Membrane proteinase 3 (mPR3) expression on neutrophils is not increased in localised Wegener's granulomatosis (WG) and Churg-Strauss syndrome (CSS)

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ABSTRACT

Objective. *The aim of this study was to analyse mPR3 expression on neutrophils in two Anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitides (AAV), namely WG (localised vs. generalised) and Churg-Strauss syndrome (CSS) and other inflammatory disorders, in order to evaluate (i) whether the pattern of mPR3 expression is specific for AAV and (ii) to assess whether the mPR3high status is associated with clinically distinct disease stages of WG (localised vs. generalised).*

Methods. *Localised WG (n=15), generalised WG (n=55), Churg-Strauss Syndrome (CSS) (n=20), systemic lupus erythematosus (SLE) (n=15), Rheumatoid Arthritis (RA) (n=22) and healthy controls (n=30) were analysed. mPR3 and CD63 expression on surface of neutrophils were assessed by flow cytometric analysis on isolated neutrophils and whole blood.*

Results. *In patients with genWG and SLE, an increased percentage of mPR3+ neutrophils and an elevated level of mPR3 expression compared to healthy controls were found (percentage: p=0.001, p=0.000; MFI ratio: p=0.038, p=0.019, respectively). There was no increased frequency of mPR3+ neutrophils in CSS. Within the group of WG, an elevated level of mPR3 expression was significantly associated with disease stage (genWG and not locWG), and in genWG with disease activity and the presence of ANCA.*

Conclusion. *The mPR3high status is associated with generalised WG and correlates with disease activity and ANCA status in generalised WG. An increased proportion of mPR3-positive neutrophils is not specific for AAV.*

Introduction

PR3 is the main target antigen of ANCA in WG (1) and is expressed on the plasma membrane of resting (2) and activated neutrophils (3). Furthermore, PR3 expression is genetically determined (4) and it is either detected on the total neutrophil population or only on a subset of it (5). Interestingly, in WG, the percentage of mPR3 "high expressors" is increased compared to the normal population (6). Moreover, the level of mPR3 expression is related to disease activity (7) and relapse (8) in WG.

Thus, an "easy access" of PR3 for antigen presenting cells (APC) and/or the display of PR3 in high amounts may be one factor for the initiation of an autoimmune response against PR3. Recently, we found that PR3 *in vitro* causes dendritic cell (DC) maturation of monocyte-derived DC and licenses PR3-"primed" DC to induce a Th1-phenotype in autologous CD4+ T-cells (9) demonstrating that Wegener's autoantigen PR3 *in vitro* is indeed able to induce an immune response. However, there is a growing body of evidence showing that high mPR3 expression is not a phenomenon restricted to WG. Increased percentages of mPR3 expression on neutrophils have also been demonstrated in RA, SLE and in septic stages such as SIRS (systemic inflammatory response) (6, 10, 11). Cytokine-mediated "priming" induces upregulation of mPR3 on the plasma membrane, which may be an explanation of upregulated mPR3 in certain inflammatory settings (12). Interestingly, in some inflammatory conditions such as bacterial endocarditis, a temporary induction of PR3-ANCA has been described (13). Although an increased expression of mPR3 in these conditions has not been assessed so far, it can be assumed that mPR3 expression may be upregulated during inflammation and additional unknown factors may then contribute to the induction of an autoimmune response directed against PR3 (14).

In the context of neutrophil mPR3 ex-

pression in WG, it is important to consider that WG is classified according to disease stages (15): Localised WG is defined as granulomatous disease in the upper and respiratory tract. Rarely, patients do not progress to systemic disease at all, so that the localised stage may also be considered as a disease variant in these rare cases (16). In localised WG, patients are often ANCA-negative, whereas generalised disease, which is consistent with often severe systemic vasculitis manifestations, is associated with a positive ANCA status (17, 18). If ANCA-production was related to mPR3-expression on neutrophils, mPR3 expression should be lower in localised WG than in generalised WG.

