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## **Increased monocyte transcription of the proteinase 3 gene in small vessel vasculitis**

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**Short title:** PR3-transcription in small vessel vasculitis

**Key words:** proteinase 3, monocytes, ANCA, systemic vasculitis, real time PCR

# Increased monocyte transcription of the proteinase 3 gene in small vessel vasculitis

## Summary

**Background:** Proteinase 3 (PR3) is a pleiotropic and destructive serine protease and it is also a major target for autoantibodies in systemic small vessel vasculitis. We have recently shown that patients in stable remission have increased circulating levels of PR3, independent of autoantibody titer, inflammation, neutrophil degranulation and renal function. Here we explore the possibility of an increased production. **Methods:** RNA was purified from peripheral blood monocytes from vasculitis patients and controls. Specific mRNA was measured by TaqMan real-time PCR. The monocyte-like cell lines THP-1 and U937 and human peripheral blood monocytes from healthy controls, were stimulated with cytokines and LPS for different time periods. PR3 protein was measured in plasma with ELISA. **Results:** The median result for PR3 mRNA was 9.6 (1.8-680) for 22 patients, compared to 1 (0.1-2.8) for the 15 healthy controls. Elastase expression was also significantly increased, whereas myeloperoxidase and interleukin-8 were not. Stimulation of monocytes with TNF- $\alpha$ , IFN- $\gamma$  or LPS did not result in any increase of PR3 or elastase transcription, whereas the IL-8 transcription was 10-folded. **Conclusions:** Circulating monocytes from patients with systemic vasculitis produce increased amounts of PR3 compared to healthy controls and patients with SLE. This may be of importance for the development of vasculitis. Our results do not favor an influence of cytokines, ANCA or immunosuppressive medication.

## ***Introduction***

Wegener's granulomatosis and microscopic polyangiitis are systemic small vessel vasculitides of unknown etiology. Due to the strong association with autoantibodies reacting with granule constituents of neutrophils and monocytes, they are often referred to as ANCA-associated small vessel vasculitis (AASV, ANCA =anti-neutrophil cytoplasmic antibodies). The major ANCA target antigens are myeloperoxidase (MPO) and proteinase 3 (PR3) (1).

PR3 is a 29-kDa serine protease stored in granules of neutrophils and monocytes. It is also present on the surface of primed or apoptotic human neutrophils in a bioactive form (2).

Extracellularly, PR3 is inhibited through binding to serine protease inhibitors (SERPINs), the most abundant in plasma being alpha-1-antitrypsin ( $\alpha$ 1-AT). The substrates of PR3 include several extracellular matrix components, and the protease has been shown to produce emphysema in hamsters when installed intratracheally (3). Besides its proteolytic effects, PR3 has influence on the proliferation of granulopoietic progenitor cells (4) and it can induce apoptosis in endothelial cells (5). PR3 is also involved in cytokine activation, chemokine activity amplification and processing of cytokine binding proteins, indicating a regulatory role in inflammatory processes (6).

Few genetic polymorphisms have reproducibly been associated with AASV. An exception is the PiZ deficiency allele of  $\alpha$ 1-AT that is associated with an increased risk to develop AASV. This association has been reported in studies from France, Austria, Holland, USA, England and Sweden (7). In our cohort we also found the PiZ allele to be linked to disease extension and a worse prognosis. These findings suggest increased protease activity to be part of the pathogenesis.

We have recently published findings of raised circulating levels of PR3 in AASV patients in remission or with chronic smoldering disease activity (8). These high levels were not an effect

of general inflammation, since there was no correlation between PR3 and C-reactive protein (CRP) or IL-6. Neither was there any correlation with clinical disease activity, using Birmingham Vasculitis Activity Score (BVAS). We could also rule out decreased renal function, using relevant disease controls and cystatin C as a marker of glomerular filtration of endogenous polypeptides. Neutrophil gelatinase-associated lipocalin (NGAL) and soluble TNF receptor 1 (sTNFr1) were measured as markers of neutrophil degranulation and monocyte activation respectively. None of them showed any correlation with PR3, indicating increased circulating PR3-levels being independent of leukocyte activation. ANCA level and ANCA specificity had no significant influence. Remaining possible explanations for the increased circulating levels include selective leakage from granules, defects in the liver or reticulo-endothelial uptake of PR3/SERPIN complexes or factors leading to increased PR3 production. The latter possibility was supported by the results from Dr J J Yang et al earlier this year, reporting upregulation of the PR3 gene in leukocytes. (9)

The monocytes play a central part in the scheme of inflammation and constitute a relatively homogenous cell population with a versatile transcription apparatus. We chose to study PR3 expression in monocytes in order to investigate the possibility of up-regulated PR3 gene transcription. Peripheral blood monocytes were isolated from patients with AASV in different stages of disease activity, healthy blood donors (HBD) and patients suffering from systemic lupus erythematosus (SLE). As we found markedly increased levels among AASV patients we also studied other granular proteins such as human leukocyte elastase (HLE) and myeloperoxidase (MPO). To explore the possibility that the PR3 upregulation was an effect of inflammation in general we also measured interleukin-8 mRNA and performed in vitro stimulation experiments using the monocyte like cell lines U937 and THP-1 (10) as well as human peripheral blood monocytes (PBMC) from healthy controls.

