Neutrophil Membrane Expression of Proteinase 3 (PR3) Is Related to Relapse in PR3-ANCA–Associated Vasculitis

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Abstract. Wegener granulomatosis (WG) is strongly associated with the presence of antineutrophil cytoplasm autoantibodies (ANCA) with specificity for proteinase 3 (PR3). Relapses of WG are frequently preceded by a rise of autoantibody titer and PR3-ANCA are able to activate primed neutrophils in vitro. Except being stored intracellularly and translocated to the cell surface upon neutrophil stimulation, PR3 can also be detected on the surface of non-stimulated neutrophils (membrane PR3 or mPR3), with an interindividual variability in percentages of mPR3-positive cells and level of mPR3 expression. This study began with the hypothesis that the presence of PR3 on the surface of non-stimulated neutrophils enables interaction with PR3-ANCA and influences clinical manifestations of the disease. It analyzed mPR3 expression on neutrophils of 89 WG patients in complete remission and 72 healthy controls to evaluate whether the presence of PR3 on the surface of resting neutrophils is related to clinical manifestations of WG and/or to the susceptibility to develop relapses. The number of patients with a bimodal mPR3 expression on resting neutrophils did not differ between patients and controls. However, in WG patients, an increased percentage of mPR3+ neutrophils and an elevated level of mPR3 expression compared with healthy individuals (P = 0.037) were found. Within the group of WG patients, an elevated level of mPR3 expression was significantly associated with an increased risk for relapse (P = 0.021) and with an increased relapse rate (P = 0.011), but not with the disease extent or particular manifestations at diagnosis or at relapse. These data support the hypothesis that PR3 expression on the membrane of neutrophils plays a role in the pathophysiology of PR3-ANCA associated vasculitis.

Wegener granulomatosiS (WG) is a systemic disease characterized by necrotizing inflammation of the respiratory tract, vasculitis, and pauci-immune glomerulonephritis (1). WG is strongly associated with the presence of antineutrophil cytoplasm autoantibodies (ANCA) with specificity for proteinase 3 (PR3) (2–4). Although the role of these autoantibodies has not been fully established yet, there is increasing evidence that PR3-ANCA are involved in the pathogenesis of WG. Remarkably, relapses of WG are frequently preceded by a rise of PR3-ANCA (5,6).

PR3 is a serine proteinase stored in azurophil granules of polymorphonuclear neutrophils (7,8). It has also been shown to localize in specific granules and secretory vesicles (9), In vitro, after priming with tumor necrosis factor-α (TNFα) PR3 is translocated to the cell surface, where it becomes accessible for interaction with PR3-ANCA (10). Cross-linking of PR3 and Fcγ receptors on the surface of primed neutrophils by PR3-ANCA induces neutrophil activation that results in the release of reactive oxygen species and proteolytic enzymes (11–14). Moreover, ANCA-activated neutrophils have been shown to be cytotoxic against vascular endothelium (15).

Recently, it has been found that in some individuals PR3 is present also on the surface of non-stimulated neutrophils (mPR3+ neutrophils) (16,17). PR3 can be detected either on the total neutrophil population or on a subset of neutrophils. The existence of two distinct, mPR3− and mPR3+, neutrophil subpopulations within one individual is called bimodal expression of PR3. The proportion of mPR3+ neutrophils varies between individuals but is highly stable in a given individual (17), suggesting a genetic background for this phenomenon. The observation that in patients suffering from vasculitis the mPR3+ neutrophil subset tends to be larger than in healthy individuals led to the hypothesis that an increased number of mPR3+ cells could be a risk factor in this disease (17).

Although the functional significance of mPR3 expression on resting neutrophils has not been elucidated yet, there is some evidence that the level of mPR3 is correlated to the degree of neutrophil activation by PR3-ANCA (18). One may hypothesize that mPR3 available on the cell surface enables interaction of PR3-ANCA with those neutrophils resulting in their activation.

The aim of this study was to evaluate whether the quantitative presence of PR3 on the surface of resting neutrophils is related to specific clinical manifestations of WG or to the susceptibility to develop relapses.