To assess whether mPR3 expression is associated with a certain disease stage or disease variant in WG and whether mPR3 expression is related only to AAV or can be found in other inflammatory diseases, we analysed the mPR3 expression in two ANCA-associated vasculitides (WG and CSS), the two disease stages/variants in WG (localised and generalised) and other inflammatory diseases (SLE and RA).

Patients and methods
Patients
WG and CSS diagnosis was based on the Chapel Hill definition (19) and the American College of Rheumatology Criteria (20, 21). Disease stages in WG were applied according to the EUVAS definition (15). Disease activity in WG and CSS was determined according to the Birmingham Vasculitis Activity Score (BVAS2003) (22). RA and SLE patients fulfilled the criteria of the American College of Rheumatology (23, 24). Disease activity was assessed by the DAS28 in RA (25) and the EC-LAM (26) in SLE.

Detection of ANCA
PR3- or MPO-ANCA were determined by indirect immunofluorescence (IIF) assay on ethanol-fixed neutrophils and by ELISA with specificity for PR3 or MPO.

Preparation of human neutrophils and whole blood
Human neutrophils were isolated as described earlier (3). For whole blood

preparation, 5 ml EDTA-anticoagulated whole blood was added to 45 ml of PBS, vortexed and then centrifuged at 1000 r.p.m. for 10 min at 200C. The supernatant was aspirated and the cells resuspended in 50 ml PBS; washing was repeated twice. The cell pellet was adjusted to 5 ml in PBS, and the whole blood preparation was ready for monoclonal antibody staining.

Staining of neutrophils and flow cytometry analysis
100 μl of washed whole blood, and isolated neutrophils (4 x 10^6) were stained with 2 μg/ml of conjugated mouse monoclonal IgG1 antibody directed against human PR3 (WGM2), human CD63, a neutrophil activation marker (EuroBioSciences GmbH, Friesoythe, Germany), or with an irrelevant IgG1 control antibody (Coulter Immunotech, Krefeld, Germany) for 30 min at 4°C according to the manucaturer's protocol. Cell surface molecule expression of cells was measured as previously described (3, 5) and performed in whole blood and isolated neutrophil samples. Stained cells were analysed by FACScalibur followed by analysis using CellQuest software (Becton Dickinson). The median fluorescence intensity (MFI) and MFI ratio of control and specific antibodies MFI values were recorded for each sample. A total of 100,000 events were analysed for each sample. Bimodal mPR3 expression was defined as the presence of 10% to 90% mPR3+ cells (5).

Statistical analysis.
Most of the results are represented as median (25th, 75th percentile) because of non-normal distribution. Proportions between groups were compared with the χ^2 test. For comparisons between more than two groups, Kruskall-Wallis test was used, and comparisons between two groups were evaluated by the Wilcoxon test or Mann-Whitney

Table I. Patient characteristics.

Wegener's granulomatosis	
Number of patients, n (locWG/genWG)	70 (15/55)
Gender (female/male), n	38/32
Mean age (years), range	56 (range 17-81)
ANCA-status (positive/negative), n	46/23
ANCA-status in locWG (positive/negative), n	2*/13
ANCA-status in genWG (positive/negative), n	44*/11
Disease activity	
remission (BVAS=0), n	16
active disease (BVAS>0), n	46
median BVAS of active loc+genWG, range	5, 1-24
median BVAS of active locWG, range	3, 1-9
median BVAS of active genWG, range	5, 1-24
Churg-Strauss syndrome	
Number of patients, n	20
Gender (female/male), n	12/8
Mean age (years), range	52 (17-89)
ANCA-status, n (positive/ negative)	3*** / 17
Disease activity,	
remission (BVAS=0), n	3
active disease(BVAS>0), n	17
median BVAS of active patients, range	3, 1-10
Rheumatoid arthritis	
Number of patients, n	22
Gender (female/male), n	11/11
Mean age (years), range	63 (38-81)
Disease activity (DAS28), median (range)	5.27 (3.00-7.17)
SLE	
Number of patients, n	15
Gender (female/male), n	14/1
Mean age (years), range	49 (34-68)
Disease activity (ECLAM), median (range)	2.00 (1.00-5.00)

*1PR3-ANCA/1MPO-ANCA. **41PR3-ANCA/3MPO-ANCA. ***3MPO-ANCA.

test appropriately. Correlation was performed using Spearman's correlation. A two-sided $p<0.05$ was considered statistically significant.

