Neutrophil extracellular traps formation in patients with eosinophilic granulomatosis with polyangiitis: association with eosinophilic inflammation

J. Natorska[1,2], M. Ząbczyk[2], J. Siudut[2], P. Krawiec[3], L. Mastalerz[4], A. Undas[1,2]

[1]Institute of Cardiology, Jagiellonian University Medical College, Krakow;
[2]John Paul II Hospital, Krakow;
[3]Department of Rheumatology, Dietl Hospital, Kraków;
[4]2nd Department of Medicine, Jagiellonian University Medical College, Kraków, Poland.

Joanna Natorska, PhD
Michał Ząbczyk, PhD
Jakub Siudut, MSc
Piotr Krawiec, MD, PhD
Lucyna Mastalerz, MD, PhD
Anetta Undas, MD, PhD

Please address correspondence to:
Dr Joanna Natorska,
Institute of Cardiology,
Jagiellonian University School of Medicine,
80 Pradnicka St.,
31-202 Kraków, Poland.
E-mail: j.natorska@szpitaljp2.krakow.pl

Received on April 25, 2016; accepted in revised form on September 2, 2016.

Clin Exp Rheumatol 2017; 35 (Suppl. 103): S27-S32.

Key words: eosinophilic granulomatosis with polyangiitis, Churg-Strauss syndrome, neutrophil extracellular traps

Funding: this study was supported by Jagiellonian University Medical College (grant no. K/ZDS/002936 to A.Undas).

Competing interests: none declared.

ABSTRACT

Objective. *Eosinophilic granulomatosis with polyangiitis (EGPA) is associated with an inflammation and the presence of antineutrophil cytoplasmic antibodies (ANCA). Thus, we investigated the impact of ANCAs and eosinophilic inflammation on neutrophil activation and extracellular traps (NETs) formation.*

Methods. *We recruited 29 patients in the remission of EGPA (17 ANCA-negative and 12 ANCA-positive, including 7 p-ANCA-positive and 5 c-ANCA-positive patients). Healthy donors' neutrophils were stimulated with EGPA patients' serum. NETs formation was assessed by immunofluorescence and scanning electron microscopy.*

Results. *EGPA patients presented enhanced ability to generate NETs compared to healthy subjects (20.3±8.2% vs. 2.7±1.5%, p=0.0036). However, there were no differences in NETs formation between ANCA-positive and ANCA-negative patients (23±11.2% vs. 17±6.1%, p=0.15). There was also no correlation between NETs generation and the amount of circulating DNA in EGPA patients. Among ANCA-positive patients, p-ANCA-positives showed the highest percentage of NETs as compared to cANCA-positive and ANCA-negative patients (27.3±10.3% vs. 17.8±10.5% and vs. 17±6.1%, both p<0.01, respectively). Eosinophils number correlated with the percentage of NETs in the whole EGPA group (r=0.53, p=0.039), but we failed to observe the correlation with an eosinophil cationic protein (r=0.49, p=0.058).*

Conclusion. *EGPA patients' serum has the ability to induce NETosis with no regard to the ANCA status in contrast to other vasculitides, where p-ANCA were considered as the main factor. Interestingly, NETs formation in EGPA patients connected with the number of eosinophils might be of major relevance. Further studies are required to assess which eosinophil-derived factors might be responsible for the neutrophils activation in EGPA patients.*

Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA), formerly named Churg-Strauss syndrome, is a rare systemic disease characterised by necrotic vasculitis involving small and medium blood vessels accompanied by eosinophilia in the peripheral blood, eosinophilic infiltration of the tissues and the presence of bronchial asthma (1, 2). The prevalence of EGPA ranges from 10-13 cases per 1 million individuals in the general population, depending on their geographical location and the classification criteria applied (3-6). Among patients with asthma the EGPA incidence is higher and is estimated from 34.6 (7) to 64.4 (4) cases per 1 million patient-years.

The current evidence indicates that EGPA develops following an inflammatory response directed at target antigens and a number of factors, including infectious agents (8), drugs (9), allergic hyposensitisations or vaccinations (10).

