

# Membrane Expression of Proteinase 3 Is Genetically Determined

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**Abstract.** Isolated human neutrophils exhibit a bimodal membrane proteinase 3 (PR3) expression. PR3 is the main target antigen in Wegener granulomatosis (WG). Cells with low expression can be easily distinguished from cell subsets with high expression. In a recent study, a large neutrophil subset expressing membrane PR3 (mPR3<sup>+</sup>) was a risk factor for systemic ANCA-associated vasculitis. PR3 membrane expression patterns are quite stable in a given individual, raising the possibility of genetic variance. The aims of this study were: (1) to investigate the association of mPR3 expression and the risk of WG in an independent German cohort; (2) to test the hypothesis that mPR3 expression on neutrophils is genetically influenced; and (3) to investigate whether or not mPR3 expression is a function of intracellular PR3 content. mPR3 expression was assessed by FACS analysis in isolated human neutrophils. Neutrophil mPR3 expression was studied in 35 patients with WG, 15 patients with other inflammatory diseases, 125 healthy volunteers, and 27 (15 monozygotic and 12 dizygotic)

pairs of twins. The intracellular PR3 content was assessed by intracellular flow cytometry and by Western blotting after separating mPR3 low and high expressing cells by FACSsort. FACS analysis in a subset of 16 healthy subjects showed a highly conserved PR3 phenotype in two independent investigations >12 mo apart ( $r = 0.937$ ). Patients with WG demonstrated a significantly higher percentage of mPR3<sup>+</sup> neutrophils than healthy controls and patients with other inflammatory diseases. The mPR3<sup>+</sup> percentage was highly correlated in MZ twins ( $r = 0.99$ ) compared with DZ twins ( $r = 0.06$ ). The intracellular PR3 content was not different in persons with low or high mPR3 expression, nor was the PR3 content different in cells with low or high mPR3 expression within a given individual. These data indicate that WG patients have a higher percentage of mPR3-expressing neutrophils. Furthermore, mPR3 expression is influenced by genetic variance. Finally, mPR3 expression is independent of intracellular PR3 content. *kettritz@frk-berlin.de*

Wegener granulomatosis (WG) is a small vessel vasculitis characterized by antineutrophil cytoplasmic antibodies (ANCA) (1). The main ANCA target antigen in WG is proteinase 3 (PR3), a neutrophil and monocyte-derived neutral serine protease (2). Proteinase 3 is located in the azurophilic granules and in the secretory vesicles of neutrophils (3). However, some PR3 is present on the outer cell membrane of resting neutrophils (3). When neutrophils are treated with subactivating concentrations of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), neutrophil “priming” results in the translocation of intracellular PR3 to the cell surface, increasing the amount of antigen that is accessible to circulating antibodies. ANCA are possibly involved in the pathogenesis of vasculitis by binding to membrane PR3 (mPR3), triggering reactive oxygen species (ROS) production, degranulation, and upregulation of surface adhesion molecules (4–10). Several studies suggested that higher amounts of mPR3 expression correlate with disease

activity. On the other hand, mPR3 expression is bimodal with the existence of two distinct neutrophil subsets, even within healthy subjects. One cell subset is characterized by low mPR3 expression (mPR3<sup>-</sup>) and the other subset by high mPR3 expression (mPR3<sup>+</sup>). The proportion of these two subsets seems to be very stable in a given individual. However, there is great inter-individual variability ranging from 0 to 100% mPR3<sup>+</sup> on the neutrophil surface. In a recent study, a high percentage of mPR3<sup>+</sup> cells was a risk factor for systemic vasculitis (11). Furthermore, a higher percentage of mPR3<sup>+</sup> cells was found to be a risk factor for disease relapse (12). From observations in two families, it was speculated that mPR3 is genetically influenced (11). We performed studies in WG patients, normal volunteers, inflammatory disease controls, and twin subjects to examine these issues further. We tested the hypothesis that higher percentage of mPR3<sup>+</sup> neutrophils is associated with WG, that mPR3 expression is constant within given individuals, and that mPR3 expression is influenced by genetic variance. We also had occasion to explore the relationship between mPR3 expression and intracellular PR3 content.

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## Materials and Methods

### Materials

Recombinant TNF- $\alpha$  was obtained from Genzyme (Rüsselsheim, FRG). The polyclonal rabbit antibody to Proteinase 3 was a gift from Wieslab AB (Lund, Sweden), the monoclonal mouse antibody to

Proteinase 3 was obtained from CLB (Amsterdam, Netherland), horseradish peroxidase-labeled donkey anti-rabbit IgG was from Amersham (Braunschweig, FRG), and FITC-conjugated F(ab)<sub>2</sub>-fragment of goat anti-mouse IgG was from DAKO (Hamburg, FRG). Dextran was purchased from Amersham Pharmacia (Amsterdam, Netherlands). Hanks balanced salt solution (HBSS), phosphate-buffered saline (PBS), and trypan blue were from Seromed (Berlin, FRG). Endotoxin-free reagents and plastic disposables were used in all experiments. Histopaque 1083 and Saponin was obtained from Sigma-Aldrich (Deisenhofen, FRG).