Results

Patients

Patient characteristics are presented in Table I. ANCA-status is displayed for the time point when the experiment was done.

Percentage and level of PR3 expression on neutrophil surface

An increased percentage of mPR3+ neutrophils in generalised WG was found compared to HC ($p=0.000$), CSS ($p=0.003$), RA ($p=0.019$), and localised WG ($p=0.004$). In SLE, a larger subset of mPR3 expressing neutrophils was found than in HC ($p=0.038$). There were no significant differences in percentages of mPR3+ neutrophils between HC and CSS, RA, and locWG (Fig. 1A).

Regarding the MFI ratio, a significantly higher ratio of mPR3+ neutrophils was detected in generalised WG compared to HC ($p=0.000$), CSS ($p=0.028$), RA ($p=0.031$), and localised WG ($p=0.028$). A higher MFI ratio of mPR3+ neutrophils was also found in patients with SLE compared to HC ($p=0.019$) (Fig. 1B). No differences in MFI ratios of PR3 expression between HC, CSS, RA and localised WG could be assessed.

Expression of CD63 and correlation with mPR3 expression on neutrophils

There was a correlation between CD63 and mPR3 expression on surface membrane of all neutrophil samples: Spearman's correlation values were $r=0.195$ for the percentages of CD63 and mPR3 positive neutrophils, and $r=0.232$ for the MFI ratios, all of which were significant ($p=0.015$, and $p=0.003$, respectively, data not shown). The median percentages of CD63 expressing neutrophils differed significantly in monomodal low (21.50% (12.50%~48.00%)), bimodal (31.00% (17.00%~53.00%)), and monomodal high mPR3 expression patterns (59.00% (34.00%~66.00%)) ($p=0.032$). There were also significant differences in the median MFI ratios of CD63 expression neutrophils, 1.56 (1.31~1.67) for monomodal low
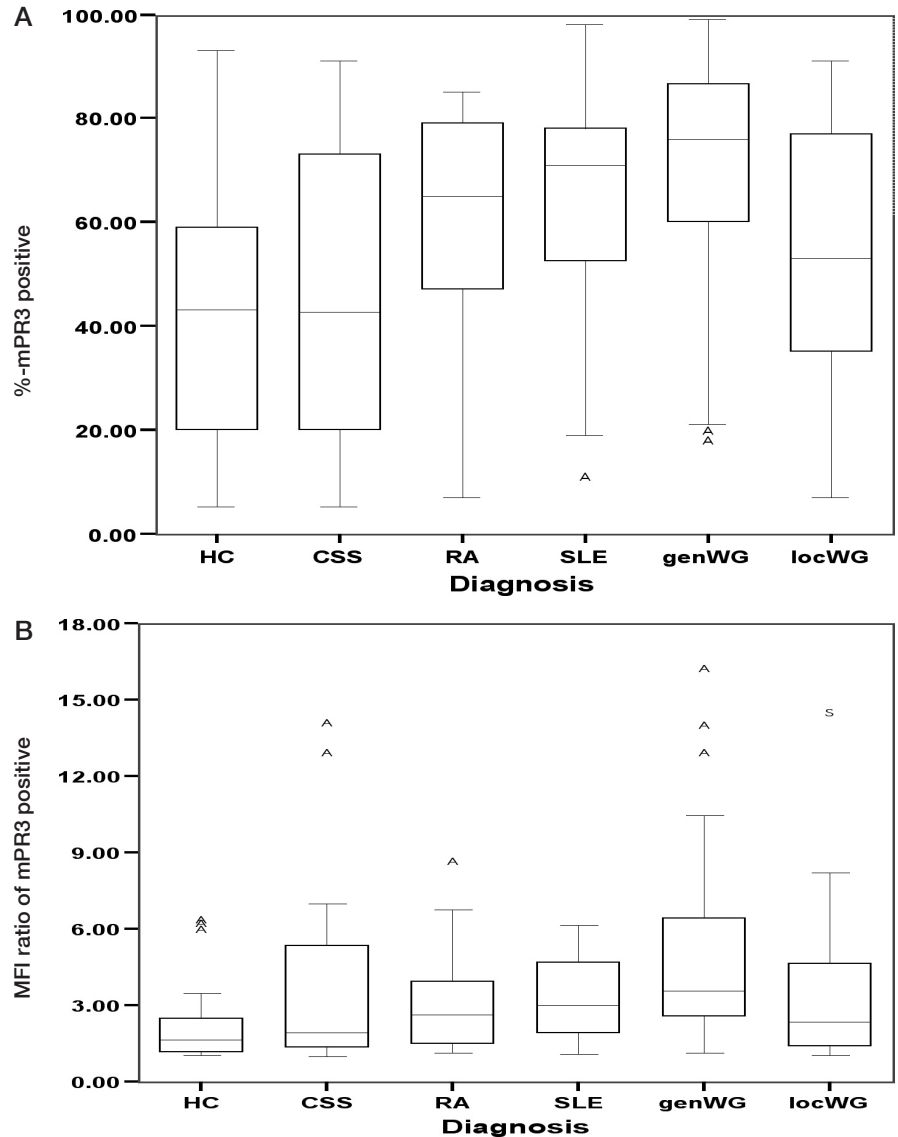