The most common laboratory abnormalities found in patients with EGPA are blood hypereosinophilia (8, 9) and high immunoglobulin E (IgE) titres that however, are non-specific and usually absent during therapy with corticosteroids (11). Another feature of EGPA is the presence of antineutrophil cytoplasmic antibodies (ANCA) among 30-40% of the patients (12, 13). ANCA-positive patients differ from those without circulating ANCA. In the former group mononeuritis multiplex is more frequent while glomerular nephritis and cardiomyopathy are less commonly observed

(9, 13). ANCA, when present, represent predominantly p-ANCA of anti-myeloperoxidase (MPO) specificity, but c-ANCA of anti-proteinase 3 (PR3) specificity can also be present (9). It is considered that pathogenic ANCA can bind to the cell surface MPO of the proinflammatory cytokine-primed neutrophils, which leads to excessive activation of neutrophils (14). It remains unclear why neutrophil-specific proteins are targeted in EGPA and other systemic autoimmune diseases. It has been suggested that this phenomenon is related to the neutrophil death at sites of inflammation where the exposure of normally sequestered antigens to the immune system may occur. There is a growing evidence that ANCA-activated neutrophils undergo degranulation and release neutrophil extracellular traps (NETs) (15-19). NET formation, which requires neutrophil death termed netosis, represents an unique, innate immune defence mechanism characterised by the destruction of the nucleus with active release of chromatin fibres decorated with histones and a variety of anti-microbial granular proteins, including MPO and PR3, (20) to bind, disarm and kill microbes. Although NETs are bactericidal, excessive NET formation as a result of non-infectious inflammatory processes, as well as a lack of DNAses to degrade them have been linked to the pathogenesis of inflammatory and autoimmune diseases such as lupus erythematosus (21, 22), rheumatoid arthritis (23) and microscopic polyangiitis (MPA) (16-19).

Recent findings have demonstrated a marked ability of serum to induce NETs in patients with MPO-ANCA associated MPA (19, 24). In the present study we sought to investigate the ability of serum derived from the EGPA patients, both ANCA-positive and ANCA-negative, to induce the NETs formation, reflecting its potential engagement in the disease.

Materials and methods

Patients

We enrolled 29 consecutive patients with EGPA on remission. EGPA was diagnosed according to the 1990 American College of Rheumatology (ACR) classification criteria (25). The patients were eligible if they met the following criteria: age over 18 years, and clinically stable disease according to the Birmingham Vasculitis Activity Score version 3 (BVAS v. 3) (25, 26). The exclusion criteria were: any signs of acute illness, known cancer, hepatic or renal dysfunction, and current anticoagulant therapy. The most frequent therapy used was oral glucocorticosteroids (n=27), while 18 subjects received immunosuppressive drugs, including cyclophosphamide (n=7, 20.5%), methotrexate (n=7, 20.5%), or azathioprine (n=4, 12%), all three in monotherapy. Peripheral blood neutrophils were obtained from 10 young healthy volunteers recruited from hospital personnel and acquaintances. The Bioethics Committee of the Jagiellonian University approved the study protocol and informed written consent was obtained from all the participants.

Laboratory investigations

After an overnight fast, venous blood was collected to determine blood cell count, glucose, and creatinine using routine laboratory assays. Blood was drawn from the antecubital vein into serum tubes (Sarstedt, Nurmbrecht, Germany), centrifuged for 10 min at 3,000 rpm (1600 ×g) at 4°C, and small aliquots were frozen at -80°C until analysis. CRP and IgE were measured by nephelometry (Siemens, Munich, Germany). To determine absolute peripheral blood eosinophil counts, a manual method and a Bürker's chamber were used.

ANCA were determined using an indirect immunofluorescence method (Euroimmun, Lübeck, Germany) according to manufacturer's protocol. Briefly, reagent slides were incubated with EGPA patients' serum diluted at 1:10 for 30 minutes, washed, and followed by an incubation with secondary fluorescein-labeled IgG. All specimens were assessed independently by 2 technicians. The end-point titre was defined as the highest dilution at which the ANCA staining pattern was visible.

Commercially available immunoenzymatic assay was used to determine plasma interleukin 6 (IL-6) (R&D Systems, Indianapolis, USA). Serum eosinophil cationic protein (ECP) was measured using a commercial fluoroimmunoassay kit (PharmaciaECP UniCAP System FEIA; Pharmacia Diagnostics, Uppsala, Sweden), with a detection limit of 2 μg/L. Technicians unaware of the sample status performed the tests. Intra- and interassay coefficients of variation ranged from 5% to 8%.

Isolation of human neutrophils

Neutrophils were isolated from peripheral blood drawn from healthy donors by Polymorphprep gradient centrifugation and dextran sedimentation, as described previously (27). Briefly, 5 ml of whole blood containing ethylenediamine tetraacetic acid (EDTA) was layered on 5 ml of Polymorphprep (Gentaur, Brussels, Belgium). After 35 minutes centrifugation at 500 xg, the neutrophils were separated from the polymorphonuclear neutrophils-rich pellet. The residual erythrocytes were than eliminated via red blood cell lysis. Neutrophils were washed with physiological saline, spun down (10 minutes, 400 xg) and resuspended in 2 ml of RPMI medium (phenol red–free) supplemented with 10 mM Hepes. Post cell counts were performed using May-Grunwald Giemsa (Sigma-Aldrich, MO, United States) counterstaining and trypan blue (Sigma-Aldrich, MO, United States) white blood cells viability analysis was performed. Neutrophil purity after isolation was at least 94% and the mean cell viability was 99%.

