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Neutrophil extracellular traps (NETs) in aortic stenosis: Comparison of methods for assessment of NETs formation

Original Study

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Abstract

Introduction. We previously showed increased neutrophil extracellular traps (NETs) formation (NETosis) in patients with severe aortic stenosis (AS). Our aim was to comprehensively assess NETosis in AS using a relatively simple and appropriate technique. We investigated circulating NETosis markers by ELISA and assessed the potential of blood neutrophils to release NETs by flow cytometry.

Materials and Methods. We enrolled 13 patients aged 66 [Q1-Q3, 60-70] years with severe isolated AS without diabetes, chronic kidney disease, and atrial fibrillation. Nine apparently healthy volunteers of similar sex and age served as controls. Serum concentrations of citrullinated histone H3 (citH3), circulating nucleosomes, myeloperoxidase (MPO), and deoxyribonuclease-1 (DNASE1) were measured using ELISAs. Peripheral blood NET-releasing neutrophils were detected by flow cytometry as MPO/citH3-positive cells.

Results. AS patients compared to controls presented 174% higher concentrations of citH3 (p<0.001), 456% higher nucleosomes (p<0.001), 136% higher MPO (p=0.021) and 19% higher DNASE1 levels (p=0.039), together with 101% elevated percentage of NET-releasing neutrophils assessed by flow cytometry (p=0.003). In AS patients, the proportion of blood NET-releasing neutrophils positively correlated with citH3 (r=0.86, p<0.001) and nucleosome (r=0.58, p=0.041) concentrations but not with MPO or DNASE1. Moreover, we observed a strong association between AS severity, measured as aortic valve area (AVA), and serum citH3 concentrations (r=-0.75, p=0.003), but not the number of NET-releasing neutrophils.

Conclusions. Our study showed increased blood neutrophil potential to release NETs together with increased levels of serum markers of NETos is in severe AS patients. However, we recommend ELISA to assess NETosis in vivo as simpler technique giving more unequivocal results compared to flow cytometry.

Keywords

neutrophil extracellular traps • aortic stenosis • flow cytometry

1. Introduction

Activated neutrophils are able to release extracellular web-like chromatin structures known as neutrophil extracellular traps (NETs) [1]. NETs contain nucleic acids, histones, and granule components such as myeloperoxidase (MPO) and neutrophil elastase [2]. They capture the pathogens and decelerate their spread, concentrating granulocyte antimicrobial activity [3]. The universal NETosis event is citrullination of histone H3 (citH3), driven by the enzyme peptidylarginine deaminase type 4 (PAD4) and the release of histones [4]. Neutrophil activation with NETs formation is usually induced *in vitro* by phorbol myristate acetate (PMA) and calcium ionophores such as ionomycin [5]. Numerous factors are *in vivo* activators of NETosis, among them lipopolysaccharides, activated toll-like receptors (TLR)4, 2, 7, 8, pathogen-associated molecular patterns (PAMPs), reactive oxygen species, oxidized lipids, proinflammatory cytokines, uric acid, high glucose levels, and autoantibodies [6]. It has been shown that NETosis not only contributes to atherosclerosis progression but also is independently associated with coronary artery disease and major adverse cardiac events [7].

Aortic stenosis (AS), the most common acquired valvular heart disease in adults, has a complex pathogenesis, with several risk factors and mechanisms shared with atherosclerosis, in particular chronic low-grade inflammation [8]. Oxidized LDL particles, oxidative stress as well as macrophage-derived proinflammatory cytokines may be important factors triggering NETosis in AS. As previously published by our group [9], NETs might, at least in part, contribute to the AS pathogenesis.

We showed that the percentage of valvular double-positive citH3/MPO cell expression was significantly higher in human stenotic aortic valves compared to healthy valves and was inversely correlated with aortic valve area (AVA), a marker of AS severity [9]. Moreover, increased concentration of plasma citH3 was associated with disease severity [9]. Although NETosis seems to be of minor importance to AS development, it can, at least partially, contribute to AS progression. Therefore,

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assessment of NETosis in AS is worth investigation. For these reasons, there is a growing practical need for accurate and rapid quantification of NETosis. A flow cytometry-based assay for NET detection on a large number of neutrophils, introduced a few years ago, could have the advantage of quickly measuring NETosis [10]. The aim of our study was to examine comprehensively NETosis in AS patients. We used the technique of cytometry to assess the potential of stimulated neutrophils to release NETs and compared the number of NET-releasing neutrophils with serum concentrations of NETosis markers.