## **Methods**

### **Patients**

22 patients with AASV, according to the Chapel Hill Consensus Conference definitions, none of which were on dialysis or suffered from any bacterial or viral infections or cancer, were consecutively included in this study (Table 1a). Based on clinical observations performed by their regular physicians at the Department of Nephrology, Lund University Hospital, their status at the time of sampling was classified either as remission, chronic smoldering activity or relapse. Clinical status, BVAS, relapse tendency as well as the development of any severe organ damage due to vasculitic complications were registered. The clinical evaluation was done without access to the results of our analyses. The patients were grouped according to ANCA specificity (PR3 or MPO). 9 patients were MPO-positive, 12 patients were PR3-positive. Patient no 22 had the diagnosis Wegener's granulomatosis (restricted to the upper airways), but had never showed any positive results in ANCA analysis and is thus classified as 'seronegative'.

Our control groups consisted of 15 HBD and 18 patients suffering from SLE. The majority of the patients with SLE were on corticosteroid therapy, 4 patients had doses > 15 mg/day (Table 1b).

The studies have been performed after approval from the Ethical committee at Lund University and written informed consent of the patients.

### **Blood samples**

48 ml venous blood from each subject was obtained in EDTA tubes. The blood was put on ice and 32 ml was immediately transferred for monocyte purification. The remaining 16 ml were

centrifuged within one hour; plasma was carefully aspirated and stored at  $-20^{\circ}\text{C}$  until assayed.

## **Elisa**

*PR3*. Elisa was performed using monoclonal antibodies as capture antibodies, as described earlier (8).

*MPO*. A microtiter plate (Nunc immunoplate) was coated overnight with  $100\ \mu\text{l/well}$  of a monoclonal anti-MPO antibody, 2B11 (11),  $1\ \mu\text{g/ml}$  in coating buffer ( $0.01\ \text{M Na}_2\text{CO}_3$ ,  $0.04\ \text{M NaHCO}_3$ ,  $0.02\ \% \text{NaN}_3$ , pH 9.5-9.7) at  $4^{\circ}\text{C}$ . The plate was blocked with coating buffer, containing  $2\ \%$  bovine serum albumin, for 30 min in room temperature and washed with  $0.9\ \% \text{NaCl}$ ,  $0.05\ \% \text{Tween 20}$  three times. All subsequent incubations were performed in  $100\ \mu\text{l}$  volumes at room temperature on a rocking table and followed by washing three times. Plasma samples diluted to 1/50, 1/100 and 1/200 in sample buffer (PBS 7.3-7.4,  $0.05\ \% \text{Tween 20}$ ,  $0.2\ \% \text{BSA}$ ) were added and the plate incubated for one hour. After washing MPO was detected by one-hour incubation with rabbit-anti-MPO (DAKO, Glostrup, Denmark) diluted to 1/2000 in sample buffer. Washing was followed by addition of the conjugate (alkaline phosphatase-labelled swine anti-rabbit IgG, DAKO, Glostrup, Denmark), diluted to 1/1000 in sample buffer and one-hour incubation. P-nitrophenyl-phosphate disodium (Sigma-Aldrich Corp, St Louis, MO, USA)  $1\ \text{mg/ml}$  in substrate buffer ( $1.0\ \text{M diethanolamine}$ , pH 9.8,  $0.5\ \text{mM MgCl}_2$ ,  $0.02\ \% \text{NaN}_3$ ) was used as substrate and optical densities were read at  $405\ \text{nm}$ . A standard curve was produced by incubation of a two-fold dilution series of MPO (Wieslab AB, Lund, Sweden), starting with  $10\ \text{ng/ml}$ .

*IL-6*, *IL-8*. A quantitative sandwich enzyme immunoassay from R&D systems (Abingdon, UK), where a monoclonal antibody specific for either IL-6 or IL-8 had been pre-coated onto a microplate, was used.

*Cystatin C, CRP, WBC*. The Clinical Chemical Laboratory at Lund University Hospital, Lund, Sweden, performed analyses on a Hitachi 917 Pluto. Kits from Roche Diagnostics (Basel, Switzerland) and Dako (Glostrup, Denmark) were used.