Stimulation of NETs formation

The resuspended neutrophils were seeded into 24-well culture plates with glass cover slips pretreated with 0.001% polylysine in a final concentration of 2×10^5 per well (28). Incubations were performed at 37°C in the presence of 5% CO_2 for 30 minutes to adhere and followed by 180 minutes incubation with EGPA serum in a final ratio 2:1 (29) or with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St Louis, MO, USA) in a final concentration 600 nM (28) to serve as a positive control. Neutrophils treated with serum derived from healthy donors served as a negative control.

Detection of NETs

• *Immunofluorescence assays*

After appropriate incubation neutrophils were fixed with 4% paraformaldehyde (28), endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol (15 min) at room temperature (RT) and unspecific background was blocked with 3% bovine albumin (Sigma Co, St. Louis, MO, US) for 30 min at RT. Then, slides were incubated with anti-2H2AB-DNA complex antibodies (HIST2H2AB, US Biological, Salem, MA, USA) conjugated with fluorescein isothiocyanate (FITC) diluted in blocking serum at final concentrations 1:500 for 60 min at RT. DNA was stained with DAPI (Invitrogen, Life Technologies, Grand Island, NY, USA). NETs quantification was performed as previously described (30). Briefly, the percentage of neutrophils releasing NETs was quantified in blinded samples by evaluating neutrophils displaying expanded nuclei and releasing DNA fibres in at least 10 random microscope fields. The NET percentage was calculated as 100x number of netting neutrophils/total number of neutrophils. The images were analysed with Olympus BX43 (Olympus America Inc., Center Valley, PA, USA) microscope and the ImageJ software v. 1.48.

• *MPO ELISA*

MPO was measured in supernatants derived from the *in vitro* stimulated-neutrophiles. Ten microliters of the supernatant was tested for MPO presence in each culture well. Using anti-MPO IgG ELISA (Euroimmun, Lübeck, Germany) according to the producer's instruction. Results were calculated as a ratio = optical density (OD) of the sample / OD of the calibrator.

• *Scanning electron microscopy (SEM)*

After appropriate incubation cells were fixed in 2.5% glutaraldehyde for 2 h at 4°C, then slides were washed three times with PBS, dehydrated using a graded ethanol followed by a tert-butyl alcohol series, and then dried in a critical point dryer. The specimen surface was coated with a gold layer using a thin layer evaporator and analysed with JEOL JCM-6000 (JEOL Ltd., Tokyo, Japan) microscope.

• *Circulating DNA measurement*

Free DNA circulating in EGPA patients and healthy controls (n=12) was assessed in serum samples as previously described (31) using the Quant-iT™ Pico Green Assay (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the manufacturer's instruction. The fluorescence of the samples was read on the fluorescence plate reader Fluoroscan Ascent (Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples were excited at 485 nm and fluorescence intensity was measured at 520 nm with a detection limit of 25 pg/mL. Intra- and interassay coefficients of variation ranged from 4% to 7%.

Statistical analysis

Data are expressed as numbers (percentage) for categorical variables and mean ± SD or median (lower-upper quartile) for continuous variables (normally or non-normally disturbed, respectively). The Shapiro-Wilk test was used to assess normality. Categorical variables were compared using the Pearson's chi-squared test or Fischer's exact test as appropriate. The two groups were compared using *t*-test for independent samples or the Mann-Whitney test, as appropriate. The Pearson coefficient was computed to study simple correlations. All tests were two-sided and *p*-values of <0.05 were considered statistically significant. Statistical analyses were performed using Statsoft 10.0 PL package (StatSoft, Inc.).

Table I. Characteristics of EGPA subjects regarding ANCA status.

