Neutrophil-related and serum biomarkers in granulomatosis with polyangiitis support extracellular traps mechanism of the disease

M. Surmiak[1,2], M. Hubalewska-Mazgaj[1,2], K. Wawrzycka-Adamczyk[1], W. Szczeklik[1], J. Musiał[1], T. Brzozowski[2], M. Sanak[1]

[1]Department of Internal Medicine, and [2]Department of Physiology, Jagiellonian University Medical College, Krakow, Poland.

Marcin Surmiak, PhD
Magdalena Hubalewska-Mazgaj, PhD
Katarzyna Wawrzycka-Adamczyk, MD
Wojciech Szczeklik, MD, PhD
Jacek Musiał, MD, PhD
Tomasz Brzozowski, PhD
Marek Sanak, MD, PhD

Please address correspondence to:
Marek Sanak, MD, PhD,
Department of Internal Medicine,
Jagiellonian University Medical College,
Skawinska 8 Street,
31-066 Krakow, Poland.
E-mail: nfsanak@cyf-kr.edu.pl

ABSTRACT

Objective. *Granulomatosis with polyangiitis (GPA) is an autoimmune disease with still unknown aetiology. Recent studies indicate that neutrophils extracellular traps participate in the pathophysiology of GPA. This study investigates the levels of circulating NET formation markers and neutrophil-platelet interaction in patients with GPA.*

Methods. *We enrolled 40 GPA patients (20 in the active stage of the disease and 20 in remission). Twenty sex- and age-matched healthy subjects served as a control group. Serum/plasma levels of serine proteases, and histone-, myeloperoxidase-, proteinase-3 DNA complexes and sP-selectin were measured using ELISA or Luminex assays. Circulating platelet-neutrophil aggregates and neutrophils activation markers expression was measured by flow cytometry.*

Results. *Patients in active stage of GPA had higher circulating levels of serine proteases, DNA-histone and myeloperoxidase -DNA complexes. In addition, platelet-neutrophil aggregates and sP-selectin were also elevated in this group. Platelet-neutrophil aggregates and myeloperoxidase -DNA complexes correlated positively with the disease activity score (BVAS).*

Conclusion. *NETs production and activation of platelets in GPA is supported by elevated myeloperoxidase-DNA complexes and platelet-neutrophil aggregates correlating positively with the disease activity score. This mechanism justifies laboratory measurements of myeloperoxidase-DNA complexes and plasma sP-selectin as biomarkers for studying GPA activity.*

Introduction

Granulomatosis with polyangiitis, described by Klinger in 1931 and by Wegener in 1936, is a rare multisystemic disease comprising necrotising granulomatous vasculitis, with respiratory tract and kidneys being primary sites of involvement. A hallmark for this disease is presence of antineutrophil cytoplasmatic antibodies (ANCA), which are serology markers of the disease (1). In the most cases a cytoplasmic pattern of reactivity of these antibodies (c-ANCA) is directed against proteinase-3 (PR-3), a protease released from azurophilic granules of the human neutrophil lysosomes. Other auto-antibodies present in the disease may have perinuclear pattern (p-ANCA) and are directed against myeloperoxidase (MPO), catepsin G, lactoferrin, elastase or other antigens (2-4).

Neutrophil extracellular traps (NETs) are neutrophil-released DNA fibres containing histones and antimicrobial molecules. Their main role is to capture and kill pathogens. Since their discovery there has been interest whether NETs can also drive autoimmunity in GPA. The current evidences suggest that NETs are directly involved in the endothelial damage and induction of ANCA (5). It has been shown that NET formation or impaired NET degradation can participate in the pathogenesis of systemic lupus erythematosus (6). In GPA, NETs containing PR3 and MPO were detected in kidneys during active phase of the disease (7). Polymorphonuclear neutrophils (PMNs) obtained from patients with ANCA-associated vasculitides (AAV) were able to produce more ROS and enhanced NET formation was observed as compared to healthy controls (8).

Recently, a platelet-neutrophil interaction was documented during NET formation and associated with pathomechanism of GPA (9). Circulating neutrophil-platelet aggregates marks

for neutrophil activation and adhesion. Moreover, platelet-derived P-selectin can induce NET formation (10, 11). NETs have the potential to promote thrombosis and incidence of thrombotic events in AAV patients is significantly increased (12–14).

Although the hypothesis on NET formation as contributing to GPA is credible, also including NET-associated auto-antigens presentation to initiate ANCA immunoreaction, the place of this mechanism in the inflammation observed during the clinical course of the disease remains to be elucidated.