Materials and Methods

Patients and Controls

The patient group consisted of 89 PR3-ANCA–positive patients with a diagnosis of WG (47 men; 42 women; mean age, 55.6 ± 16 yr; range, 21 to 85 yr) seen at our outpatient clinic between March 7 and
December 31, 2001. All patients gave informed consent before participation in the study. The diagnosis was established according to the Chapel Hill criteria (19), and all patients fulfilled the American College of Rheumatology criteria for WG (20). At diagnosis, renal involvement was present in 52 (58%) of 89 patients and pulmonary involvement in 47 (53%) of 89 patients. The mean Birmingham Vasculitis Activity Score (BVAS) of the patient population at diagnosis was 23 ± 10.5. At the time of determination of mPR3 expression, all patients were in complete remission (BVAS = 0). Patients had been followed for 81.4 ± 66.8 mo since diagnosis and had received standard treatment with a combination of cyclophosphamide and prednisolone (6). During follow-up, 50 patients (56%) experienced one or more relapses. Relapsing disease was defined as described previously (22). PR3-ANCA were determined by indirect immunofluorescence (IIF) assay on ethanol-fixed neutrophils and by PR3-specific enzyme-linked immunosorbent assay (ELISA), as described previously (22). Median cANCA titer at diagnosis was 1:320 (range, 1:40 to 1:1280).

Seventy-two gender- and age-matched healthy volunteers (33 men; 39 women; mean age, 49.2 ± 13.3 yr; range, 24 to 79 yr) were included in the control group.

### Neutrophil Isolation

Neutrophils were freshly isolated from EDTA-anticoagulated blood by centrifugation on Polymorphprep (Nycomed, Oslo, Norway) and hypotonic lysis of contaminating erythrocytes with ice-cold ammonium chloride buffer. Cells were washed with cold Hanks' balanced salt solution (HBSS) without Ca²⁺/Mg²⁺ (Life Technologies, Breda, The Netherlands) and resuspended in cold phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% sodium azide. The isolation of neutrophils from healthy controls crossmatched isolation from WG patients. (MCGl; IQProducts, Groningen, The Netherlands) for 30 min. Next, nonbound antibodies were washed with PBS–1% BSA–0.1% sodium azide. This step was followed by 30 min of incubation with phycoerythrin (PE)–conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) in the presence of 0.5 mg/ml heat-aggregated goat IgG, followed by a subsequent washing step. Fluorescence was analyzed on an ELITE flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA), and fluorescence intensity was standardized using Standard Brite beads (Coulter, Hialeah, FL).

Bimodal mPR3 expression was defined as the presence of 10% to 90% mPR3⁺ cells. The level of mPR3 was expressed in arbitrary units (AU) as calculated as the mean fluorescence intensity (MFI PR3) of mPR3⁺ cells corrected for the nonspecific binding of isotype control antibody (MFI NSB) and multiplied by the percentage of cells within the mPR3⁺ subset.

### Statistical Analyses

Differences in continuous variables between two groups were analyzed by means of the Mann Whitney test. For comparisons between more than two groups, one-way ANOVA and Kruskall-Wallis tests were used. Proportions between groups were compared with the χ² test. Correlations were analyzed using Spearman rank test. Actuarial relapse-free survival was calculated from diagnosis to the first relapse (n = 50), death (n = 1), or December 31, 2001 (n = 38), whichever came first, and compared between groups with the log-rank test. A two-sided P < 0.05 was considered to indicate statistical significance.

### Results

#### Distribution of mPR3-Expressing Neutrophils in Healthy Controls and Patients with WG

We found a high interindividual variability in the percentage of mPR3-expressing neutrophils ranging from 0 to 100% of total neutrophils (Figures 1 and 2). Bimodal expression of PR3 (the presence of both mPR3-negative and mPR3-positive subpopulations within one individual) was detected in 22 healthy individuals (30%). The remaining 50 healthy controls (70%) had one uniform population of neutrophils with an interindividually variable level of mPR3 expression. This group included two individuals who had a monomodal neutrophil population without detectable mPR3. In WG patients, the

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**Figure 1.** Patterns of mPR3 expression detected by flow cytometry. The thin line represents nonspecific binding of isotype-matched control monoclonal antibody. The bold line shows binding of monoclonal anti-PR3 antibody. Monomodal negative mPR3 expression (left), monomodal positive mPR3 expression (middle), and bimodal mPR3 expression (right) are shown.
distribution of phenotypes was comparable, with 26 WG patients (29%) displaying a bimodal mPR3 pattern and 63 WG patients (71%) with a monomodal mPR3 expression, including one patient without detectable mPR3 on the total neutrophil population. Although the number of individuals with a bimodal expression of mPR3 in these two groups did not differ, WG patients with a bimodal mPR3 expression had an increased percentage of mPR3* neutrophils compared with healthy individuals (P = 0.048).