Variable	ANCA-negative (n=17)	ANCA-positive (n=12)	*p*-value
Age, years	47.83 ± 12.02	45.91 ± 8.43	0.9
Women, n (%)	11 (64.7)	9 (75)	0.7
Laboratory parameters			
Glucose, mmol/l	4.79 ± 1.35	4.35 ± 1.18	0.9
Creatinine, µmol/l	61.27 ± 20.76	69 ± 22.72	0.9
WBC, $x10^3/\mu L$	7.68 ± 2.73	7.74 ± 3.90	0.8
Neutrophils, %	75.7 ± 13.6	62.7 ± 12.9	0.6
Eosinophils, μL^{-1}	206.5 (0-1151)	378 (0-3304)	0.045
IgE, IU/mL	19.9 (18.7-269)	35.9 (18.7-352)	0.043
CRP, mg/L	1.06 (0.15-6.51)	2.5 (0.25-8.67)	0.052
ECP, µg/L	70.9 ± 23.5	80.3 ± 31.3	0.7
IL-6, pg/mL	1.27 (0.86–2.04)	1. 41 (073.-2.35)	0.6
pANCA, n (%)	-	7 (58.33)	-
cANCA, n (%)	-	5 (41.6)	-

Values are given as number (%), mean ± SD or median (lower-upper quartile).

Results

A total of 29 patients with EGPA were included in the final analysis in which 17 (59%) patients were ANCA-negative and 12 (41%) were ANCA-positive (among them 7 patients represented p-ANCA and 5 patients cANCA-positive specificity). The median disease duration was 3 years (IQR, 5 months to 12 years). Clinical data are summarised in Table I. Additionally, p-ANCA-positive patients were characterised by higher eosinophil count and IgE levels when compared with c-ANCA-positive patients (437 [6–3304] *vs*. 378 [0–872], *p*=0.045 and 40 [19–352] *vs*. 23 [7–47] IU/ml, *p*=0.042, respectively).

Serum samples obtained from the EGPA patients presented enhanced ability to stimulate neutrophils from healthy donors to generate NETs (Fig. 1A, Fig. 2) compared to those treated with healthy subjects serum (20.3±8.2% *vs*. 2.7±1.5%, *p*=0.0036). When EGPA patients were divided into subgroups regarding the ANCA status, there were no differences in the mean rate of netting neutrophils between ANCA-positive and ANCA-negative patients (23±11.2% *vs*. 17±6.1%, *p*=0.15). Among ANCA-positive patients, p-ANCA-positive patients showed a higher percentage of NET forming cells compared with both c-ANCA-

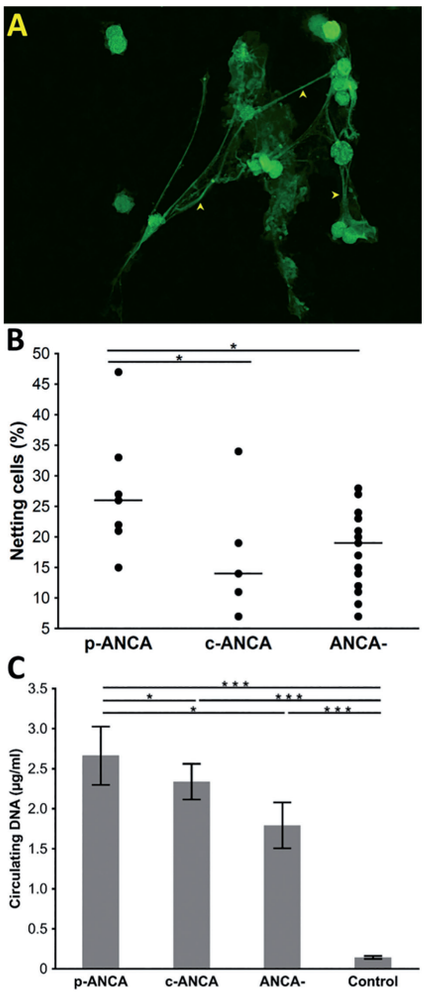

Fig. 1A. NETs generation under stimulation with serum obtained from EGPA patient. Immunostaining for anti–2H2AB–DNA complex antibodies conjugated with fluorescein isothiocyanate (FITC). Magnification 400x.

Fig. 1B. Comparision of ability to NETs generation under stimulation with serums obtained from EGPA patients with positive anti-neutrophil cytoplasm antibodies (ANCA; divided into anti-myeloperoxidase ANCA called p-ANCA and anti-proteinase 3 ANCA called c-ANCA) or negative ANCA status. *p<0.05

Fig. 1C. Level of circulating DNA in EGPA patients' serum with positive anti-neutrophil cytoplasm antibodies (ANCA; divided into anti-myeloperoxidase ANCA called p-ANCA and anti-proteinase 3 ANCA called c-ANCA) or negative ANCA status. *p<0.05.