*ANCA*. Wieslab AB, Lund, Sweden, performed analyses of PR3-ANCA and MPO-ANCA by routine methods (12).

## **Cell separation**

Peripheral blood monocytes were isolated by means of a monocyte isolation technique based on the OptiPrep density-gradient medium (Axis-Shield PoC AS, Oslo, Norway) (13). The method is carried out at 4°C, during sterile conditions, using sterile solutions. Briefly OptiPrep working solution (WS) was added to whole blood. A centrifugation gradient was created by mixing WS and solution B (DMEM medium supplemented with 10% serum; Invitrogen, Carlsbad, CA, USA). 5 ml blood was pipetted into a 15 ml test tube, after that 5 ml gradient was carefully added and finally 0.5 ml solution B on top (in order to avoid banding of the cells at a liquid/air interface). During the following centrifugation (700g, 30 min, 4°C, no brake during deceleration) the monocytes float to the top of the gradient layer. After collection, the cells were gently diluted with 2 vol Solution B, harvested by centrifugation and resuspended in Solution B. 25 µl cell suspension was then mixed with Türk's solution (methyl-violet) and counted in a Bürker chamber. A cell smear from each sample was also stained with May Grünwald for differential counting. The monocyte purity was 85-95%, with single contaminating lymphocytes.

## **RNA extraction**

Total RNA was extracted with RNeasy Mini kit (Qiagen, VWR International, West Chester, PA, USA) using the supplied protocol. High purity and good integrity were determined in two ways. First by optical density, 260/280 nm spectrophotometric ratios and then by the Agilent



2100 Bioanalyzer, using the RNA 6000 Nano Assay reagent kit (Agilent Technologies, Palo Alto, CA, USA). After adding a gel-dye mix together with the RNA sample (25-500 ng) to the RNA 6000 Nano Chip channel system, the bioanalyzer uses electrophoretic and electro-osmotic forces to drive fluids through capillaries to produce a virtual gel image and an electropherogram. In the electropherogram RNA of good quality shows up with clear 18 S and 28 S rRNA peaks and a flat baseline, whereas in the gel you see the corresponding sharp bands – the larger ribosomal band being more intense. Only RNA samples that met these criteria were accepted for further analyses.

### **Quantitative PCR assay**

Total RNA was transcribed into cDNA, using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations. In short, random hexamers were used as template and put into the mastermix together with MultiScribe Reverse Transcriptase, RNase inhibitor, dNTPs, 5.5 mM MgCl<sub>2</sub> and Reverse Transcription Buffer. 500 ng total RNA was added in each 50- $\mu$ l reaction and put in the thermocycler, set at 25°/10 min, 48°/30 min, 95° 5 min. For determination of gene expression, quantitative PCR assays were performed on an ABI PRISM 7000 Sequence Detector (Applied Biosystems) with Taqman Universal Master Mix UNG, using the standard conditions determined by the company. After UNG incubation for two min at 50° and AmpliTaq Gold activation for 10 min at 95°, 40 cycles were run with denaturing temperature 95° (15 seconds) and annealing/extension temperature 60° (1 minute). Assay on Demand, a unique combination of forward and reverse primers and fluorescent MGB-probes designed by the company, was used for each target gene.  $\beta$ -actin expression levels were used for normalization. cDNA corresponding to 10 ng RNA was used per 25  $\mu$ l reaction and each reaction was performed in triplicate. The level of expression was calculated based upon the PCR cycle number (Ct) at which the exponential growth in fluorescence from the probe

passes a certain threshold value. Relative expression was determined by the difference in the Ct values for the target genes after normalization to RNA input level, using  $\beta$ -actin Ct values. Relative quantification was determined by standard  $2^{(-\Delta\Delta Ct)}$  calculations (14). Data are presented in relation to the median value of the HBD, set as 1.

## **In vitro stimulation assay**

### **Stimulation of cell-lines U937/THP1 cells**

The monocyte-like cell lines THP-1 and U937-4, a subclone of U937, were stimulated with cytokines and LPS for different time periods (10). The cells were cultured in suspension in RPMI 1640 with 10% FCS (Invitrogen, Carlsbad, CA, USA) and exposed to IFN $\gamma$  100 U/ml (Boehringer, Ingelheim, Germany), LPS 10  $\mu$ g/ml (Sigma-Aldrich Corp, St Louis, MO, USA), TNF $\alpha$  20 ng/ml (ICN Biomedichals, Aurora, Ohio, USA) and LPS 10  $\mu$ g/ml plus TNF $\alpha$  20 ng/ml. The cells were harvested, after stimulation and incubation for 6 and 20 hours respectively. Total RNA was extracted using the Trizol LS reagent (Invitrogen). Real time data are presented in relation to cells that have been incubated for 6 vs. 20 hours, without stimuli, set as 1.

