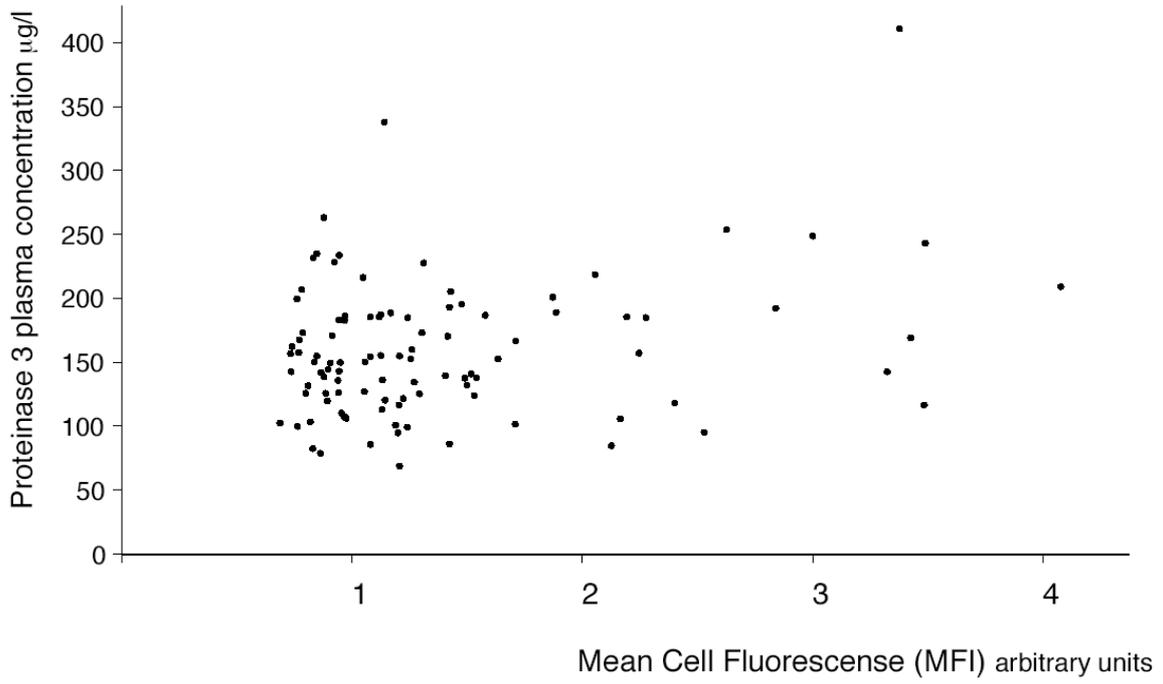


Figure 5

A



B