positive (27.3±10.3% *vs*. 17.8±10.5%, p=0.044) and ANCA-negative patients (27.3±10.3% *vs*. 17±6.1%, p=0.009, Fig. 1B). This trend was followed by the *in vitro*-released MPO concentration, which was 1.7-fold higher in the p-ANCA *vs*. c-ANCA and ANCA-negative groups (p<0.05) and 2.9-fold higher in the p-ANCA group versus control group (p<0.01). The ability of NETs formation did not correlate with the titre of p-ANCA or c-ANCA antibodies, however the highest value of netosis (47%) was observed in one individual patient with the highest p-ANCA titre (1:160). However netosis intensity was positively correlated with the eosilophil numbers (r=0.53, p=0.039). The amounts of circulating DNA, measured in serum of EGPA patients, were higher in p-ANCA-positive subjects as compared to c-ANCA-positive as well as ANCA-negative patients (2.84±0.2 µg/ml *vs*. 2.01±0.29 µg/ml, p=0.04 and 2.84±0.2 µg/ml *vs*. 1.98±0.13 µg/ml, p=0.038, respectively; Fig. 1C). Of note, healthy subjects showed the lowest concentrations of circulating DNA, even if DNA levels were compared with ANCA-negative patients (0.143±0.018 *vs*. 1.98±0.13 µg/ml, p<0.0001). However, the percentage of *in vitro* NET releasing cells did not correlate with the amount of *in vivo* circulating DNA in any of examined subgroups. Similar order was observed between the percentage of NETs releasing neutrophils and IgE level, which correlated only in p-ANCA-positive patients (r=0.41, p=0.018) but not in c-ANCA-positive or ANCA-negative patients (r=0.39, p=0.083; r=0.45, p=0.103, respectively). Serum CRP level correlated with the percentage of NET releasing cells in the whole EGPA group (r=0.57, p=0.046). There were no significant correlations between the percentage of netting neutrophils and either IL-6 or ECP levels and other laboratory parameters or medication used (data not shown).

Discussion

Although the ability of p-ANCA to induce the NETs formation in vasculitis has been reported previously (19, 24), to our knowledge, this study is the first to show that the intensity of NETosis in EGPA patients is related to eosinophil number.

Interestingly, we demonstarted that in patients with EGPA the ability to generate NETs is present not only in subjects positive for c-ANCA but also for p-ANCA patients. So far, only PR3 and PR3-ANCA induced enhancement of inflammation has been demonstrated in an animal model (32, 33). Surprisingly, we observed the formation of NETs in ANCA-negative patients as well. In our opinion, the ability of the EGPA serum to induce NETs formation regardless of the ANCA status might be of major importance, suggesting that in EGPA patients netosis is related rather to eosinophilic inflammation than to the presence of ANCA like in other vasculitides (19, 24). Especially, the correlation between eosinophil number and NETosis intensity was observed. As the pathology of EGPA includes not only ANCA-related vasculitis, but mainly eosinophil-rich granulomatous inflammation. It could be speculated that eosinophil-derived factors such as ECP, major basic protein (MBP), eosinophil peroxidase (EPO), and lysophospholipase, which determine a pro-inflammatory environment and are implicated in the vascular damage (34) could be responsible for activation of NETs formation in EGPA patients. We failed to show that ECP correlates with netosis intensity. However, the other proteins, especially MBP, which induces release of interleukin-8, the main neutrophils activator (35) and NET formation (20), might potentially be implicated in the NET generation. Moreover, it is known that MBP contributes to the pathophysiology of acute asthma (35). In EGPA patients the intensity of NETs formation is correlated with inflammation reflected by the level of circulating CRP. In our opinion CRP itself does not contribute to the process of NET formation, as we observed using high level CRP serum from patients without EGPA (data not shown). The correlation of CRP with netosis rate suggests the important role of the inflammatory status in EGPA patients. As shown by Tadie *et al*., the level of HMGB-1 (high mobility group box 1), which is closely correlated with circulating CRP (36) increases the NET formation *in vitro* in ANCA associated vasculitis patients (36, 37). However, it seems that in EGPA patients, factors other than ANCA are able to induce NETs as well. In our opinion the lack of the correlation between DNA concentration and NETosis intensity is not surprising since the stimulation of netosis was performed *in vitro*, whereas