Materials and methods
Patients and study design
We enrolled 36 GPA patients for this study (20 in the active stage of the disease, 6 of whom were reevaluated during remission, and 14 patients in the remission) Samples were collected between October 2012 and June 2015). Severity of the disease was evaluated using the Birmingham Vasculitis Activity Score (BVAS version 3.0). In all patients the disease diagnosis was confirmed using The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. Twenty sex- and age-matched healthy subjects served as a control group (HC). Peripheral blood samples were collected without anticoagulant or with potassium ethylenediamine tetraacetic salt using uniform blood collection system (Venous Blood - S-Monovette®, Sarstedt, Germany). Serum or plasma was separated using standard laboratory procedures, then aliquoted and frozen in -80°C for further analyses. In patients with the active stage of GPA all blood samples were taken prior to immunosuppressive drug administration. All participants in this study had basic laboratory tests done (CBC, CRP level, creatinine and LDH level) and measured both anti-PR3 IgG and anti-MPO IgG (ELISA, EUROIMMUN, Lübeck, Germany). All GPA patients were anti-PR3 positive and anti-MPO negative. The study was approved by the Bioethical Committee of Jagiellonian University and informed consent was obtained from all the subjects.

Serum nucleases activity assay
Total serum activity of nucleases was evaluated using DNAse detection kit as recommended by the manufacturer protocol (Jena Bioscience, Jena, Germany). In brief, 10 μL of serum was mixed with 10 μL of the master mix containing reaction buffer and a fluorescent dye (FAM) labelled DNA probe. Data collection was done using a real-time thermal cycler (7900HT Fast Real-Time PCR System – Life Technologies, Carlsbad, CA) with a two-step cycling protocol: 36°C for 10 sec and 37°C for 50 sec for 30 cycles. The results were presented as a relative fluorescence.

Serum levels of MPO and circulating MPO-DNA, PR3-DNA, DNA-histone complexes
Measurements of circulating complexes of neutrophil proteases/peroxidase-DNA and DNA-histones were done using ELISA. Serum DNA-histone complexes we measured using a commercially available EIA kit (DNA-Death plus, BS, Switzerland, Roche). Determination of MPO-DNA and PR3-DNA complexes was performed with use of ELISA as previously described (7). Briefly, 96-well ELISA plates were coated with anti-MPO or anty-PR3 antibodies (anti -MPO - 5μg/ml, AbDSerotec, NC, USA; anti-PR3- 5-20 μg/ml Hycult Biotech,Uden, NL). All buffers and secondary HRP-conjugated antibody were from DNA-Death plus EIA kit. Results were calculated from the calibration curve for MPO (mg/mL) or expressed as optical densities for MPO-DNA, PR3-DNA, DNA-histone complexes.

Serum levels of PR3, neutrophil elastase and plasma level of soluble P-selectin
Determination of plasma level of soluble P-selectin (sP-selectin), serum level of PR3 and neutrophil elastase (NE) was done using Luminex microbeads fluorescent assays (sP-selectin - Bio-Plex Pro™ Human Chemokine Panel, Bio-Rad, Hercules, CA; PR3 and NE - Merck Millipore, Darmstadt, Germany) and MAGPIX fluorescent-based detection system (Luminex Corp., Aus-

tin, TX). Results were calculated from calibration curves in pg/mL.

Circulating platelet-neutrophil aggregates and measurements of neutrophil activation
Evaluation of circulating platelet-neutrophil aggregates and neutrophil activation was carried out in the whole anticoagulated blood (sodium citrate) with Epic XL Beckman flow cytometer. During neutrophil activation studies, cells were stained with PE-Cy5- labelled anti-CD16 (BD Biosciences, NJ), FITC-anti-CD11b (SIGMA, St. Louis, MO) or PE-anti-CD66b (BD Biosciences) antibodies or appropriate isotype controls for 30 minutes, fixed with FACS Lysing Solution (BD Biocsiences), washed and counted. Events positive for both CD16 and CD11b or CD66b were considered activated neutrophils. For platelet-neutrophil aggregates, a platelet marker (CD42a; PE-anti-CD42a, BD Biosciences) was used concurrently with the neutrophil CD16 marker. Neutrophils were gated according to forward/side scatter signal. Events positive for both CD16 and CD42a were defined as platelet-neutrophil aggregates.

Statistical analysis
Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc, San Diego, CA). All comparisons were done using one-way analysis of variance (ANOVA) with Tukey's post hoc test or Kruskal-Wallis test for not-normally distributed variables with Dunn's post hoc test. Descriptive statistics were presented as mean and standard deviation (SD) or median with 25th-75th percentile range, depending on the distribution. Type I statistical error $p<0.05$ was considered significant.

Results
Clinical characteristic of the study groups
Patients stratified according to the presence of symptoms of the active GPA (BVAS>0) or remission (BVAS=0) had no differences in their age or gender distribution. In patients with the active disease, higher doses of oral glucocor-

ticoids were administered, however this difference was not significant. The active disease was also characterised by elevated white blood cells count, platelets and PMN leukocytes count, higher serum levels of C-reactive protein and creatinine. Serum lactic dehydrogenase was elevated both in the active and inactive GPA, possibly indicating an on-going tissue damage. Details on the study groups are summarised in Table I.