### **Stimulation of human PBMC from healthy controls**

The cells were purified using Optiprep as described above and  $10^6$  cells/well were cultured in RPMI 1640 with 10% FCS (Invitrogen, Carlsbad, CA, USA), 10 ng/ml IL-4 and 20 ng/ml GM-CSF for one hour. The cells were then exposed to IFN $\gamma$  or IFN $\alpha$  100 U/ml (Boehringer, Ingelheim, Germany), LPS 10  $\mu$ g/ml (Sigma-Aldrich Corp, St Louis, MO, USA) and TNF $\alpha$  10 ng/ml (ICN Biomedichals, Aurora, Ohio, USA) respectively, and harvested after six hours. Total RNA was extracted using the RNeasy Mini kit. Real time data are expressed in relation to non stimulated cells, set as 1.

## **Statistical analysis**

All statistics were performed in StatView 5.01. Due to not normally distributed parameters, the non-parametric Spearman's rank correlation test was used for correlation analysis in order to reduce the impact of outliers. Analysis of variance was done using the nonparametric Kruskal-Wallis test and Mann Whitney U-test.

## **Results**

### **PR3 and MPO levels in plasma**

We found increased circulating levels of the PR3 protein in patients with AASV, regardless of ANCA specificity as indicated in Table 2. MPO-levels were also found to be higher in the patients with AASV as compared to HBD. The SLE patients had higher levels of PR3 in plasma than the HBD, but not to the same extent as in AASV, whereas MPO levels were similar to AASV. IL-6 and IL-8 were measured in plasma as inflammatory markers, showing similar inflammatory activity in the two disease groups.

### **mRNA levels in monocytes**

Compared to both HBD ( $p < 0.0001$ ) and SLE patients ( $p = 0.01$ ), we found a 10-fold increase of PR3 expression in monocytes from patients with ANCA-associated vasculitis (Table 3). As shown in Figure 1, all but two of the ANCA-positive patients had a PR3 expression greater than the maximum HBD expression. Four of our patients had no ongoing immunosuppressive treatment, whereas the others had different combinations of corticosteroids and other immunosuppressants. The four SLE patients with the most active disease had high PR3-expression. These patients had high doses of corticosteroids, at similar levels as AASV-patients no. 2 and 5 (Table 1a,b). The other patients had low doses of corticosteroids. A 4-fold increase of HLE expression was seen among patients with AASV and a 3-fold among SLE patients ( $p < 0,05$  when compared to HBD). MPO and BPI expression did not differ from that

in HBD for patients with AASV. IL-6 and IL-8 were measured in plasma as inflammatory markers (Table 2). On the RNA-level, IL-6 expression was very low and data therefore unreliable and not shown. This observation indirectly contradicts an unspecific activation of the cells during the purification process. IL-8 expression was increased in the SLE group, but not in the AASV group, indicating a difference in inflammatory mobilization, as shown in Table 3.

In patients with AASV, plasma levels of PR3 tended to reach higher levels in patients with greater expression, but the correlation was not statistically significant (Figure 2). No significant correlation was seen between plasma levels of CRP, IL-6, or IL-8 and PR3-expression. The correlation coefficients were  $-0.01$ ,  $0$  and  $0.4$  respectively. PR3-ANCA titers showed negative correlation with PR3-expression ( $\rho -0.7$ ,  $p < 0.05$ ), whereas the opposite was seen with white blood cell counts (WBC,  $\rho 0.6$ ,  $p < 0.05$ ).

In the ANCA-patients, PR3 expression co-varied to some extent with HLE, and to a minor and degree with MPO and BPI. The SLE patients, however, exhibited an apparent co-variation between all four measured granular proteins (Table 4).

### **In vitro stimulation of monocyte like cell lines and PBMC**

Stimulation of the monocyte like cell lines U937 and THP1 and human PBMC from healthy controls with various cytokines and LPS resulted in activation, since a substantial up regulation of the IL-6 and IL-8 mRNA levels were seen. This was, however, not accompanied by any increase in the mRNA for the granule constituents PR3 or HLE, as shown in Table 5a,b.

## ***Discussion***

This study demonstrates a strong relative increase in mRNA levels for PR3 in monocytes from patients with AASV as compared to both HBD and disease controls. The median result for PR3 mRNA was a 10-fold increase, and the separation between HBD and AASV was remarkable. The correlation between plasma PR3 concentration and monocyte PR3 mRNA was positive, but weak and failed to reach statistical significance when using a non-parametric test. This is, however, not surprising considering that there are several steps between mRNA levels and circulating protein. PR3 is also produced by neutrophils, which are more abundant, and our data concerns normalized RNA and thus indicate PR3 production per cell rather than the total production in the body. Furthermore, autoantibodies against PR3 might influence half-life of PR3 in the circulation and/or the measurement of PR3 in plasma. In the present study higher levels of PR3-ANCA were associated with lower levels of circulating PR3 (data not shown). There was also a negative correlation between ANCA levels and PR3 mRNA in the monocytes, which argue against a direct causative effect of the autoantibodies on the PR3 mRNA production. An *in vitro* study by Yang and co-workers did not find PR3 among genes up regulated in neutrophils treated with ANCA-IgG (15).