### Isolation of Human Neutrophils

Polymorphonuclear neutrophils of healthy human volunteers were isolated from heparinized whole blood by red blood cell sedimentation with dextran 1%, followed by Ficoll-Hypaque density gradient centrifugation and hypotonic lysis of erythrocytes for 15 s using sodium chloride solution. Neutrophils were centrifuged (10 min at 1050 rpm) and resuspended in HBSS with calcium and magnesium (HBSS<sup>Ca++</sup>). The cell viability was detected in every cell preparation by trypan blue exclusion and found to be greater than 99%. The percentage of neutrophils in the suspension was >95% by Wright-Giemsa staining and by light microscopy.

### Assessment of ANCA-Antigen Expression by Flow Cytometry

FACS was used as described previously (7) to evaluate the PR3 expression on neutrophils. Briefly, cells were stimulated with 2 ng/ml TNF- $\alpha$  or buffer control for 20 min at 37°C and spun down at 200 g for 7 min at 4°C. Pellets were resuspended in 1 ml of ice-cold PBS/0.5% paraformaldehyde and stored on ice for 20 min. Cells were pelleted again and resuspended in HBSS without Ca<sup>2+</sup>/Mg<sup>2+</sup> before they were incubated with dilutions of mab to PR3 or an isotype control followed by a secondary FITC-conjugated F(ab)<sub>2</sub> fragment of goat anti-mouse IgG. Flow cytometry was performed on the same day using a FACSort (Becton Dickinson, Heidelberg, FRG), and 10,000 events per sample were collected. Data are reported either as percent-

age of the total neutrophil population that were mPR3<sup>+</sup> or as mean fluorescence intensity (MFI), reflecting the total amount of mPR3. For the latter, the marker was set to include both mPR3<sup>-</sup> and the mPR3<sup>+</sup> cells.

### Flow Cytometry of Intracellular PR3

Neutrophils were permeabilized as described previously (13). Briefly, 10<sup>6</sup> cells were pelleted (200 g; 5 min; 4°C) and resuspended in 250  $\mu$ l of 4% paraformaldehyde in PBS. Cells were stored on ice for 20 min, washed once in PBS plus 1% BSA (buffer A), and resuspended in 100  $\mu$ l of permeabilization buffer containing PBS, 1% BSA, and 0.2% Saponin (buffer B). Monoclonal antibody to PR3, or an equal amount of mouse IgG1-negative control (isotype control), or PBS only was added, and samples were kept for 30 min on ice. Cells were washed and incubated with FITC-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse Ig (1:50). After washing, cells were resuspended in 500  $\mu$ l of buffer A and stored on ice in the dark until analyzed using a FACSort flow cytometer.

### Subjects

We investigated the heritability of PR3 membrane expression on neutrophils in 27 pairs of monozygotic (MZ = 15) and dizygotic (DZ = 12) healthy German twins. Twin pairs were recruited by advertisement in public print media. All subjects underwent a medical history and physical examination before the study. Persons receiving medications were excluded from the study. Zygosity was determined by use of five microsatellite markers coamplified by PCR. In addition we investigated 125 healthy, 35 patients with WG, and 15 patients with other inflammatory diseases. Clinical and laboratory data describing patients with WG are listed in Table 1. The demographic data of the twin pairs are given in Table 2. Healthy controls included 75 female patients and 50 male patients with a mean age of 41.4  $\pm$  13.8 yr. The 15 patients of the inflammatory disease control group included 4 women and 11 men with a mean age of 72.3  $\pm$  8.1. Diagnoses were sepsis for nine patients, pneumonia in five, and erysipelas in two. Leukocyte count was 19.3  $\pm$  8.8 Gpt/L, and CRP levels were 169.3  $\pm$

Table 1. Clinical and laboratory data for the patients with Wegener granulomatosis (WG)<sup>a</sup>

Parameter	Mean	Minimum	Maximum
Age	59 $\pm$ 14	23	76
CRP (mg/L)	20.5 $\pm$ 38.6	0	144.5
WBC (Gpt/L)	9.5 $\pm$ 4.2	3.6	21.0
Hb (g/dl)	10.8 $\pm$ 2.6	6.5	15.1
Crea (mg/dl)	2.6 $\pm$ 3.5	0.69	12.2
BVAS	4.9 $\pm$ 9.2	0	25.0
Thrombocytes (GPT/L)	281 $\pm$ 105	136	579
Proteinuria (g/d)	0.8 $\pm$ 1.0	0	3.4
ANCA-IF (1: )	314 $\pm$ 1290	0	5120
ANCA-ELISA (kU/L)	193 $\pm$ 487.0	0	1740
Number of patients receiving glucocorticoids	23		
Number of patients on cyclophosphamide	15		
disease extent			
renal	26		
lung	24		
CNS	5		
ENT	24		

<sup>a</sup>16 female patients and 19 male patients.