Whole blood platelet-neutrophil aggregates and neutrophil activation
Higher level of platelet-neutrophil (P-N) aggregates and expression of CD11b on neutrophils were observed only in patients with the active stage of GPA (P-N aggregates: active GPA 15.6% (12.9–24.5) *vs.* remission GPA 11.4% (9.5–13) but not in healthy controls (HC 8.9%(6.7–11.1)), *p*<0.05, Fig. 1B; CD11b expression: active GPA 33.2±8.2 MFI *vs.* remission GPA 21.3±5.4 and HC 22±4.7, *p*<0.05, Fig. 1A). Percentage of circulating platelet-neutrophil aggregates also correlated positively with BVAS (ϱ=0.72, *p*<0.05, Fig. 1C). Similar correlation was found for the level of MPO-DNA complexes in active GPA (ϱ=0.65, *p*<0.05, Fig. 1D) and for all study subjects (ϱ=0.66, data not shown); *p*<0.05). No differences in neutrophil expression of CD66b were noted in either of the studied groups (data not shown).

Serum levels of MPO, PR3, NE and circulating MPO-DNA, PR3-DNA or DNA-histone complexes
MPO and NE levels were elevated in patients with active GPA as compared to the patients in remission or to healthy controls (MPO: active GPA 860±448 *vs.* remission GPA 412.6±200 and HC 323.9±156 ng/mL, *p*<0.05, Fig. 2B; NE: active GPA 159.4±50.8 *vs.* remission GPA 109.9±46.8 pg/mL and HC 82.55±39.6 pg/mL, *p*<0.05, Fig. 2D). Serum PR3 concentration was higher in GPA patients as compared to HC, but no differences between the active or GPA in remission were observed (active GPA 505 (330.5–958) *vs.* remission 383 (245–770), and HC 263.5 (157.3–303) pg/mL, *p*<0.05, Fig. 2C).

Table I. Basic characteristics of the study participants.

	Active stage of GPA	GPA remission group	Controls
n	20	20#	20
Age (mean±SD)	58.2 ± 13.4	54.2 ± 13.3	55.9 ± 12.6
Gender (F/M)	11/9	10/10	11/9
BVAS (min-max)	6-28	0	0
GC treatment (YES/NO)	6/14	15/5	0/20
GC dose [mg/day] (min-max)	3-20	2-8	0
cANCA (anti-PR3) [RU/mL] (min-max)	<8-200	<3.2-140	<2
CRP [mg/mL]	<5.0-140	<5.0-6.3	<5.0
PBMC [10^3/μL]	10.6 ± 3.9*	7.6 ± 2.49	5.7 ± 2.5
PMN [10^3/μL]	7.76 ± 3.4*	5.38 ± 2.3	3.1 ± 1.1
PLT [10^3/μL]	251 ± 221.3*	218.8 ± 76.3	202.5 ± 36.5
Creatinine [μmol/L]	188 ± 308*	120 ± 81.4	81 ± 25
LDH [U/L]	521 ± 126.4*	516.2 ± 95.53*	391 ± 89

#6 GPA patients were sampled twice: during exacerbation and during remission of the disease.
BVAS: Birmingham Vasculitis Activity Score; *p*<0.05, in comparison with controls; GC: glucocorticosteroids. Platelet count is presented as median with interquartile range.
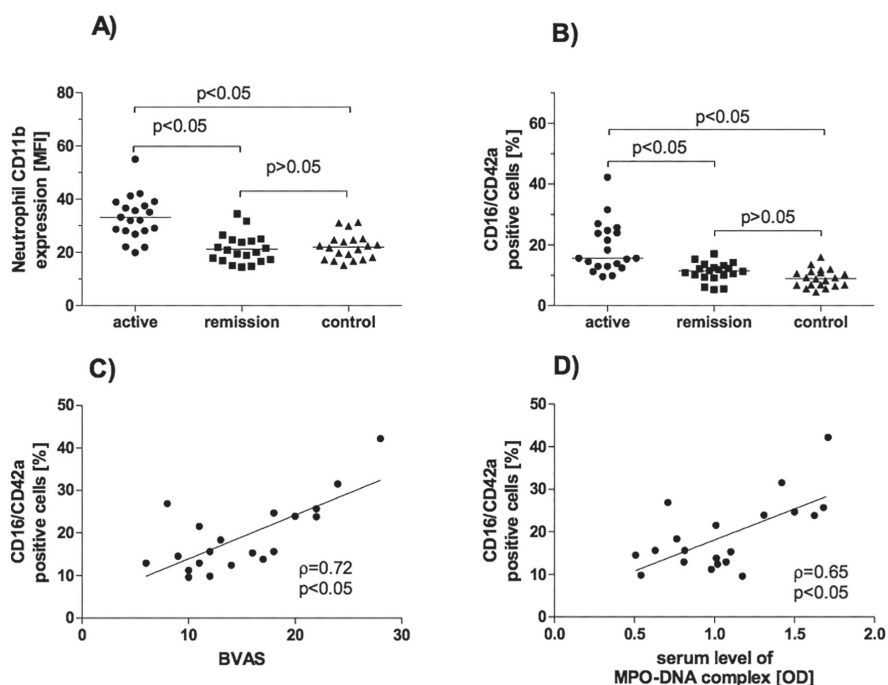

Fig. 1. Neutrophil activation and platelet-neutrophil aggregates in patients with granulomatosis with polyangiitis. **A)** Expression of CD11b on neutrophils, **B)** Platelets-neutrophil aggregates, **C)** Correlation between platelet-neutrophil aggregates and the disease activity (BVAS) in patients with the active stage of GPA. **D)** Correlation between platelet-neutrophil aggregates and MPO-DNA complexes in patients with the active stage of GPA. Platelet-neutrophil aggregates presneted as median (horizontal line), CD11b presented as the geometric mean of fluorescence intensity.