Fig. 1. mPR3 expression on neutrophils from patients with AAV, SLE, RA and healthy controls (box and wisker plots indicating the overall range – error bars –, 25-70 ranges-boxes- and median value – horizontal lines – of the surface expression). Percentage of mPR3 positive neutrophils is shown in (**A**), the MFI ratio of mPR3 positive neutrophils is given in (**B**).

mPR3, 1.66 (1.43~1.98) for bimodal mPR3, and 1.79 (1.57~2.02) for monomodal high mPR3 expression pattern ($p=0.041$).

Relationship between mPR3 expression on neutrophils and ANCA-status and BVAS in generalised WG

In PR3-ANCA-positive generalised WG, (n=41), there was an elevated MFI ratio of mPR3 expressing neutrophils (3.55 (2.51~5.78)) compared to ANCA-negative genWG (n=9) (2.07 (1.20~4.99), $p=0.042$), but not a significant difference in the percentages of mPR3+ neutrophils (69.00% (56.00%~86.50%) *vs.* 46.00%

(20.00%~80.00%), $p=0.059$). Furthermore, in generalised WG both percentage and MFI ratio of mPR3+ neutrophils correlated significantly with BVAS ($r=0.352$, $p=0.012$; $r=0.414$, $p=0.003$, respectively).