Table 2. Demographic data of twin pairs<sup>a</sup>

Parameter	Twins	
	Monozygotic	Dizygotic
<i>n</i>	30	24
Gender (M/F), <i>n</i>	8/22	11/13
Age, yr	32 ± 9	31 ± 7
Height, cm	171 ± 8	173 ± 11
Weight, kg	65 ± 10	67 ± 14

<sup>a</sup>Values are mean ± SD.

105.5 mg/L. Written informed consent was obtained before study entry as required by our institutional review board.

### Separation of PR3<sup>+</sup> and PR3<sup>-</sup> Neutrophils by Flow Cytometry Sorting

After staining ( $3 \times 10^7$ ) for PR3 as described, PR3<sup>+</sup> and PR3<sup>-</sup> cells were separated by FACSORT (Becton Dickinson, Heidelberg, FRG). Cells were stained for membrane PR3 expression as described. After gating in the light-scatters cells were separated by gating for mPR3<sup>+</sup> and mPR3<sup>-</sup> neutrophils. Cells were collected and counted after centrifugation for 10 min at  $300 \times g$  with Trypan blue-light microscopy.

### Western Blot Analysis of Proteinase 3

Unseparated neutrophils before FACSORTing, mPR3<sup>+</sup> and mPR3<sup>-</sup> neutrophils separated by FACSORT ( $5 \times 10^4$ ), and Jurkat cells were centrifuged at 8000 rpm for 1 min at 4°C, and pellets were lysed with 20 μl of ice-cold lysing buffer (20 mM Tris-HCl, pH 8.0, containing 138 mM NaCl, 1% Triton X-100, 2 mM ethylenediaminetetraacetate (EDTA), 10% glycerol, 0.2 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM quercetin, 5 mM Iodoacetamide). Samples were kept on ice for 5 min, supernatant was recovered by centrifugation at  $13,000 \times g$  for 5 min at 4°C, and protein concentration was measured by BCA protein assay (Pierce, Munich, FRG). Loading buffer (250 mM Tris-HCL, pH 6.8, with 4% SDS, 20% glycerol, 0.01% bromphenol blue) was added, and samples were heated for 5 min at 95°C. Each sample containing 5 μg of protein per lane was loaded on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel, electrophoresed, and transferred to nitrocellulose membranes. The membranes were blocked in TBS-T + nonfat dry milk 10% for 1 h and incubated overnight with an antibody to Proteinase 3 (gift from Wieslab AB, Lund, Sweden) (1:2000 dilution) in TBS-T + nonfat dry milk 1%. Membranes were washed and incubated with a secondary Ab (horseradish peroxidase-labeled donkey anti-rabbit IgG [1:5000]; Amersham, Amsterdam, Netherlands). Blot was developed by incubation in a chemiluminescence substrate (ECL, Amersham) and exposed to a x-ray film. Equal loading of protein was confirmed by stripping and reprobings the blots for total p38 MAPK. Densitometry of the PR3 bands was performed with scanned x-ray films and the NIH image program.

### Statistical and Quantitative Genetics

Statistical analyses were conducted by use of the SPSS program. All data are expressed as mean ± SD. Relationship between parameters was assessed by linear regression analysis. Interindividual differences of mean group values were tested with unpaired *t* test. A value for *P* < 0.05 was considered to be statistically significant.

Parameters of the quantitative genetic models were estimated by structural equation modeling by use of the MX program developed by Neale (14). Variability of any given phenotype within a population can be decomposed into genetic influences (VaraddGen), environmental influences shared by the twins within the family (VarsharedEnv), and effects of random environment (Varenv), as follows:

$$\text{Var} = \text{VaraddGen} + \text{VarsharedEnv} + \text{Varenv}.$$

For MZ and DZ, the covariance of their phenotype is given by:

$$\text{CovMZ} = \text{VaraddGen} + \text{VarsharedEnv};$$

$$\text{CovDZ} = 0.5 \text{VaraddGen} + \text{VarsharedEnv}.$$

Heritability analysis in twin studies can estimate additive components of genetic variability as well as two environmental influences. These values estimate the relative amount of the influence of the variable on interindividual differences up to a sum of 1. Genetic and environmental effects were estimated by the best-fit model as selected by  $\chi^2$  value.

To test differences among patients with WG, disease controls, and healthy controls, we applied ANOVA with post-hoc Bonferroni testing.

## Results

### mPR3 Expression in Normal Persons and WG Patients

First, we investigated the stability of the neutrophil PR3 expression on the surface membrane over prolonged periods of time. We selected 16 individuals with membrane PR3 expression ranging from 0 to 100% and investigated the PR3 expression on two occasions with a time interval of at least 12 mo. Figure 1 indicates that the mPR3 expression was reproducible and highly stable with a correlation factor of 0.937.