The highest level of DNA-histone complexes was found in patients with the active stage of GPA (active GPA 1.2 (0.84–2.3) *vs.* remission 0.89 (0.32–1.6), and HC 0.33 (0.17-0.63) OD; *p*<0.05, Fig. 2A). Similar results were observed for MPO-DNA complex quantification. In patients with active stage of GPA, serum MPO-DNA complexes level was the highest (active GPA 1.1±0.3 *vs.* remission 0.64±0.25, and HC 0.35±0.13 OD; *p*<0.05, Fig. 3A).

Analysis of correlations revealed that BVAS was positively associated with MPO-DNA complexes along with MPO or NE serum concentrations (BVAS and MPO-DNA, ϱ=0.79, *p*<0.05; NE and MPO-DNA, ϱ=0.566, *p*<0.05; MPO and MPO-DNA, ϱ=0.78,
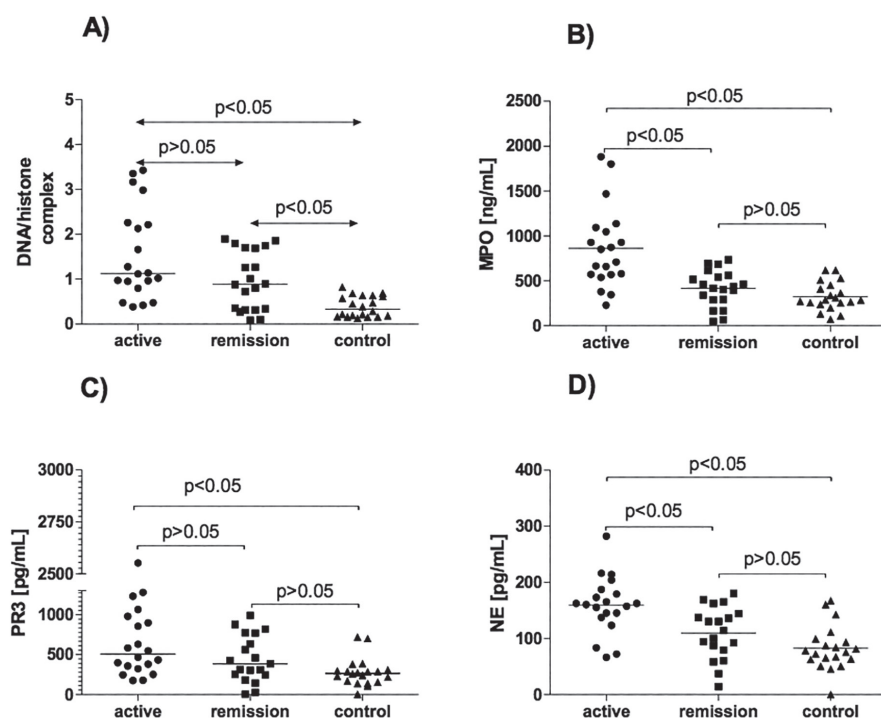
Fig. 2. Neutrophils serine proteases and DNA-histone complex in patients with granulomatosis with polyangiitis. **A)** Serum level of DNA-histone complexes, **B)** Myeloperoxidase serum concentration **C)** Proteinase-3 serum concentratiion, **D)** Neutrophil elastase serum concentration. For MPO and NE plots the horizontal line represents mean, for PR3 and DNA-histone complex the horizontal line presents median. Results are presented as the mean (MPO and NE levels) or the median (PR3 and DNA-histone complexes).
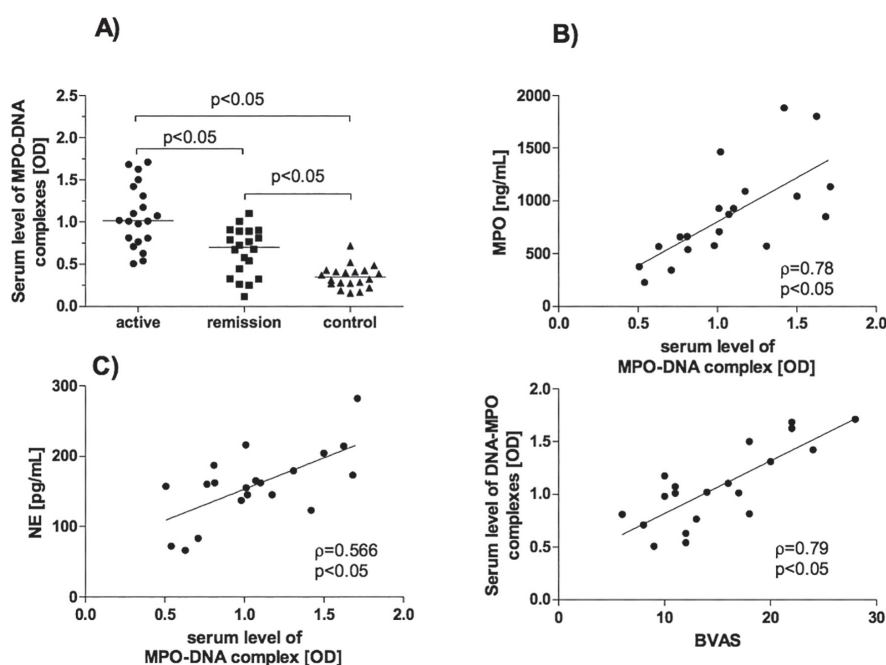