PR3 is normally transcribed during myelopoiesis and is supposed to be turned off in mature leucocytes (16). Constitutive expression of PR3 is a feature of many haematopoietic malignancies where a differentiation block have prevented the maturation and kept the cells in a proliferative state (17). An alternative name for PR3 is myeloblastin, which was first described as a substance that maintained the proliferative capacity of myeloblasts. Antisense treatment blocking PR3 transcription led to growth arrest and differentiation of promyelocytic leukaemia cells (18).

Recently there have been several reports showing that PR3 production can occur in more mature cells. Zhou *et al* have published a study showing de novo synthesis of PR3 by circulating mononuclear cells that were cultured and stimulated with TNF $\alpha$ . This study was performed using mononuclear cells from healthy donors and the percentage monocytes/lymphocytes was 40/60, leaving a potential lymphocyte influence on the results (19). Another study, by Just *et al*. showed an up-regulation of PR3 mRNA expression in circulating monocytes, but not in neutrophils, in cystic fibrosis patients, correlating with pulmonary exacerbation (20). Brockman *et al* found increased PR3 expression in macrophages at inflammatory sites in lung tissue from patients with WG (21). Yang *et al* earlier this year demonstrated increased PR3 transcription in circulating leukocytes from patients with AASV compared with HBD and SLE patients, correlating with disease activity (9). These reports indicate that our results might be a result of cytokine action caused by general inflammation. There are several findings in our study that argues against this notion: 1) there was no correlation between circulating IL-6, IL-8 or CRP-levels and PR3 expression, 2) there was no significant IL-6 or IL-8 up regulation on the mRNA level in the monocytes, 3) there was no general up regulation of granular proteins, 4) in vitro stimulation of monocyte-like cell lines and healthy PBMC with TNF $\alpha$ , IFN $\gamma$  or LPS for 6 or 20 hours did not result in any increase of PR3 production, whereas the IL-8 expression was highly up regulated.

In addition to the increased PR3 levels we also found a minor increase in HLE mRNA levels when compared to HBD, but we found no significant alteration of MPO or BPI. These results differ from those of Dr Yang, which indicated a more general upregulation of granular proteins (9). What we did see was that the SLE patients exhibited a strong co-regulation of the transcription of the granular enzymes. Thus there were both quantitative and qualitative differences between SLE and AASV monocyte gene transcription in the present study. There are reports that G-CSF (granulocyte colony stimulating factor) can up regulate PR3

transcription (22). Indeed we found a positive correlation between total white blood cell count and monocyte PR3 mRNA. The G-CSF was shown to stimulate through the G-CSF receptor and the transcription factor PU.1. Since PU.1 response elements are present in the vast majority of promoters for granule constituents, high G-CSF levels are unlikely to be responsible for the qualitative difference between AASV and SLE patients.

Another concern is that of immunosuppressive drugs. This was a major reason for choosing SLE patients as disease controls. Fifteen out of 22 patients with AASV had low doses of corticosteroids and three patients had high doses (exceeding 15 mg per day). The latter three had high PR3 expression, but otherwise there was no correlation with the corticosteroid dose. Four of the SLE patients also had high doses of corticosteroids and they exhibited high expression of all measured granular mRNAs, indicating that above a certain dose corticosteroids could influence PR3 expression. Three of our AASV patients were treatment naïve, but still demonstrated considerably raised PR3 expression.

A skewed distribution of a polymorphism in the promoter region of the PR3 gene, involving a transcription factor binding site, have recently been described (23). Since the difference in allele frequency between patients and controls was relatively small it is very unlikely that this polymorphism could explain our present findings.

If the increased PR3 production is not caused by inflammation, medication, autoantibodies or germline genetic variants, what could then be the cause? A highly speculative hypothesis would be that a somatic mutation occurs in a stem cell leading to very late maturation block with increased PR3 production as a major feature. This is a bold hypothesis in need of extensive work in order to be thoroughly evaluated.

In conclusion, circulating monocytes from patients with systemic vasculitis produce increased amounts of PR3. Considering the great toxic potential of PR3, this may be of

importance to the development of vasculitis. The origin of this increased production remains obscure. Our results do not favor an influence of cytokines, ANCA or immunosuppressive medication. All of these aspects, however, need to be further studied.