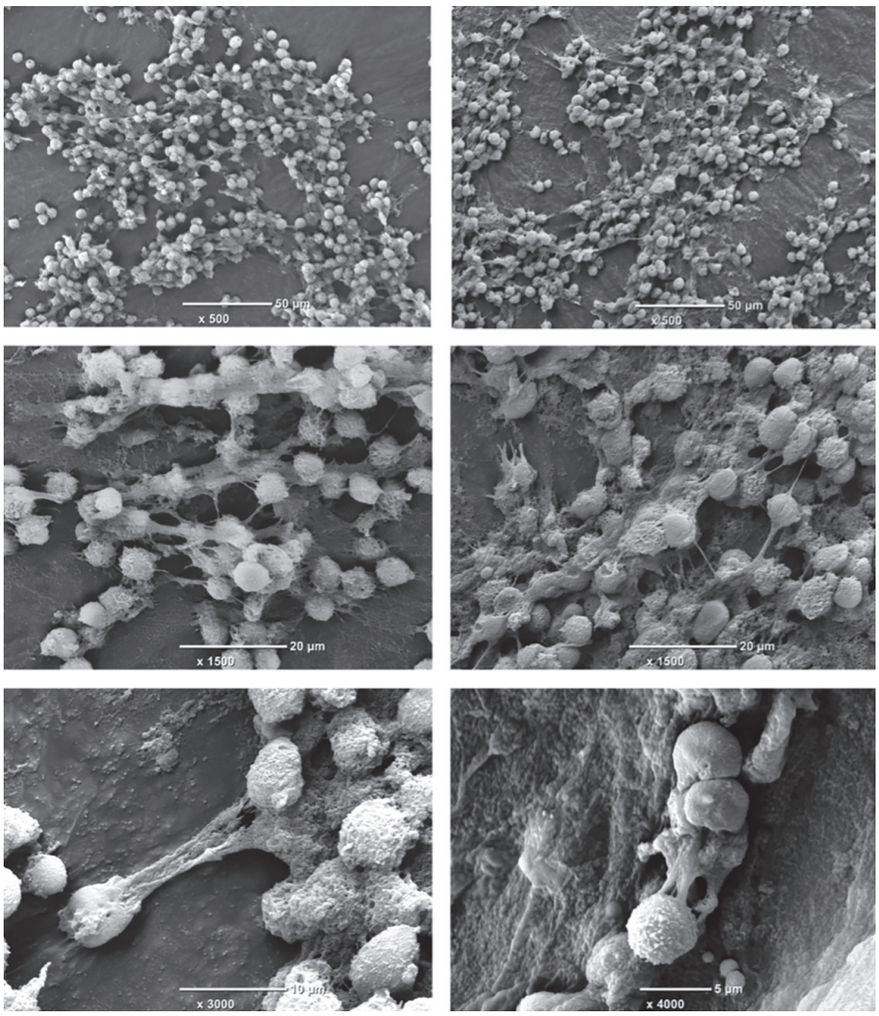

Fig. 2. NETs generation under stimulation with serum obtained from EGPA patient visualised by SEM.

the level of free DNA was measured in the EGPA patients serum where the free DNA might be not only neutrophils-derived but also could come from eosinophil traps (ETosis) (38), mast cells or monocytes and macrophages which were also shown to release extracellular traps, but to a lesser extent when compared to neutrophils (39, 40). Moreover, in EGPA patients resulting eosinophils activation epithelial damage-derived DNA should also be included to the pool of free DNA. Further studies with EGPA serums depleted of ANCA IgG are needed to validate this hypothesis.

Study limitations

This study has several limitations. First, the number of subjects is low, especially in the subgroups regarding the ANCA status, however EGPA is a rare disease. Second, the stimulation of neutrophils was performed with serum. On one hand this "global model" does not allow to estimate precisely which single factor is involved in NET generation. On the other hand, this model enabled to assess the role of the other factors present in the patients' sera, including proinflammatory cytokines, and eosinophilic proteins that might contribute to NET formation. It would be also of major importance to examine naeutrophils stimulation regarding the G-protein coupled receptors CXCR1, CXCR2. In generally, studies of NET formation are difficult to design. Third, serum contained DNases, which might diminish the amount of NETs. To our knowledge, there have been no reports showing differences in this enzyme activity in EGPA compared with other vasculitides. The issue of a potential impact of DNases was beyond the scope of the current study.

Conclusions

In conclusion, we showed that serum obtained from the EGPA patients has the ability to induce NETs formation regardless of the ANCA status, which might be associated with the unique eosinophil activation *in vivo* in contrast to other vasculitides, where it seems that the main factor determining NETs formation is the presence of p-ANCA. In our opinion eosinophilic-derived proteins can be involved in NETosis induction or eosinophils itselfs can undergo so called ETosis triggering NETosis, which can enhance inflammation in a vicious circle and might contribute to thromboembolic complications (41). The important question remains to be answered of which eosinophil-derived factors might be responsible for the neutrophils activation.

Acknowledgement

We thank all patients and physicians who supported this project.