Fig. 3. MPO-DNA complexes in patients with GPA. A) Serum level of MPO-DNA complexes. Correlation between serum MPO-DNA complexes and serum MPO level (B) and serum NE level (C), or BVAS (D). Graphs B-D show patients with the active stage of GPA.

$p<0.05$, Fig. 3B-D). PR3-DNA complexes were detectable only in six patients with the recently diagnosed and treatment naïve GPA, these re- sults were obtained using ELISA plate coated with anti-PR3 antibody at the higher 20 μl/mL concentration (data not shown).

Plasma sP-selectin level and serum nucleases activity

Plasma sP-selectin was elevated only in the patients with active GPA (active GPA 22.45±8.2 *vs.* remission 17.66±3.8, and HC 15.54±3 μg/mL, $p<0.005$, Fig. 4A). Moreover, in the patients with active GPA concentration of sP-selectin positively correlated with BVAS ($\varrho=0.68$, $p<0.05$, Fig. 4B) and circulating MPO-DNA complexes ($\varrho=0.73$, $p<0.05$, Fig. 4C). Measurements of serum total nucleases activity did not reveal any differences between the studied groups (active GPA 298.1±153; remission GPA 272.7±105; HC 264.5±141 RFU, Fig. 4D).

Discussion

Neutrophil is the most important cell involved in the pathophysiology of granulomatosis with polyangiitis. Neutrophil stimulation with cANCA leads to activation of several pathways including cytokines release and prostaglandins production, calcium signalling, generation of reactive oxygen species and secretion of several proteases from granules (15, 16). These neutrophil proteases, like PR3 and NE participate in regulation of several immune response steps like cytokines maturation, differentiation of immune cells or bacteria killing (17, 18). Elevated serum levels of neutrophil proteases were observed in patients with various diseases like type-1 diabetes, acute coronary syndrome, hypertension and ANCA-associated vasculitis (19–22).

Studies of Kessenbrock *et al.* showed that both MPO and PR3 can form complexes with DNA and MPO-DNA complexes were considered a circulating marker of neutrophil extracellular traps formation (7). In our previous study we also described elevated levels of free circulating mitochondrial and genomic DNA as a serum marker of GPA (23). However, in the current study only MPO-DNA complexes were detectable in all clinical samples, whereas PR3-DNA complexes were present only in a few samples collected from recently diagnosed GPA patients. It might be speculated that unlike other proteases, binding of PR3 to DNA is not as specific as complex formation between DNA
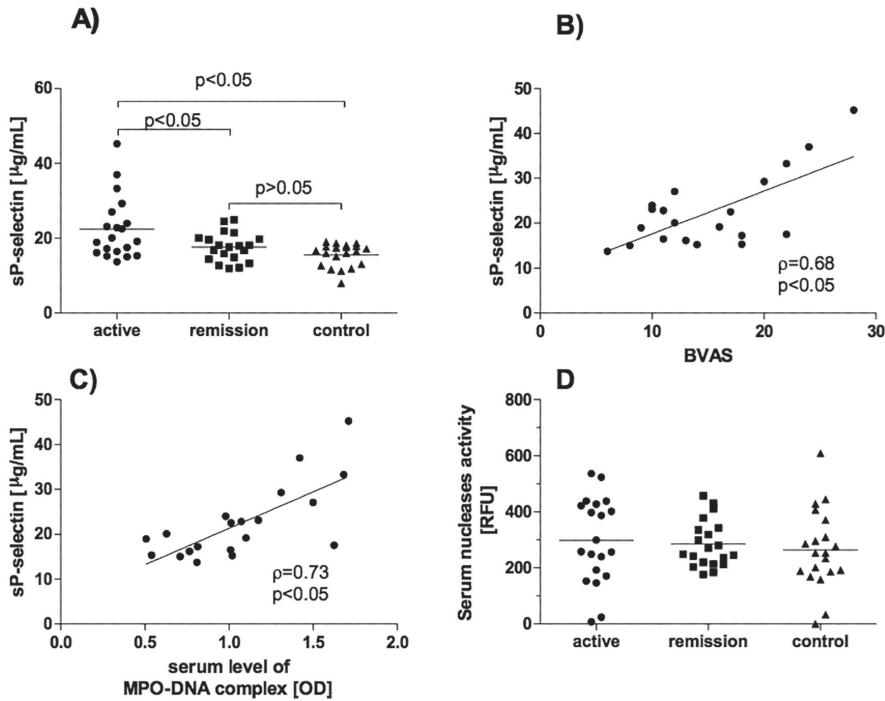
Fig. 4. sP-selectin level and total serum nucleases activity in the studied groups. **A**) plasma level of sP-selectin. **B**) Correlation between plasma sP-selectin level and BVAS (patients with active GPA). **C**) Correlation between plasma sP-selectin level and serum level of MPO-DNA complexes (patients with active GPA). **D**) Total activity of serum nucleases in the study groups.