The percentage of neutrophils expressing PR3 on their surface was stable in all seven healthy controls tested twice within half a year and in all 14 WG patients tested two times within 1 yr (Figure 4).

In contrast to PR3, human leukocyte elastase (HLE)—another serine protease stored in azurophil granules and homologous to PR3—could not be detected on the surface of resting neutrophils, as determined using indirect staining with monoclonal anti-HLE antibody, HLEG-1.

To analyze whether the level of mPR3 expression on the surface of neutrophils was related to in vivo or isolation-induced degranulation, we assessed cell membrane expression of CD63 by staining with anti-CD63 monoclonal antibody. CD63 is a protein detectable on the neutrophil surface and is upregulated upon degranulation of azurophil granules (24). CD63 expression did not correlate to the mPR3 bimodality. We always found CD63 to be uniformly expressed on the neutrophil surface, also in individuals with bimodal mPR3 expression, and we never observed two distinct subsets of neutrophils with different levels of CD63.

**Level of PR3 Expression on the Neutrophil Surface**

Next to percentages of mPR3* neutrophils, we analyzed also the MFI of the mPR3* subset. In case of bimodal mPR3 expression, the total level of PR3 expression was calculated by multiplying the relative MFI of the mPR3* subset by the percentage of cells within this population. We found that the median expression of PR3 on resting neutrophils was higher in WG patients compared to healthy individuals (P = 0.037). Interestingly, the difference between patients and healthy controls was seen only in individuals with a monomodal mPR3 expression (P = 0.004), but not in individuals with a bimodal mPR3 expression (P = 0.764, Figure 5). In both WG patients and healthy controls with a bimodal mPR3 expression, the median expression of mPR3 was much higher compared with individuals with a uniform neutrophil population (WG patients: 94 versus 43 arbitrary units [P < 0.0001]; healthy controls: 90 versus 22 arbitrary units [P < 0.0001]). We did not find a correlation between the percentage of mPR3* cells and the level of PR3 present on the surface of these cells.

**mPR3 Expression and Clinical Manifestations of WG**

To analyze the relation between PR3 expression on the surface of neutrophils and the clinical features we divided the
WG patient population arbitrarily in three groups: patients with a monomodal low mPR3 expression (MFI PR3 of WG patients with a monomodal mPR3 expression; n = 32), patients with a monomodal high mPR3 expression (MFI PR3 > median MFI PR3 of WG patients with a monomodal mPR3 expression; n = 31) and patients with a bimodal mPR3 expression (n = 26). Data of these groups are given in Table 1. Age, ANCA titer, and BVAS at diagnosis as well as the follow-up time were comparable between the groups. Organ involvement at diagnosis did not differ between these three groups, except for a slightly increased incidence of skin involvement in WG patients with monomodal high and bimodal mPR3 expression (P = 0.027). Also the clinical manifestations at the moment of the first relapse of vasculitis were comparable between groups (results not shown).

The number of patients receiving immunosuppressive treatment at the moment of mPR3 measurement differed between WG patients with a monomodal low mPR3 expression (19%) and WG patients with a monomodal high (58%) or bimodal (58%) mPR3 expression (Table 1). Although all patients were in remission at the moment of mPR3 measurement, 50% of WG patients with a monomodal low mPR3 expression, 52% of WG patients with a monomodal high mPR3 expression, and 54% of WG patients with a bimodal mPR3 expression were tested positive for ANCA by indirect immunofluorescence. Median ANCA titer did not differ between groups (Table 1).

Compared with only 38% of WG patients with a monomodal low mPR3 expression, 68% of WG patients with a monomodal high mPR3 expression and 65% of WG patients with a bimodal mPR3 expression experienced one or more relapses during the comparable follow-up time (Table 1). Moreover, the disease-free survival time between diagnosis and the first relapse was significantly shorter in WG patients with a monomodal high mPR3 expression (median disease-free survival, 30.8 mo) and bimodal mPR3 expression (median disease-free survival, 36.6 mo) than in WG patients with monomodal low mPR3 expression (median relapse-free survival, 104.5 mo; P = 0.011; Figure 6). Compared with WG patients with a monomodal low mPR3-expression, the relative risk for relapse was 2.20 (95% CI, 1.07 to 4.99) for WG patients with a bimodal mPR3 expression and 2.45 (95% CI, 1.24 to 4.97) for WG patients with a monomodal high mPR3 expression. In WG patients with a bimodal mPR3 expression, relapse-free survival time was independent of the percentage of mPR3+ neutrophils and the level of mPR3 expression on mPR3+ neutrophils.