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**Table 1a. Patients with ANCA-associated small vessel vasculitis**

| <i>Patient</i> | <i>Age</i> | <i>Sex</i> | <i>Status</i> | <i>Spec</i> | <i>Titre</i> | <i>BVAS</i> | <i>CRP</i> | <i>WBC</i> | <i>Immunosuppressive treatment at the day of sampling</i> |
|----------------|------------|------------|---------------|-------------|--------------|-------------|------------|------------|---|
| 1              | 70         | F          | G             | MPO         | 580          | 2           | 18         | 7.6        | MTX12.5; P 2.5  |
| 2              | 55         | M          | R             | MPO         | >320         | 10          | <5         | 8.4        | CP 150; P 80  |
| 3              | 60         | M          | R             | MPO         | >320         | 24          | 13         | 6.9        | none  |
| 4              | 62         | M          | R             | MPO         | 184          | 13          | 74         | 7.3        | none  |
| 5              | 52         | F          | R             | MPO         | 147          | 15          | <5         | 5.8        | none  |
| 6              | 83         | F          | Q             | MPO         | 41           | 0           | <5         | 5.2        | AZ 100; P 7.5   |
| 7              | 84         | F          | Q             | MPO         | 37           | 0           | 12         | 4.6        | P 5   |
| 8              | 52         | F          | R             | MPO         | 25           | 8           | <5         | 8.0        | CP 150; P 60  |
| 9              | 68         | F          | Q             | MPO         | 0            | 0           | <5         | 7.6        | MMF 500 ; P 7.5   |
| 10             | 67         | F          | Q             | PR3         | 85           | 0           | <5         | 4.7        | AZA 100   |
| 11             | 59         | F          | Q             | PR3         | 74           | 1           | <5         | 6.1        | CyA 150; P 2.5  |
| 12             | 68         | F          | G             | PR3         | 37           | 2           | 9          | 5.9        | MTX 12.5; P 10  |
| 13             | 51         | F          | Q             | PR3         | 23           | 0           | 13         | 7.3        | CP 75; P 7.5  |
| 14             | 26         | M          | G             | PR3         | 17           | 3           | <10        | 3.6        | MTX 22.5; P 10  |
| 15             | 48         | M          | Q             | PR3         | 0            | 0           | <5         | 5.8        | CP 100; P 7.5   |
| 16             | 68         | M          | Q             | PR3         | 0            | 0           | <9         | 6.0        | AZA 100; P 7  |
| 17             | 45         | M          | Q             | PR3         | 0            | 1           | 11         | 8.7        | MTX 20; P 10  |
| 18             | 57         | M          | Q             | PR3         | 0            | 0           | <5         | 8.6        | CP 125; P 20  |
| 19             | 74         | M          | Q             | PR3         | 0            | 0           | 6          | 6.3        | MTX 10; P 5   |
| 20             | 65         | M          | G             | PR3         | ND           | 3           | 16         | 7.9        | AZ 100; P 10  |
| 21             | 61         | M          | G             | PR3         | ND           | 3           | 10         | 6.4        | CyA 200; MMF 1000   |
| 22             | 28         | M          | Q             | seroneg     | ND           | 0           | <5         | 5.9        | none  |

M = male; F = female. G =grumbling activity; Q = quiescent disease/remission; R =relapse;  
 Spec = ANCA-specificity; MPO = antibodies to myeloperoxidase (MPO-ANCA) at  
 diagnosis; PR3 = antibodies to proteinase 3 (PR3-ANCA) at diagnosis; Titre = Results of  
 ANCA determinations at the day of sampling (ELISA units); ND = not done; CRP = C-  
 reactive protein (mg/l); WBC = white blood cell count (x10<sup>9</sup>/l); MTX= methotrexate  
 mg/week; P=prednisolone mg/day; CP =cyclophosphamide mg/day; AZ= azathioprine  
 mg/day; CyA = cyclosporin A mg/day; MMF = mycophenolatmophetil mg/day

**Table 1b. Control group, patients with SLE**

| <i>Patient</i> | <i>Age</i> | <i>Sex</i> | <i>Immunosuppressive treatment at the day of sampling</i> |
|----------------|------------|------------|---|
| <b>1</b>       | 25         | F          | P 30; CP, pulse therapy                                   |
| <b>2</b>       | 60         | F          | P 6   |
| <b>3</b>       | 28         | F          | P 5; AZ 50  |
| <b>4</b>       | 59         | F          | AZ 25   |
| <b>5</b>       | 51         | F          | P 5; AZ 100   |
| <b>6</b>       | 35         | F          | P 7.5; AZ 150   |
| <b>7</b>       | 30         | F          | P 10; AZ 50   |
| <b>8</b>       | 59         | F          | None  |
| <b>9</b>       | 28         | F          | None  |
| <b>10</b>      | 51         | F          | P 8.75  |
| <b>11</b>      | 27         | M          | P 30; AZ 150  |
| <b>12</b>      | 42         | F          | P 5   |
| <b>13</b>      | 59         | F          | None  |
| <b>14</b>      | 21         | F          | P 10; MMF 500   |
| <b>15</b>      | 25         | M          | P 20  |
| <b>16</b>      | 57         | F          | P 20; MMF 500   |
| <b>17</b>      | 60         | F          | P 10; AZ 50   |
| <b>18</b>      | 24         | F          | P 2.5; MMF 2000   |