and NE or cathepsin G (24). Despite the presence of PR3, MPO and NE in NETs, only MPO and NE were reported to contribute to NET formation by enhancement of nuclear chromatin decondensation (25–27). The positive correlation between serum level of NE or MPO and MPO-DNA complexes that we found seems to confirm this hypothesis. Nuclear DNA is tightly bound to histones. A partial proteolysis of histones is required before proteases-DNA complexes are formed. Thus, DNA-histone complexes are released into the extracellular space during the cellular death or are secreted during formation of NETs (28, 29). Elevated levels of nucleosomes were observed in patients with sickle cell disease, cancer, and autoimmune disorders (30-32). It has also been suggested that quantification of circulating DNA-histone complexes could be a marker of NETs formation, however, DNA-MPO complexes are more commonly used for this purpose (14). We noticed a good correlation between DNA-histone and DNA-MPO complexes. However, DNA-histone complexes were also elevated in patients in the remission of GPA at the

level not different from the active GPA. Moreover, DNA-histone complexes did not correlate with BVAS widely used in clinics to evaluate the severity of symptoms. In our opinion, measurements of DNA-MPO complexes are superior to DNA-histone complexes because are more specific for the active phase of the disease and reflect actual NETs formation better.

Beside the main function of trapping and clearing bacteria, NETs might be a source for autoantigens instigating autoimmune disorders and can incite coagulation cascade causing thrombosis (33). Protein C activation by histones was described through a thrombin/thrombomodulin complex binding and more directly a platelet activation and aggregation on NETs was reported (14, 34). Platelet function goes far beyond coagulation process. Activated platelets can release nucleotides, adhesion molecules, coagulation factors and proteases inhibitors (35). There were some suggestions that platelet count is also a biomarker of GPA (36). In line with this report, we observed a higher platelet count in GPA patients as compared with controls but no dif-

ferences were noticed between the active or remission stages. Since platelet can aggregate with neutrophil by an interaction of specific receptors (*e.g.* P-selectin and its ligand PSGL), this may modify neutrophil activity. Fibrinogen bound to activated platelet can activate neutrophil and contribute to the oxidative burst (37). Such an interaction also enhances neutrophil transendothelial migration (38). It is a limitation of the current study that no direct method for platelet activation analysis was used, however, plasma level of soluble P-selectin was suggested a surrogate marker of platelet activation (39, 40). Recent studies are also indicating that P-selectin has a direct impact on platelet-neutrophil aggregation and NET formation (11, 41). Results of the current study seem to confirm this observation because a robust positive correlation between sP-selectin and MPO-DNA complexes was found. We also observed that patients with the active stage of GPA had higher counts of platelet-neutrophil aggregates and this parameter correlated with the disease activity score. The number of reports that document a high frequency of venous thrombotic events in GPA is growing (13, 42). Thus, platelet-neutrophil interactions could be a very important clinical aspect of GPA pathophysiology.

Neutrophil activation and formation of NETs is counterbalanced by extracellular traps removal. NETs are preprocessed by DNAse and are cleared by macrophages. Deficiency of DNAse I mediated NET degradation was observed in patients with acute thrombotic microangiopathies, dermatomyositis and lupus nephritis (6, 43, 44). Based on experiments in vitro, however, even physiological concentration of DNAse I is not sufficient for total NETs degradation, suggesting other mechanisms participating in this process (45). Interestingly, a recent publication by Nakazawa *et al.* (46) showed that M1 subpopulation is responsible both for initial enhancement of NETs DNA content due to a release from macrophages, and subsequent clearance of NETs. In the present study we measured total serum nuclease activity, but failed to

show any difference between GPA and healthy controls. Likewise, complement C3 and C4 did not differ between patients with the active and remission stage of the disease (data not shown). This rather precludes disturbance of NET degradation in GPA. However in our study we did not performed *in vitro* experiments in which we could evaluate a direct impact of serum form GPA patients on NETs degradation and the hypothesis would require future studies. In conclusion, we observed that patients with the active stage of GPA have higher circulating levels of serine proteases, DNA-histone and MPO-DNA complexes. In addition, platelet-neutrophil aggregates and sP-selectin levels were elevated. Since MPO-DNA complexes and platelet-neutrophil aggregates correlated positively with the disease activity score, a conclusion can be drawn on a pertinent NETs production and simultaneous activation of platelets in GPA. These parameters might be ascertained as a standard laboratory test, hence we propose serum MPO-DNA complexes and plasma sP-selectin level as biomarkers for studying GPA activity.