References

1. COMARMOND C, PAGNOUX C, KHELLAF M *et al*.: Eosinophilic granulomatosis with polyangiitis (Churg-Strauss): clinical characteristics and long-term follow-up of the 383 patients enrolled in the French Vasculitis Study Group cohort. *Arthritis Rheum* 2013; 65: 270-81.
2. PAGNOUX C, GUILPAIN P, GUILLEVIN L: Churg-Strauss syndrome. *Curr Opin Rheumatol* 2007; 19: 25-32.
3. MAHR A, GUILLEVIN L, POISSONNET M, AYMÉ S: Prevalences of polyarteritis nodosa, microscopic polyangiitis, Wegener's granulomatosis, and Churg-Strauss syndrome in a French urban multiethnic population in 2000: a capture-recapture estimate. *Arthritis Rheum* 2004; 51: 92-9.
4. MARTIN RM, WILTON LV, MANN RD: Prevalence of Churg-Strauss syndrome, vasculitis, eosinophilia and associated conditions: retrospective analysis of 58 prescription-event monitoring cohort studies. *Pharmacoepidemiol Drug Saf* 1999: 8: 179-89.
5. WATTS RA, LANE SE, BENTHAM G, SCOTT DG: Epidemiology of systemic vasculitis: a ten-year study in the United Kingdom. *Arthritis Rheum* 2000; 43: 414-9.
6. WATTS RA, LANE S, SCOTT DG: What is known about the epidemiology of the vasculitides? *Best Pract Res Clin Rheumatol* 2005; 19: 191-207.
7. HARROLD LR, ANDRADE SE, GO AS *et al*.: Incidence of Churg-Strauss syndrome in asthma drug users: a population-based perspective. *J Rheumatol* 2005; 32: 1076-80.
8. GUILLEVIN L, PAGNOUX C, MOUTHON L: Churg-Strauss syndrome. *Semin Respir Crit Care Med* 2005; 25: 535-45.