In the 125 healthy control subjects, the PR3-membrane

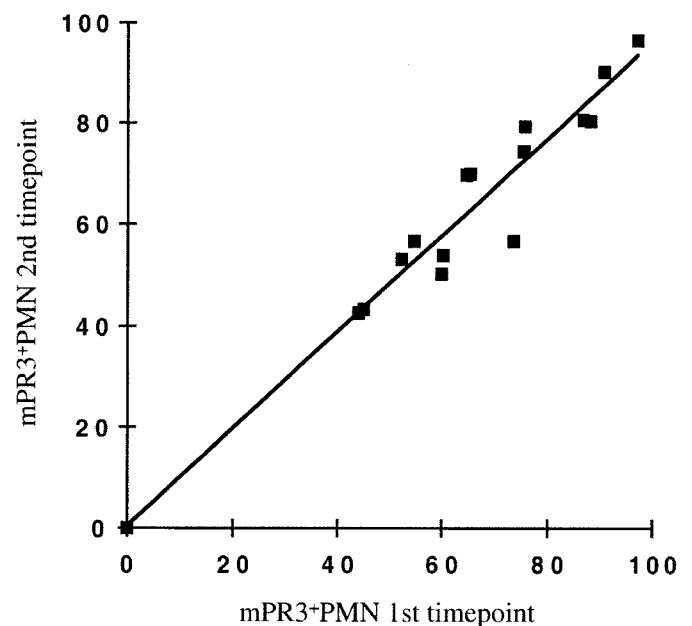


Figure 1. Stability and reproducibility of membrane proteinase 3 (PR3) expression (mPR3) measured over prolonged time periods. Isolated neutrophils from 16 unrelated donors were stained for mPR3 and analyzed by FACS analysis. The percentage of mPR3 positivity at the first evaluation is shown on the x axis, with the percentage at the second evaluation on the y axis. Percentage of mPR3<sup>+</sup> was highly stable, with a correlation factor of *R* = 0.937.

expression distribution percentage showed a normal bell-shaped curve. In contrast, the mPR3<sup>+</sup> expression distribution percentage in patients with WG was significantly skewed to a higher mPR3<sup>+</sup> phenotype as shown in Figure 2A. The mean mPR3<sup>+</sup> percentage was 56.1% in the healthy cohort compared with 76.8% in the WG cohort ( $P < 0.001$ ) given in Figure 2B. These results indicate that the percentage of mPR3<sup>+</sup> was highly conserved in any given individual over time and that the percentage of mPR3<sup>+</sup> was significantly higher in our German cohort of patients with WG compared with healthy controls. In addition, we investigated the mPR3 expression in a cohort of 15 patients with other inflammatory diseases. We observed no difference in the percentage of the mPR3<sup>+</sup> population compared with healthy controls (59.4% versus 56.1%). However, this number was significantly lower compared with 76.8% in patients with WG ( $P < 0.05$ ). When we assessed total amount of mPR3 by measuring mean fluorescence intensity (MFI), we found a value of  $278 \pm 206$  for healthy controls,  $457 \pm 310$  for patients with WG, and  $461 \pm 257$  for patients with idiopathic inflammation. MFI values in both WG and the disease control

were significantly higher compared with healthy individuals ( $P < 0.05$ ). We did not see a significant difference in the percentage of PR3 positivity or in the MFI for the total amount of PR3 between WG patients with CRP-levels below versus above 5 mg/L. However, when we compared patients with a BVAS below versus above a score of 2, we observed a trend toward a higher total amount of expressed membrane PR3 in patients with a BVAS  $>2$  ( $360 \text{ MFI} \pm 270$  for patients with a BVAS  $< 2$  versus  $562 \pm 315$  for those with a BVAS  $> 2$ ;  $P = 0.08$ ), whereas the percentage of PR3 positivity was similar. These data indicate that patients with WG are characterized by higher percentages of PR3<sup>+</sup> neutrophils compared with healthy controls as well as with patients with other inflammatory diseases. In contrast, the total amount of mPR3 may also be upregulated during the course of other inflammatory processes than WG.

### PR3 Membrane Expression in Monozygotic and Dizygotic Twins

MZ and DZ twins were demographically similar (Table 2). We phenotyped 15 MZ and 12 DZ twin pairs according to their membrane PR3 expression. The percentage of mPR3<sup>+</sup> was significantly correlated in MZ twin pairs with a correlation factor of 0.99, as shown in Figure 3A. In contrast, no correlation in the percentage of PR3 expression was observed in the DZ twin pairs (Figure 3B). Here, the correlation factor was 0.06. The heritability percentage of mPR3<sup>+</sup> was estimated as 99% (Table 3). In addition, we found that the absolute amount of PR3 that is expressed on the outer cell membrane, as assessed by the mean channel intensity, was also correlated in MZ twins with a regression coefficient of 0.96 as shown in Figure 4A. In the DZ twins, shown in Figure 4B, no correlation was found. The heritability estimate for this parameter was 96.7%. These results suggest a strong genetic effect on mPR3 expression.