**Discussion**

This study demonstrates that patients with PR3-ANCA–associated vasculitis have an increased expression of PR3 on the surface of resting neutrophils compared with healthy controls and that the elevated level of mPR3 expression in vasculitis patients is associated with an increased incidence and rate of relapse.

PR3 is the main autoantigen recognized by antineutrophil cytoplasm antibodies in patients suffering from WG (2–4). PR3 is stored intracellularly in neutrophil granules and secretary vesicles (7–9), and recently it has been found also on the surface of resting neutrophils (16). Further analysis (16,17) revealed in some individuals a uniform pattern of mPR3 expression, and in other individuals the existence of two distinct mPR3+ and mPR3− subpopulations of neutrophils. The latter phenomenon, designated as bimodal expression of PR3, has been shown to be a stable feature in a given individual not related to age and gender, and it has been suggested to be a risk factor for the development of vasculitis (17). In our study, the number of WG patients with a bimodal PR3 expression did not differ from healthy individuals, suggesting that the presence of two distinct subsets of neutrophils cannot be considered to be a vasculitis-related feature. This discrepancy between our results and results elsewhere could be caused by differences in methodology, such as differences in donor inclusion criteria, definition of bimodality, or data analysis.

Although, in contrast to the results of Witko-Sarsat et al. (17), we did not find any difference between our WG population and healthy individuals in the number of subjects with a bimodal PR3 expression; we did find that WG patients with a bimodal mPR3 expression had an increased percentage of mPR3+ cells (Figures 2 and 3). Bimodal mPR3 expression did not result from degranulation of a neutrophil subpopulation, and the percentage of mPR3+ cells was stable in time within a given individual (Figure 4). These results suggest that an increased number of cells within the mPR3+ neutrophil subset can be a risk factor in PR3-ANCA-related vasculitis.

We also analyzed the level of PR3 expression on the surface of the mPR3+ subpopulation. In WG patients, the level of PR3 expression on the surface of neutrophils was significantly increased compared with healthy controls (Figure 5). Interestingly, the difference in the level of mPR3 expression between
WG patients and healthy controls was strongly pronounced in individuals with a monomodal neutrophil population but absent when considering individuals with a bimodal pattern of PR3 expression. On the other hand, we noticed that the mPR3/H11001 subset in WG patients and healthy controls with a bimodal mPR3 expression showed a significantly higher level of mPR3 expression than neutrophils from individuals with a monomodal mPR3 expression.

The observation that neutrophils of WG patients are characterized by an increased membrane PR3 expression supports our hypothesis on the possible pathophysiologic role of mPR3. We suggest that neutrophils of the mPR3\(^+\) phenotype are more susceptible to activation by PR3-ANCA than mPR3\(^-\) neutrophils because the autoantigen is directly accessible on the cell surface, also without priming. As a result, increased numbers of mPR3\(^+\) neutrophils and/or an increased level of mPR3 expression might be associated with more severe disease. To test this possibility, we looked for possible correlations between membrane PR3 expression and clinical features. We demonstrate that WG patients with a monomodal high membrane expression of PR3 are significantly more at risk for relapse of vasculitis than those with low level of mPR3 expression. Moreover, the relapse rate in patients with a monomodal high mPR3 expression, but also in patients with a bimodal mPR3 expression, was much higher compared with patients with monomodal low mPR3 expression (Figure 6). According to our expectations, median relapse-free survival of WG patients with a bimodal mPR3 expression was comparable to the median relapse-free survival of WG patients with a monomodal high mPR3 expression. This can be explained by the observation that the level of mPR3 expression on the mPR3\(^+\) subset in individuals with a bimodal PR3 expression tends to be even higher than the level of mPR3 expression in individuals with a monomodal high mPR3 expression, resulting in relatively high total mPR3 expression of the neutrophil population.