9. SABLÉ-FOURTASSOU R, COHEN P, MAHR A *et al*.: French Vasculitis Study Group. Antineutrophil cytoplasmic antibodies and the Churg-Strauss syndrome. *Ann Intern Med* 2005; 143: 632-8.
10. GUILLEVIN L, GUITTARD T, BLETRY O, GODEAU P, ROSENTHAL P: Systemic necrotizing angiitis with asthma: causes and precipitating factors in 43 cases. *Lung* 1987; 165: 165-72.
11. MOUTHON L, DUNOGUE B, GUILLEVIN L: Diagnosis and classification of eosinophilic granulomatosis with polyangiitis (formerly named Churg-Strauss syndrome). *J Autoimmun* 2014; 48-49: 99-103.
12. MOOSIG F, BREMER JP, HELLMICH B *et al*.: A vasculitis centre based management strategy leads to improved outcome in eosinophilic granulomatosis and polyangiitis (Churg-Strauss, EGPA): monocentric experiences in 150 patients. *Ann Rheum Dis* 2013; 72: 1011-7.
13. SINICO RA, DI TOMA L, MAGGIORE U *et al*.: 2005. Prevalence and clinical significance of antineutrophil cytoplasmic antibodies in Churg-Strauss syndrome. *Arthritis Rheum* 2005; 52: 2926-35.
14. ELEFANTE E, TRIPOLI A, FERRO F, BALDINI C: One year in review: systemic vasculitis. *Clin Exp Rheumatol* 2016; 34 (Suppl. 97): S1-6.
15. HU N, WESTRA J, KALLENBERG CG: Dysregulated neutrophil--endothelial interaction in antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides: implications for pathogenesis and disease intervention. *Autoimmun Rev* 2011; 10: 536-43.
16. CARTIN-CEBA R, PEIKERT T, SPECKS U: Pathogenesis of ANCA-associated vasculitis. *Curr Rheumatol Rep* 2012; 14: 481-93.
17. SCHÖNERMARCK U, CSERNOK E, GROSS WL: Pathogenesis of anti-neutrophil cytoplasmic antibody-associated vasculitis: challenges and solutions 2014. Nephrol *Dial Transplant* 2015; 30 (Suppl. 1):i46-52.
18. TANG S, ZHANG Y, YIN SW *et al*.: Neutrophil extracellular trap formation is associated with autophagy-related signalling in ANCA-associated vasculitis. *Clin Exp Immunol* 2015; 180: 408-18.
19. NAKAZAWA D, SHIDA H, TOMARU U *et al*.: Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. *J Am Soc Nephrol* 2014; 25: 990-7.
20. BRINKMANN V, REICHARD U, GOOSMANN C *et al*.: 2004. Neutrophil extracellular traps kill bacteria. *Science* 2004; 303: 1532-5.
21. 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.
22. VILLANUEVA E, YALAVARTHI S, BERTHIER CC *et al*.: Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol* 2011; 187: 538-52.
23. KHANDPUR R, CARMONA-RIVERA C, VIVEKANANDAN-GIRI A *et al*.: NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med* 2013; 5: 178ra40.
24. KESSENBROCK K, KRUMBHOLZ M, SCHÖNERMARCK U *et al*.: 2009. Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* 2009; 15: 623-5.
25. FRIES JF, HUNDER GG, BLOCH DA *et al*.: The American College of Rheumatology 1990 criteria for the classification of vasculitis. Summary. *Arthritis Rheum* 1990; 33: 1135-6.
26. FLOSSMANN O, BACON P, DE GROOT K *et al*.: 2007. Development of comprehensive disease assessment in systemic vasculitis. *Ann Rheum Dis* 2007; 66: 283-92.
27. DEGEL J, SHOKRANI M: Validation of the efficacy of a practical method for neutrophils isolation from peripheral blood. *Clin Lab Sci* 2010; 23: 94-8.
28. BRINKMANN V, LAUBE B, ABU ABED U, GOOSMANN C, ZYCHLINSKY A: Neutrophil extracellular traps: how to generate and visualize them. *J Vis Exp* 2010; 36pii: 1724.
29. VIA CS, ALLEN RC, WELTON RC: Direct stimulation of neutrophil oxygenation activity by serum from patients with systemic lupus erythematosus: a relationship to disease activity. *J Rheumatol* 1984; 11: 745-53.
30. BRINKMANN V, GOOSMANN C, KÜHN LI, ZYCHLINSKY A: Automatic quantification of in vitro NET formation. *Front Immunol* 2013; 3: 413.
31. NEUMANN A, BROGDEN G, JERJOMICEVA N, BRODESSER S, NAIM HY, VON KÖCKRITZ-BLICKWEDE M: 2014. Lipid alterations in human blood-derived neutrophils lead to formation of neutrophil extracellular traps. *Eur J Cell Biol* 2014; 93: 347-54.
32. PFISTER H, OLLERT M, FRÖHLICH LF *et al*.: 2004. Antineutrophil cytoplasmic autoantibodies against the murine homolog of proteinase 3 (Wegener autoantigen) are pathogenic in vivo. *Blood* 2004; 104: 1411-8.
33. KESSENBROCK K, FRÖHLICH L, SIXT M *et al*.: Proteinase 3 and neutrophil elastase enhance inflammation in mice by inactivating antiinflammatory progranulin. *J Clin Invest* 2008; 118: 2438-47.
34. KOJIMA K, SASAKI T: Venoocclusive disease in hypereosinophilic syndrome. *Intern Med* 1995; 34: 1194-7.
35. PAGE SM, GLEICH GJ, ROEBUCK KA, THOMAS LL: Stimulation of neutrophil interleukin-8 production by eosinophil granule major basicprotein. *Am J Respir Cell Mol Biol* 1999; 21: 230-7.
36. WANG C, GOU SJ, CHANG DY, YU F, ZHAO MH, CHEN M: 2013. Association of circulating level of high mobility group box 1 with disease activity in antineutrophil cytoplasmic autoantibody-associated vasculitis. *Arthritis Care Res* (Hoboken) 2013; 65: 1828-34.
37. TADIE JM, BAE HB, JIANG S *et al*.: HMGB1 promotes neutrophil extracellular trap formation through interactions with Toll-like receptor 4. *Am J Physiol Lung Cell Mol Physiol* 2013; 304: L342-9.
38. SIMON D, HOESLI S, ROTH N, STAEDLER S, YOUSEFI S, SIMON HU: Eosinophil extracellular DNA traps in skin diseases. *J Allergy Clin Immunol* 2011; 127:194-9.
39. WEBSTER SJ, DAIGNEAULT M, BEWLEY MA *et al*.: Distinct cell death programs in monocytes regulate innate responses following challenge with common causes of invasive bacterial disease. *J Immunol* 2010; 185: 2968-79.
40. BARTNECK M, KEUL HA, ZWADLO-KLARWASSER G, GROLL J: 2010. Phagocytosis independent extracellular nanoparticle clearance by human immune cells. *Nano Lett* 2010; 10: 59-63.
41. MASTALERZ L, CELIŃSKA-LÖWENHOFF M, KRAWIEC P, BATKO B, TŁUSTOCHOWICZ W, UNDAS A: Unfavorably altered fibrin clot properties in patients with eosinophilic granulomatosis with polyangiitis (Churg-Strauss Syndrome): association with thrombin generation and eosinophilia. *PLoS One* 2015; 10: e0142167.