Distribution of mPR3-expressing neutrophils

When comparing the distribution of mPR3 phenotype in patients (n=127) and healthy controls (n=30), we found a high inter-individual variability in the percentage of mPR3-expressing neutrophils ranging from 0 to 100% of total neutrophils. Regarding the percentage of mPR3 expressing neutrophils three
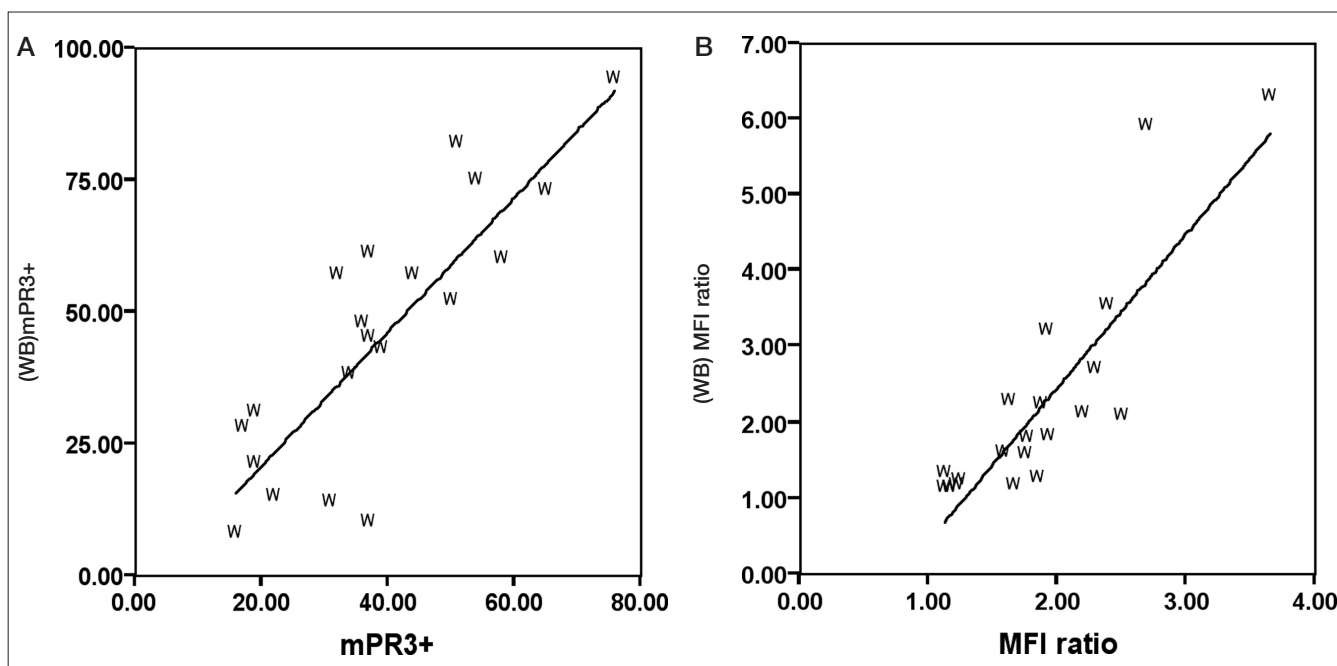
Fig. 2. Comparison of mPR3 expression obtained from isolated PMN and whole blood (WB) methods in 20 healthy controls. Percentages of mPR3+ neutrophils are shown in (**A**) and MFI ratio of mPR3 expressing neutrophils are given in (**B**). Spearman's r correlation values are shown and all were significant.

expression patterns were found: firstly, monomodal low mPR3 expression: 18 individuals had an mPR3+ subset <10% of the total neutrophil circulating pool (23.33% healthy controls, 25.00% CSS, 4.55% RA, 6.67% SLE, 3.64% generalised WG, and 13.33% locWG); secondly, a bimodal mPR3 expression: 122 individuals had between 10 to 90% mPR3+ cells and the presence of both mPR3- and mPR3+ subpopulations within one individual could be detected (73.33% healthy individuals, 70.00% CSS, 95.45% RA, 80% SLE, 74.55% genWG, and 80.00% locWG); thirdly a monomodal high mPR3 expression: 17 individuals had an higher mPR3 expression (>90% mPR3+ cells) (3.33% healthy control, 5% CSS, 13.33% SLE, 21.82% genWG, and 6.67% locWG patients) (Fig. 2A). A higher proportion of monomodal high mPR3 expressing patterns were found in generalised WG, compared to HC (p=0.003), and CSS (p=0.008), respectively. No statistical differences in the proportions of mPR3 phenotype were found between HC, CSS, RA, SLE, and localised WG. Similar to observations of mPR3 expression on isolated neutrophils, the percentage of mPR3 expressing neutrophils in washed whole blood is highly variable

among individuals (from 0 to 100% of total neutrophils).