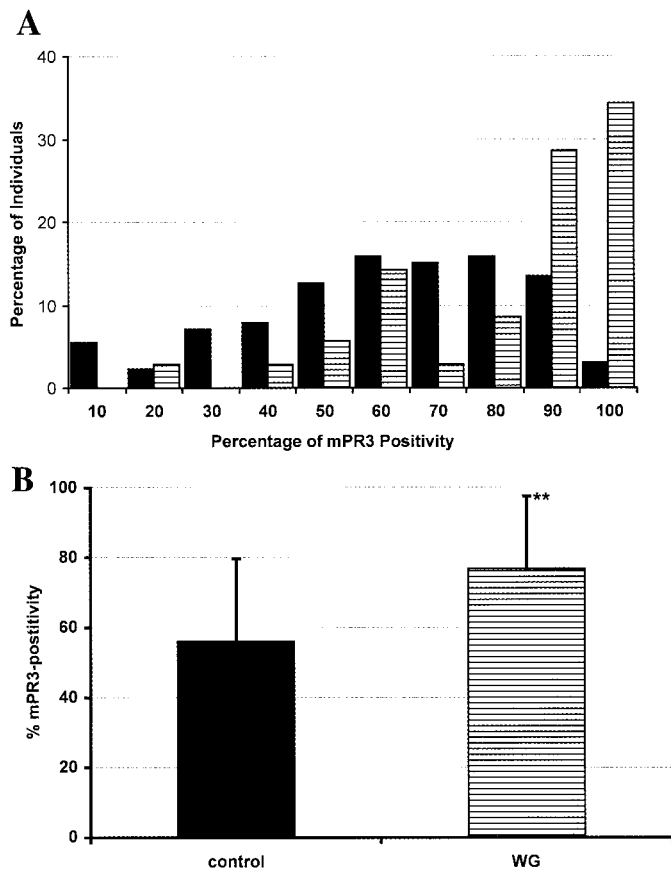


Figure 2. (A) The distribution of mPR3<sup>+</sup> in a cohort of 125 healthy German subjects compared with 35 patients with Wegener granulomatosis (WG). Isolated neutrophils were stained with a monoclonal antibody against PR3, a secondary FITC-conjugated goat anti-mouse antibody, and analyzed by flow cytometry. Healthy individuals are shown as black bars, WG patients as black hatched bars. (B) The mean percentage of neutrophils expressing PR3 on their membrane is shown. The differences were highly significant (\*\*  $P < 0.001$ ).

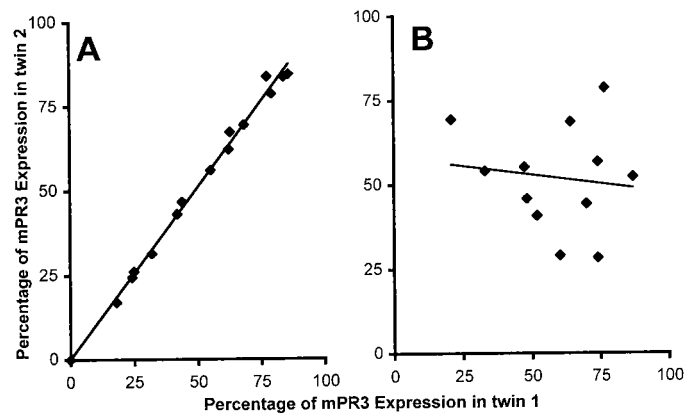
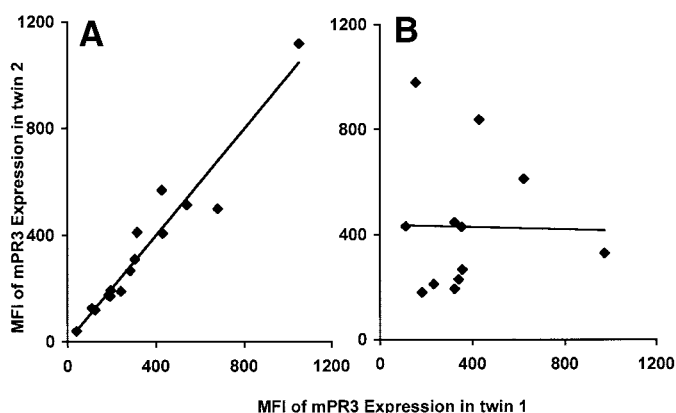


Figure 3. The percentage of mPR3<sup>+</sup> neutrophils was assessed in monozygotic (MZ) and dizygotic (DZ) twin pairs. The percentage of mPR3<sup>+</sup> of one twin (x axis) is plotted against the other twin (y axis) of the pair. (A) 15 pairs of MZ twins; the percentage of mPR3<sup>+</sup> was highly correlated ( $R = 0.99$ ). (B) 12 pairs of DZ pairs; No correlation was found ( $R = 0.06$ ).