M = male; F = female. P=prednisolone mg/day; CP =cyclophosphamide mg/day; AZ= azathioprine mg/day; MMF = mycophenolatmophetil mg/day

**Table 2. PR3 and MPO protein levels in plasma**

|                | <i>PR3</i><br>( $\mu\text{g/l}$ ) | <i>MPO</i><br>( $\mu\text{g/l}$ ) | <i>IL-6</i><br>( $\text{ng/l}$ ) | <i>IL-8</i><br>( $\text{ng/l}$ ) |
|----------------|-----------------------------------|-----------------------------------|----------------------------------|----------------------------------|
| <b>AASV</b>    | 560 (110-3940)                    | 74 (14-120)                       | 3.2 (1.6-8.6)                    | 8.3 (2.4-18.9)                   |
| <b>MPO-pos</b> | 560 (380-1770)                    | 68 (14-103)                       | 3.9 (1.8-7.8)                    | 7.2 (2.4-18.9)                   |
| <b>PR3-pos</b> | 570 (110-3940)                    | 74 (45-120)                       | 3.1 (1.6-8.6)                    | 9.1 (7.7-17.1)                   |
| <b>HBD</b>     | 350 (110-580)                     | 15 (12-18)                        | 1.0 (0.3-6.1)                    | 4.7 (2.4-8.8)                    |
| <b>SLE</b>     | 435 (138-959)                     | 69 (49-90)                        | 3.0 (1.2-8.7)                    | 5.7 (3.7-12.0)                   |

AASV= all patients with ANCA associated vasculitis; MPO-pos = patients with MPO-ANCA at diagnosis; PR3-pos= patients with PR3-ANCA at diagnosis; HBD = healthy blood donors; SLE = disease controls with systemic lupus erythematosus. Data presented as ‘median (range)’.

**Table 3. Monocyte expression profile, relative mRNA levels**

|                      | <i>PR3</i>       | <i>HLE</i>       | <i>MPO</i>       | <i>BPI</i>       | <i>IL-8</i>       |
|----------------------|------------------|------------------|------------------|------------------|-------------------|
| <b>AASV, n= 22</b>   | 9.6<br>(1.8-680) | 4.0<br>(0.6-52)  | 2.1<br>(0.4-14)  | 1.4<br>(0.3-12)  | 0.9<br>(0.03-13)  |
| <b>MPO-pos, n=9</b>  | 15.4<br>(5.4-70) | 5.7<br>(0.6-12)  | 2.1<br>(0.8-8.1) | 1.4<br>(0.7-12)  | 1.3<br>(0.2-13)   |
| <b>PR3-pos, n=12</b> | 8.1<br>(1.8-680) | 2.5<br>(0.9-52)  | 1.6<br>(0.4-14)  | 1.5<br>(0.3-7.6) | 0.3<br>(0.03-6.4) |
| <b>ANCA-neg, n=1</b> | 0.8              | 0.6              | 0.3              | 0.3              | 2.0               |
| <b>HBD, n=15</b>     | 1.0<br>(0.1-2.8) | 1.0<br>(0.2-4.8) | 1.0<br>(0.4-8.1) | 1.0<br>(0.3-23)  | 1.0<br>(0-42)     |
| <b>SLE, n=18</b>     | 1.4<br>(0.1-480) | 3.1<br>(0.4-540) | 5.7<br>(1.0-150) | 1.2<br>(0.3-34)  | 5.2<br>(0.2-33)   |

AASV= all patients with ANCA associated vasculitis; MPO-pos = patients with MPO-ANCA at diagnosis; PR3-pos= patients with PR3-ANCA at diagnosis; HBD = healthy blood donors; SLE = disease controls with systemic lupus erythematosus. The real time data are corrected according to the  $2^{-\Delta\Delta CT}$  formula and then expressed in relation to the median value of the healthy controls. Results are shown as ‘median (range)’.