Table 1. Patient characteristics\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Monomodal Low mPR3 (n = 32)</th>
<th>Monomodal High mPR3 (n = 31)</th>
<th>Bimodal mPR3 (n = 26)</th>
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<tbody>
<tr>
<td><strong>At diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>15 (47%)</td>
<td>14 (45%)</td>
<td>18 (69%)</td>
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<tr>
<td>F</td>
<td>17 (53%)</td>
<td>17 (55%)</td>
<td>8 (31%)</td>
</tr>
<tr>
<td>Mean age (yr)</td>
<td>49.4 ± 16.1</td>
<td>50.9 ± 18.4</td>
<td>47.7 ± 15.4</td>
</tr>
<tr>
<td>Median ANCA titer (IIF)</td>
<td>1:320</td>
<td>1:640</td>
<td>1:640</td>
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<tr>
<td>Mean BVAS</td>
<td>22.2 ± 11</td>
<td>22.7 ± 9.8</td>
<td>22.7 ± 10.9</td>
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<tr>
<td><strong>Organ involvement</strong></td>
<td></td>
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<tr>
<td>Nose</td>
<td>30 (94%)</td>
<td>30 (97%)</td>
<td>26 (100%)</td>
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<tr>
<td>Ear</td>
<td>12 (38%)</td>
<td>18 (58%)</td>
<td>15 (58%)</td>
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<tr>
<td>Trachea</td>
<td>4 (13%)</td>
<td>5 (16%)</td>
<td>1 (4%)</td>
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<tr>
<td>Eye</td>
<td>11 (34%)</td>
<td>16 (52%)</td>
<td>9 (35%)</td>
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<tr>
<td>Lung</td>
<td>18 (56%)</td>
<td>16 (52%)</td>
<td>13 (50%)</td>
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<tr>
<td>Kidney</td>
<td>19 (59%)</td>
<td>17 (55%)</td>
<td>16 (62%)</td>
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<tr>
<td>Skin</td>
<td>6 (19%)</td>
<td>15 (48%)</td>
<td>12 (46%)</td>
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<td>Joints</td>
<td>25 (78%)</td>
<td>28 (90%)</td>
<td>23 (88%)</td>
</tr>
<tr>
<td>Mononeuritis</td>
<td>13 (41%)</td>
<td>12 (39%)</td>
<td>13 (50%)</td>
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<tr>
<td><strong>Follow-up</strong></td>
<td></td>
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<tr>
<td>Follow-up (mo)(b)</td>
<td>70.5 ± 56</td>
<td>83.5 ± 68</td>
<td>92.2 ± 77.8</td>
</tr>
<tr>
<td>No relapses during follow-up</td>
<td>20 (62%)</td>
<td>10 (32%)</td>
<td>9 (35%)</td>
</tr>
<tr>
<td>(\geq) 1 relapse during follow-up</td>
<td>12 (38%)</td>
<td>21 (68%)</td>
<td>17 (65%)</td>
</tr>
<tr>
<td>Median relapse-free survival (mo)</td>
<td><strong>104.5</strong></td>
<td><strong>30.8</strong></td>
<td><strong>36.6</strong></td>
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<tr>
<td><strong>At the moment of mPR3 measurement</strong></td>
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<tr>
<td>Mean age (yr)</td>
<td>54 ± 16.3</td>
<td>57.3 ± 16.3</td>
<td>54.8 ± 14.7</td>
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<td>ANCA positivity (IIF)</td>
<td>16 (50%)</td>
<td>16 (52%)</td>
<td>14 (54%)</td>
</tr>
<tr>
<td>Median ANCA titer (IIF)</td>
<td>1:80</td>
<td>1:80</td>
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<tr>
<td>Mean BVAS</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP, AZA, MTX or MMF</td>
<td>6 (19%)</td>
<td>18 (58%)</td>
<td>15 (58%)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>6 (19%)</td>
<td>17 (55%)</td>
<td>14 (54%)</td>
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</table>

\(a\) Patients were divided into three groups on the basis of the mPR3 expression on their neutrophils. Significant differences are shown in bold. CP, cyclophosphamide; AZA, azathioprine; MTX, methotrexate; MMF, mycophenolate mofetil, IIF, indirect immunofluorescence assay; BVAS, Birmingham Vasculitis Activity Score. \(b\) Time between diagnosis and the moment of mPR3 measurement.
Compared with WG patients with a monomodal low mPR3 expression, more patients with a monomodal high and bimodal mPR3 expression received immunosuppressive treatment at the moment of mPR3 measurement. The necessity to use immunosuppressive drugs to keep particularly these patients in remission might be caused by an increased risk for relapse related to an increased level of mPR3 expression in combination with persistent PR3-ANCA positivity.

In conclusion, this study demonstrates that the neutrophil population of WG patients is characterized by an increased incidence over, in WG patients elevated PR3 expression on the surface of resting neutrophils is associated with an increased incidence and rate of relapse. Our findings support the hypothesis that the availability of PR3 for interaction with circulating autoantibodies plays a role in the pathophysiology of PR3-ANCA associated vasculitis.

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References


Access to UpToDate on-line is available for additional clinical information at http://www.jasn.org/