**Table 3.** Group mean values of percentage of membrane proteinase 3–positive PMN (Mean % PR3<sup>+</sup>) and mean channel fluorescence intensity (MFI) of membrane proteinase 3 (Mean MFI mPR3) on PMN for monozygotic and dizygotic twins<sup>a</sup>

Parameter	Twins	
	Monozygotic	Dizygotic
Mean % mPR3 <sup>+</sup>	55 ± 4	55 ± 4
Correlation % mPR3 <sup>+</sup>	0.997	0.058
Heritability % mPR3 <sup>+</sup>	99.7 (66.9 to 99.8)	
Mean MFI mPR3	341 ± 49	358 ± 49
Correlation MFI mPR3	0.961	0.001
Heritability MFI mPR3	96.7 (68.3 to 98.6)	

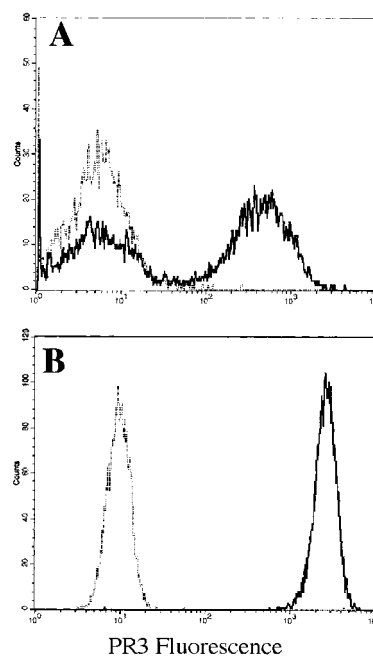
<sup>a</sup>Values are mean ± SEM. No significant difference between MZ and DZ twins was seen. The heritability was a maximum likelihood estimate of heritability.



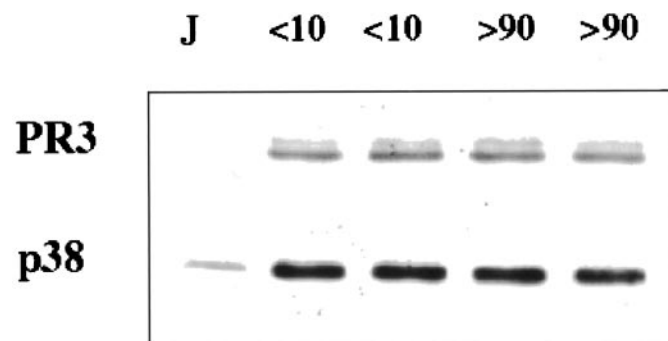
**Figure 4.** The mean PR3 expressed on the neutrophil plasma membrane measured as the mean fluorescent intensity (MFI) was compared between MZ and DZ twin pairs. The MFI of membrane PR3 of one twin (x axis) is plotted against the other twin (y axis). (A) 15 pairs of MZ twins; the mean membrane PR3 amount was highly correlated ( $R = 0.96$ ). (B) Results from 12 pairs of DZ twin pairs; no correlation was found ( $R = -0.11$ ).

#### Intracellular Proteinase 3 Content in Neutrophils

To investigate whether or not mPR3 expression is a function of different intracellular PR3 concentrations, we assessed the intracellular PR3 content in neutrophils from individuals with approximately 50% mPR3 expression by flow cytometry. PR3 staining was either performed in unpermeabilized cells or, to detect intracellular PR3, after detergent permeabilization. Figure 5 shows that the typical bimodal type of mPR3 expression was not seen in cells after permeabilization. This observation suggests that the amount of mPR3 is not dependent on the intracellular content of PR3. Next, we performed Western blot analysis for total PR3, comparing neutrophils from donors with less than 10% mPR3<sup>+</sup> and more than 90% mPR3<sup>+</sup>. Figure 6 indicates no difference in the total amount of PR3 between the different donors. Finally, to exclude interindividual variations,