References

1. TARABISHY AB, SCHULTE M, PAPALIODIS GN, HOFFMAN GS: Wegener's granulomatosis: clinical manifestations, differential diagnosis, and management of ocular and systemic disease. *Surv Ophthalmol* 2010; 55: 429-44.
2. KALLENBERG CGM: Pathogenesis and treatment of ANCA-associated vasculitides. *Clin Exp Rheumatol* 2015; 33: S11-4.
3. SAVIGE JA, DAVIES DJ: Anti-neutrophil cytoplasm antibodies (ANCA). *Aust N Z J Med* 1990; 20: 271-4.
4. SAVIGE JA, YEUNG SP, GALLICCHIO M, DAVIES DJ: Two ELISAs to detect anti-neutrophil cytoplasm antibodies (ANCA) in various vasculitides. *Pathology* 1989; 21: 282-7.
5. CARMONA-RIVERA C, ZHAO W, YA-LAVARTHI S, KAPLAN MJ: Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. *Ann Rheum Dis* 2015; 74: 1417-24.
6. HAKKIM A, FÜRNROHR BG, AMANN K *et al.*: Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci USA* 2010; 107: 9813-8.
7. KESSENBROCK K, KRUMBHOLZ M, SCHOU *et al.*: Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* 2009; 15: 623-5.
8. OHLSSON SM, OHLSSON S, SÖDERBERG D *et al.*: Neutrophils from vasculitis patients exhibit an increased propensity for activation by anti-neutrophil cytoplasmic antibodies. *Clin Exp Immunol* 2014; 176: 363-72.
9. LOONEY MR: Platelets Induce Neutrophil Extracellular Traps in Transfusion-Related Acute Lung Injury. *Blood* 2014; 124: SCI – 18.
10. KORNERUP KN, SALMON GP, PITCHFORD SC, LIU WL, PAGE CP: Circulating platelet-neutrophil complexes are important for subsequent neutrophil activation and migration. *J Appl Physiol* 2010; 109: 758-67.
11. ETULAIN J, MARTINOD K, WONG SL *et al.*: P-selectin promotes neutrophil extracellular trap formation in mice. *Blood* 2015; 126: 242-6.
12. EMMI G, SILVESTRI E, SQUATRITO D *et al.*: Thrombosis in vasculitis: from pathogenesis to treatment. *Thromb J* 2015; 13: 15.
13. STASSEN PM, DERKS RPH, KALLENBERG CGM, STEGEMAN CA: Venous thromboembolism in ANCA-associated vasculitis- incidence and risk factors. *Rheumatology* (Oxford) 2008; 47: 530-4.
14. FUCHS TA, BRILL A, DUERSCHMIED D *et al.*: Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA* 2010; 107: 15880-5.
15. KETTRITZ R: How anti-neutrophil cytoplasmic autoantibodies activate neutrophils. *Clin Exp Immunol* 2012; 169: 220-8.
16. SURMIAK M, KACZOR M, SANAK M: Expression profile of proinflammatory genes in neutrophil-enriched granulocytes stimulated with native anti-PR3 autoantibodies. *J Physiol Pharmacol* 2012; 63: 249-56.
17. PHAM CTN: Neutrophil serine proteases fine-tune the inflammatory response. *Int J Biochem Cell Biol* 2008; 40: 1317-33.
18. KORKMAZ B, MOREAU T, GAUTHIER F: Neutrophil elastase, proteinase 3 and cathepsin G: physicochemical properties, activity and physiopathological functions. *Biochimie* 2008; 90: 227-42.
19. BALDUS S, HEESCHEN C, MEINERTZ T *et al.*: Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. *Circulation* 2003; 108: 1440-5.
20. EL-ESHMAWY MM, EL-ADAWY EH, MOUSA AA *et al.*: Elevated serum neutrophil elastase is related to prehypertension and airflow limitation in obese women. *BMC Womens Health* 2011; 11: 1.
21. OHLSSON S, WIESLANDER J, SEGELMARK M: Increased circulating levels of proteinase 3 in patients with anti-neutrophilic cytoplasmic autoantibodies-associated systemic vasculitis in remission. *Clin Exp Immunol* 2003; 131: 528-35.
22. WANG Y, XIAO Y, ZHONG L *et al.*: Increased neutrophil elastase and proteinase 3 and augmented NETosis are closely associated with β-cell autoimmunity in patients with type 1 diabetes. *Diabetes* 2014; 63: 4239-48.
23. SURMIAK MP, HUBALEWSKA-MAZGAJ M, WAWRZYCKA-ADAMCZYK K *et al.*: Circulating mitochondrial DNA in serum of patients with granulomatosis with polyangiitis. *Clin Exp Immunol* 2015; 181: 150-5.
24. DURANTON J, BELORGEY D, CARRÈRE J *et al.*: Effect of DNase on the activity of neutrophil elastase, cathepsin G and proteinase 3 in the presence of DNA. *FEBS Lett* 2000; 473: 154-6.
25. PAPAYANNOPOULOS V, METZLER KD, HAKKIM A, ZYCHLINSKY A: Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol* 2010; 191: 677-91.
26. BJÖRNSDOTTIR H, WELIN A, MICHAÉLSSON E *et al.*: Neutrophil NET formation is regulated from the inside by myeloperoxidase-processed reactive oxygen species. *Free Radic Biol Med* 2015; 89: 1024-35.
27. METZLER KD, GOOSMANN C, LUBOJEMSKA A, ZYCHLINSKY A, PAPAYANNOPOULOS V: A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. *Cell Rep* 2014; 8: 883-96.
28. JAHR S, HENTZE H, ENGLISCH S *et al.*: DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001; 61: 1659-65.
29. BRINKMANN V, REICHARD U, GOOSMANN C *et al.*: Neutrophil extracellular traps kill bacteria. *Science* 2004; 303: 1532-5.
30. SCHIMMEL M, NUR E, BIEMOND BJ *et al.*: Nucleosomes and neutrophil activation in sickle cell disease painful crisis. *Haematologica* 2013; 98: 1797-803.
31. HOLDENRIEDER S, EICHHORN P, BEUERS U *et al.*: Nucleosomal DNA fragments in autoimmune diseases. *Ann NY Acad Sci* 2006; 1075: 318-27.
32. HOLDENRIEDER S, NAGEL D, SCHALHORN A *et al.*: Clinical relevance of circulating nucleosomes in cancer. *Ann NY Acad Sci* 2008; 1137: 180-9.
33. VAN MONTFOORT ML, STEPHAN F, LAUW MN *et al.*: Circulating nucleosomes and neutrophil activation as risk factors for deep vein thrombosis. *Arterioscler Thromb Vasc Biol* 2013; 33: 147-51.
34. AMMOLLO CT, SEMERARO F, XU J, ESMON NL, ESMON CT: Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation. *J Thromb Haemost* 2011; 9: 1795-803.
35. ZARBOCK A, POLANOWSKA-GRABOWSKA RK, LEY K: Platelet-neutrophil-interactions: linking hemostasis and inflammation. *Blood Rev* 2007; 21: 99-111.
36. WILLEKE P, KÜMPERS P, SCHLÜTER B *et al.*: Platelet counts as a biomarker in ANCA-associated vasculitis. *Scand J Rheumatol* 2015; 44: 302-8.
37. KORNERUP KN, SALMON GP, PITCHFORD SC, LIU WL, PAGE CP: Circulating platelet-neutrophil complexes are important for subsequent neutrophil activation and migration. *J Appl Physiol* 2010; 109: 758-67.
38. LAM FW, BURNS AR, SMITH CW, RUMBAUT RE: Platelets enhance neutrophil transendothelial migration via P-selectin glycoprotein ligand-1. *Am J Physiol Heart Circ Physiol* 2011; 300: H468-75.
39. FERRONI P, MARTINI F, RIONDINO S *et al.*: Soluble P-selectin as a marker of *in vivo* platelet activation. *Clin Chim Acta* 2009; 399: 88-91.
40. CHUNG I, CHOUDHURY A, PATEL J, LIP GYH:

Soluble, platelet-bound, and total P-selectin as indices of platelet activation in congestive heart failure. *Ann Med* 2009; 41: 45-51.

41. POLANOWSKA-GRABOWSKA R, WALLACE K, FIELD JJ *et al*.: P-selectin-mediated platelet-neutrophil aggregate formation activates neutrophils in mouse and human sickle cell disease. *Arterioscler Thromb Vasc Biol* 2010; 30: 2392-9.

42. MERKEL PA, LO GH, HOLBROOK JT *et al*.: Brief communication: high incidence of venous thrombotic events among patients with Wegener granulomatosis: the Wegener's Clinical Occurrence of Thrombosis (WeCLOT) Study. *Ann Intern Med* 2005; 142: 620-6.

43. ZHANG S, SHU X, TIAN X *et al*.: Enhanced formation and impaired degradation of neutrophil extracellular traps in dermatomyositis and polymyositis: a potential contributor to interstitial lung disease complications. *Clin Exp Immunol* 2014; 177: 134-41.

44. JIMÉNEZ-ALCÁZAR M, NAPIREI M, PANDA R *et al*.: Impaired DNase1-mediated degradation of neutrophil extracellular traps is associated with acute thrombotic microangiopathies. *J Thromb Haemost* 2015; 13: 732-42.

45. FARRERA C, FADEEL B: Macrophage clearance of neutrophil extracellular traps is a silent process. *J Immunol* 2013; 191: 2647-56.

46. NAKAZAWA D, SHIDA H, KUSUNOKI Y *et al*.: The responses of macrophages in interaction with neutrophils that undergo NETosis. *J Autoimmun* 2016; 67: 19-28.