Comparison of whole blood and neutrophils separation methods
Flow cytometry analysis of mPR3 on neutrophils was performed on both isolated and whole blood neutrophils. The whole blood method has been used before as a non-activating approach (7), however, a comparison between the two methods has not been done. The percentage and the MFI ratio of neutrophils in whole blood expressing mPR3 correlated well to purified neutrophils isolated from the same blood sample (Fig. 2A and B). There was a strong correlation between two methods regarding mPR3 expression: Spearman's correlation values are r=0.8 for the percentages of mPR3+ neutrophils and r=0.8 for the MFI ratios and they were all significant (p<0.001) (Fig. 1). FACS plots of stained neutrophil populations prepared by whole blood or neutrophil isolation methods were highly comparable (data not shown).

Discussion
High mPR3 expression on neutrophils has been associated with WG and linked to disease activity and relapse

(7, 8), however, an elevated expression of mPR3 has also been found in RA, SLE and SIRS. We analysed whether mPR3 expression is associated with disease stages in WG (localised and generalised). Furthermore, we investigated whether and increased mPR3 expression is restricted to WG or occurs in other AAV (such as CSS) or other rheumatic autoimmune diseases.

We confirm previous results showing that mPR3 expression is higher in WG patients compared to controls and correlates with disease activity (assessed by BVAS) in generalised WG. Moreover, mPR3 expression correlates with expression of the neutrophil activation marker CD63. Importantly, we found that mPR3 expression on neutrophils is higher in generalised WG than in localised WG. Moreover, increased numbers of mPR3+ neutrophils were found not only in WG but also in SLE, but not in RA and CSS.

The association of percentage and level of mPR3 expression with disease activity in generalised WG and the fact that increased mPR3 expression correlates with the neutrophil activation marker CD63, underline the influence of an inflammatory stage on mPR3 expression. The increased expression of mPR3 in

generalised WG compared to localised WG may suggest that a high amount of accessible antigen (mPR3) may be associated with the generation of PR3-ANCA, which is more frequently detectable in generalised WG compared to localised WG. Interestingly, the difference in level of mPR3 expression in ANCA-positive and ANCA-negative generalised WG patients compared to controls was clearly pronounced in favour of ANCA-positive WG.

Regarding mPR3 expression on neutrophils in RA and SLE, our data are in line with the study of van Rossum *et al.* (10), who found elevated percentages of mPR3+ neutrophils in WG and SLE, but not in RA. Additionally, we did not find an increased expression of mPR3 in Churg-Strauss syndrome, which is – like WG – considered as AAV. Whether the facts that ANCA are detected in only around 40% of CSS patients or that the main target antigen of ANCA in CSS is MPO and not PR3 could be an explanation for these findings remains speculative. We have to admit that in our study, only 3 of 20 CSS patients were MPO-ANCA positive. In the future, mPR3 expression should be investigated in ANCA-positive and ANCA-negative CSS separately to clarify this issue. Clearly, an increased mPR3 expression is not specific for WG or AAV.

We also investigated whether the established flow cytometric analysis of mPR3 expression on isolated neutrophils is applicable to whole blood samples and found the portion of WB mPR3+ neutrophils obtained from patients and controls that correlated well to that of isolated neutrophils. In fact, the percentage and MFI ratio of mPR3+ neutrophils in washed whole blood was very similar to that of mPR3 expressing isolated non-primed neutrophils within a given individual. The whole blood method is fast and reproducible to quantify mPR3 on neutrophils and requires a little amount of blood to detect mPR3 on circulating neutrophils.

In summary, the amount and the percentage of mPR3 expression is increased in generalised WG but not localised WG. A high amount of autoantigen may be one factor for the induction of autoimmunity and the generation of ANCA in WG. However, a high mPR3 expression is not specific for WG and occurs in other inflammatory disorders and may be related to the activation state of neutrophils. Additional factors apart from high mPR3 expression are necessary to induce an autoimmune response including the generation of PR3-ANCA.

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