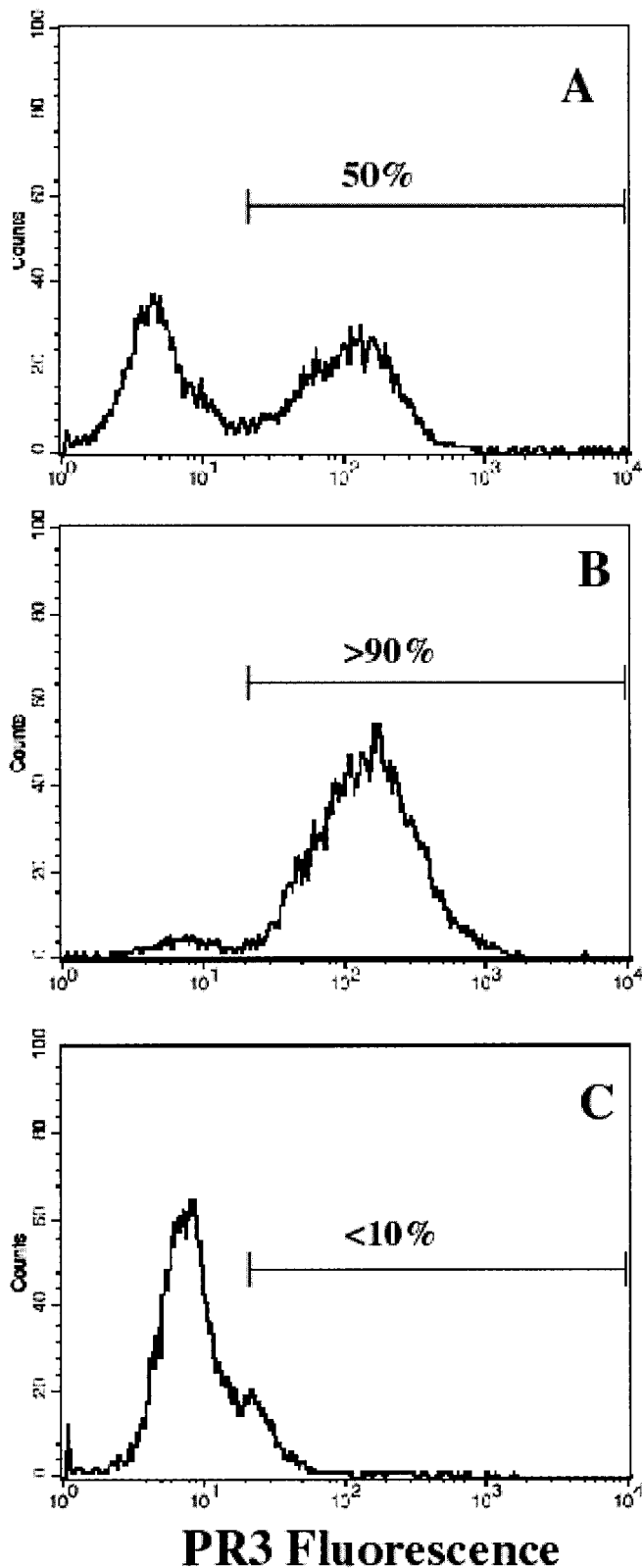


**Figure 5.** (A) The mPR3 staining was compared with intracellular PR3 staining. A donor with approximately 50% mPR3<sup>+</sup> neutrophils was stained for membrane PR3 (black graph). The isotype control (gray-hatched graph) showed no significant membrane staining. (B) Neutrophils from the same donor were permeabilized and stained for intracellular PR3 (black graph). With isotype control, no significant staining was observed (gray hatched graph). As shown after permeabilization, a homogeneous population staining for PR3 was observed, suggesting an equal intracellular content of PR3 in both subsets.



**Figure 6.** The PR3 content in neutrophils was compared by immunoblotting with a rabbit antibody against PR3 between donors with <10% mPR3<sup>+</sup> neutrophils and donors with >90% mPR3<sup>+</sup> neutrophils. Jurkat cells (J) that do not express PR3 served as negative control. The upper row shows staining for PR3. Membranes were stripped and restained for p38 MAPK to show equal protein loading (p38). No difference in intracellular PR3 amount was observed.

we physically separated mPR3<sup>+</sup> and mPR3<sup>-</sup> neutrophils from five different donors by FACSsort. Figure 7 shows that sorting based on mPR3 expression resulted in two populations with either less than 90% mPR3<sup>+</sup> or more than 90% mPR3<sup>+</sup> neutrophils, respectively. Unseparated cells containing 50% mPR3<sup>+</sup> and 50% mPR3<sup>-</sup> cells, and cells after sorting were



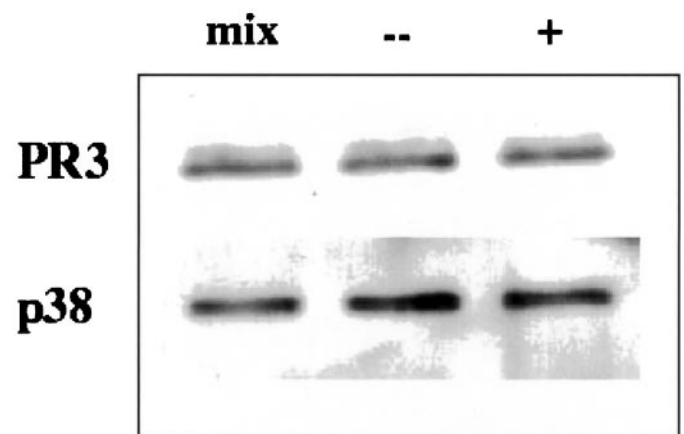
**Figure 7.** mPR3<sup>+</sup> and mPR3<sup>-</sup> neutrophils from a single donor were separated by flow cytometry sorting. Before sorting, approximately 50% of neutrophils expressed PR3 on their plasma membrane (A). After sorting for mPR3<sup>+</sup> neutrophils, a population with more than 90% mPR3<sup>+</sup> neutrophils was identified (B). After sorting for mPR3<sup>-</sup> cells, a population of less than 10% mPR3<sup>+</sup> neutrophils was identified (C).

analyzed for total PR3 by Western blot analysis (Figure 8). When we used densitometry on scanned x-ray films to quantify the PR3 bands, we found no significant difference among unseparated neutrophils, mPR3<sup>+</sup>, and mPR3<sup>-</sup> cells (respective OD values were 68.9, 68.0, and 66.6).