**Table 4. Co-variation of the relative mRNA levels of some monocyte genes.**

|                         | <i>IL-8</i> | <i>BPI</i> | <i>MPO</i> | <i>HLE</i> |
|-------------------------|-------------|------------|------------|------------|
| <b><i>PR3, AASV</i></b> | 0.2 ns      | 0.5*       | 0.6*       | 0.7**      |
| <b><i>SLE</i></b>       | -0.4 ns     | 0.8 **     | 0.8**      | 0.9**      |
| <b><i>HLE, AASV</i></b> | 0.5*        | 0.7*       | 0.7*       |            |
| <b><i>MPO, AASV</i></b> | 0.4*        | 0.6*       |            |            |
| <b><i>BPI, AASV</i></b> | 0.5*        |            |            |            |

AASV= ANCA-associated systemic vasculitis; SLE, systemic lupus erythematosus.

Correlations are expressed as Rho; \* p<0,05; \*\* p<0,001; ns p>0.05.

**Table 5a. Cytokine stimulation of PR3, HLE and IL-8 expression in THP1 and U937 cell lines**

|                       | <i>PR3</i> | <i>HLE</i> | <i>IL-8</i> | <i>IL-6</i> |
|-----------------------|------------|------------|-------------|-------------|
| <b>THP1, 6 hours</b>  |            |            |             |             |
| unstimulated          | 1          | 1          | 1           | 1           |
| IFN $\gamma$          | 1.5        | 1.3        | -           | -           |
| TNF $\alpha$          | 0.8        | 0.6        | 10.5        | 3.0         |
| LPS+TNF $\alpha$      | 0.8        | 0.7        | 4.0         | 2.8         |
| <b>THP1, 21 hours</b> |            |            |             |             |
| unstimulated          | 1          | 1          | -           | -           |
| IFN $\gamma$          | 0.5        | 0.5        | -           | -           |
| TNF $\alpha$          | 0.3        | 0.3        | -           | -           |
| LPS                   | 0.6        | 0.8        | 19.3*       | -           |
| IFN $\alpha$          | 0.3        |            |             |             |
| <b>U937, 6 hours</b>  |            |            |             |             |
| unstimulated          | 1          | -          | -           | 1           |
| IFN $\gamma$          | 0.7        | -          | -           | 24.3        |
| TNF $\alpha$          | 0.8        | -          | -           | 4.7         |
| LPS                   | 0.9        | -          | -           | 2.5         |
| <b>U937, 21 hours</b> |            |            |             |             |
| unstimulated          | 1          | -          | -           | -           |
| TNF $\alpha$          | 0.1        | -          | -           | -           |
| LPS                   | 0.3        | -          | -           | -           |
| IFN $\alpha$          | 0.3        | -          | -           | -           |
| <b>Mono, 21 hours</b> |            |            |             |             |
| unstimulated          | 1          | -          | -           | -           |
| IFN $\alpha$          | 1.1        | -          | -           | -           |

The cells were incubated for 6 and 21 hours respectively. The real time RT-PCR data are corrected according to the  $2^{-\Delta\Delta CT}$  formula and expressed in relation to unstimulated cells, incubated for the same length of time. \* unstimulated THP1 incubated 6 h set as 1 in this experiment. - = not done; IFN $\gamma$  =interferon gamma 100 U/ml; TNF $\alpha$  = tumor necrosis factor alpha 20 ng/ml; LPS = lipopolysaccharide 10  $\mu$ g/ml. Mono = human monocytes; IFN $\alpha$  = interferon alpha, 1000 U/ml.

**Table 5b. Cytokine stimulation of PR3, HLE and interleukin expression in healthy peripheral blood monocytes**

|                               | <i>PR3</i> | <i>HLE</i> | <i>IL-8</i>         | <i>IL-6</i>         |
|-------------------------------|------------|------------|---------------------|---------------------|
| <b>Culture medium</b>         | 0.4        | 0.3        | 2.3*10 <sup>4</sup> | 0.4*10 <sup>4</sup> |
| <b>LPS</b>                    | 0.2        | 0.2        | 10 *10 <sup>4</sup> | 1.2*10 <sup>4</sup> |
| <b>TNF<math>\alpha</math></b> | 0.3        | 0.2        | 3.2*10 <sup>4</sup> | 0.2*10 <sup>4</sup> |
| <b>IFN<math>\gamma</math></b> | 0.2        | 0.1        | 3.5*10 <sup>4</sup> | 0.4*10 <sup>4</sup> |

The cells were incubated with stimuli for six hours. Data are presented as mean values of PBMC from three separate donors, in relation to the corresponding freshly drawn, non stimulated samples.

Culture medium = RPMI with 10% FCS, 10 ng/ml IL-4 and 20 ng/ml GM-CSF; LPS = lipopolysaccharide, 10  $\mu$ g/ml; TNF $\alpha$  = tumor necrosis factor alpha, 10  $\mu$ g/ml; IFN $\gamma$  = interferon gamma, 100 u/ml.