**Discussion**

We confirm in a second independent cohort of patients with WG that a high percentage of mPR3 expressing neutrophils is a risk factor for ANCA vasculitis. In addition, using the twin model, we provide solid genetic evidence that mPR3 expression is genetically controlled. Furthermore, our experimental studies extend our previous knowledge by demonstrating that differences in the mPR3 expression do not reflect different intracellular PR3 content. Our data demonstrate the influence of genetics in the occurrence of systemic ANCA vasculitis and also underscore the importance of mPR3 surface expression rather than total PR3 cell content in terms of disease risk.

PR3 is the main autoantigen in WG (15–17). PR3 is stored intracellularly in azurophilic and secretory vesicles (3). However, some PR3 can be detected on the cell membrane of isolated resting neutrophils. Neutrophil activation *in vitro* or during active vasculitis results in increased mPR3 expression (4,18). Recently, a bimodal expression pattern of mPR3 was shown. Two distinct populations of mPR3<sup>+</sup> and mPR3<sup>-</sup> neutrophils were distinguished in isolated neutrophils (19,20). The clinical relevance of this finding was demonstrated by the fact that a large percentage of mPR3<sup>+</sup> on the cell surface is a risk factor for vasculitis and a risk factor for disease relapse in patients with WG (11,12). Our data confirm and extend these findings. Documentation in a second, independent cohort renders this association important credibility. Furthermore, the powerful twin model confirmed the suggestion of genetic vari-



**Figure 8.** PR3 content in separated neutrophils was assessed by Western blot analyses. Shown are the unseparated cells with approximately 50% mPR3<sup>+</sup> cells (mix), the neutrophils sorted for mPR3<sup>-</sup> expression (-), and neutrophils sorted for mPR3<sup>+</sup> expression (+). The first row shows staining for PR3. The second row shows the staining for p38 MAPK after stripping of membranes to show equal protein loading (p38). PR3 content in all three populations was similar.

ance for PR3 membrane expression based on two families in the earlier study.

Several consequences of high membrane PR3 expression are conceivable in the setting of ANCA vasculitis. A higher mPR3 could possibly trigger the generation of ANCA itself. A recent investigation demonstrated that injection of apoptotic neutrophils into rats resulted in ANCA production (21). Apoptotic neutrophils express higher levels of ANCA antigens (22,23). Furthermore, membrane PR3 is enzymatically active and was shown to be partially resistant to the naturally occurring inhibitor alpha1-antitrypsin and elafin (24). Thus, a higher PR3 membrane expression could accelerate tissue lesions, including those found during vasculitic inflammation. Finally, it is conceivable that more mPR3 allows for more interaction with PR3-ANCA. Increased ANCA binding could initiate more neutrophil activation followed by endothelial cell injury (25,26). Further studies will be necessary to address these issues.

We considered the possibility that the low and high mPR3-expressing neutrophil subsets may be a consequence of differences in intracellular PR3 amounts. We observed that the typical bimodal type of PR3 membrane staining vanished after cells were permeabilized, allowing for intracellular PR3 staining. In addition, the results of Western blot analyses in donors with membrane PR3 percentage of more than 90% and less than 10% showed no difference in PR3 content. Both observations suggested that mPR3 expression was not a direct consequence of intracellular PR3 content. After separating membrane PR3<sup>+</sup> and PR3<sup>-</sup> neutrophils physically, we also investigated this issue within individuals, as opposed to inter-individual comparisons. Our data indicate that the intracellular amount of PR3 was similar in both subsets. These results clearly show that the two different subsets of membrane PR3<sup>+</sup> and PR3<sup>-</sup> neutrophils are not a function of variances in the intracellular PR3 content.

We used a twin approach to test whether or not mPR3 expression is genetically controlled. The twin approach allows detection and quantification of genetic effects in relatively small subjects groups. MZ twins share all genes in common, whereas DZ twins are related as siblings and share half their genes on average. The heritability of the percentage of mPR3<sup>+</sup> cells was high; however, we are aware that twin studies overestimate genetic influences and interpret these findings only as showing a robust genetic effect. For WG, several genetic factors involved in the pathogenesis have been suggested. A defective PIZ-allele of the PR3 inhibitor  $\alpha$ 1-antitrypsin was associated with WG (27,28). A common variant of the CD18 gene confers increased risk for ANCA-associated vasculitis (29,30). In a recent work from Gencik *et al.* (31), the authors found certain PR3 epitope variants in 79 patients with WG. Furthermore, they identified polymorphisms in the promoter region of PR3 change transcription factor recognition sites that may lead to altered transcriptional activity of the PR3 gene and possibly an increased expression of PR3 on the neutrophil surface. However, the investigators did not phenotype their subjects in terms of membrane PR3 expression so that the hypothesis remains to be tested.

To our knowledge, we are the first to demonstrate that the neutrophil PR3 membrane expression in neutrophils is genetically regulated. The specific gene loci and genes that are responsible for mPR3 expression need now to be identified. We are studying larger numbers of DZ twins and have also recruited their parents. With this approach, we intend an identity-by-descent linkage analysis to find the gene loci that are responsible. This approach has been successful in identifying cardiovascularly relevant genes by our group in earlier studies (32).

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