2. Materials and methods

2.1. Patients

We enrolled 13 patients with isolated symptomatic AS recruited between November 2019 and July 2020 in the Department of Cardiovascular Surgery and Transplantology at John Paul II Hospital, Krakow. Data on demographics, medical history, and current treatment were collected using a standardized questionnaire. Severe AS was defined, as previously [10], as mean transvalvular pressure gradient (PG_{mean})>40mmHg, peak transvalvular velocity (V_{max})>4.0m/s, and AVA<1cm^2 based on transthoracic echocardiography performed by an experienced cardiologist on a Toshiba APLIO 80 (Toshiba, Tokyo, Japan) ultrasound machine. The exclusion criteria for AS patients were: atherosclerotic vascular disease requiring revascularization, acute infection including infective endocarditis, rheumatic AS, diabetes mellitus, advanced chronic kidney disease, need for concomitant valvular surgery (e.g., mitral valve repair), percutaneous coronary intervention, recent (<3 months) acute coronary syndrome or cerebrovascular episode, known malignancy, and pregnancy [9]. The control group comprised 9 healthy volunteers recruited among random hospital personnel who were of similar age and sex, had no chronic diseases or acute infection, and were not taking any medications.

2.2. Laboratory analysis

After an overnight fasting, venous blood was drawn from the antecubital vein into serum and EDTA tubes. Blood was centrifuged at 1600*g* at 4°C for 10 minutes and serum was stored in aliquots at -80°C. To determine blood cell count, lipid profile, glucose and creatinine, routine laboratory assays were used. C-reactive protein (CRP) was determined using immunoturbidimetry (Roche Diagnostics, Mannheim, Germany).

2.3. Enzyme-linked immunosorbent assay (ELISA)

Human citH3, circulating nucleosomes, MPO, and deoxyribonuclease-1 (DNASE1) were assayed quantitatively in serum samples using commercial ELISA kits (Cayman Chemical Company, Ann Arbor, MI, USA; Active Motif, La Hulpe, Belgium; Wuhan Fine Biotech CO., Wuhan, China, respectively) in accordance with manufacturers' instructions. Inter- and intra-assay variability was <7%.

2.4. Flow cytometry

Whole blood samples of 4mL were collected into EDTA tubes from AS patients and healthy donors. Neutrophils were isolated using density gradient of Polymorphprep (cat. no. 04-03-09393/01, AXIS-SHIELD AS, Oslo, Norway) according to the manufacturer's instruction. Neutrophil purity after isolation was 91%. Initially, a series of experiments were carried out with different stimulants (ionomycin and MBA), noting their concentrations as well as incubation times.

Finally, numbers of 5x10^5 neutrophils suspended in 1mL of Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, Life Technologies, Paisley, UK) were stimulated with 4μM ionomycin (cat. no. IO634-1MG, Sigma-Aldrich) for 1 hour (37°C, 5% CO_2) in the low attachment 6-well plates (Costar, Washington, DC, US) for flow cytometry or with glass cover slip placed on the bottom of the well for immunofluorescence staining. Cells were fixed in 2% paraformaldehyde in phosphate buffered saline (PBS Biowest, Nuaillé, France), and incubated for 30 minutes with 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS (BSA/PBS) to block unspecific staining. The neutrophils were incubated subsequently with anti-citH3 primary (unconjugated) antibody (cat. no. ab5103, Abcam, Cambridge, MA, USA) at 1:300 dilution (50 minutes), Alexa Fluor700-conjugated secondary antibody (cat. no. A-21038, Thermo Scientific, Rockford, IL, USA) at 1:300 dilution (30 minutes), and FITC-conjugated anti-MPO antibody (cat. no ab11729, Abcam) at 1:20 dilution (30 minutes). Each incubation was followed by a wash with BSA/PBS and a centrifugation at 300 g and 4°C for 10 min. The flow cytometer BD FACSCanto™II (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) with Kaluza Analysis 1.2. software was used.

We used the neutrophil gating strategy based on a logarithmic scale for light side scatter in order to visualize optimally the neutrophil plot (parameters set: linear forward scatter, FSC, *versus* logarithmic side scatter, SSC). Such data presentation defines neutrophils as a clear distinct plot of dots (cells) and differentiates them from cell clumps, contaminated eosinophils, mononuclears and cell debris.

The flow cytometry results were finally presented as a percentage of double-positive (MPO+citH3+) neutrophils, as a measure of NETs releasing cells.

2.5. Immunofluorescent staining of NETs

Cells were fixed in ice-cold methanol-acetone (1:1) mixture (Sigma-Aldrich), washed three times with PBS and labeled with immunofluorescence using primary antibody against citH3 (Abcam, cat. no ab5103, at 1:250 dilution) and followed by the corresponding secondary antibody conjugated with fluorochrome AlexaFluor 488 (Abcam, cat. no ab 150077, at

1:250 dilution), as previously described [9]. The experiment was performed using neutrophils from healthy individuals, each time in 3 repetitions.

The percentage of NET-releasing cells was quantified per 100 randomly selected neutrophils by evaluating cells displaying expanded nuclei and releasing fibers in at least 10 random microscope fields. The analyses were performed using an Olympus BX 43 microscope.

2.6. Statistical Analysis

All statistical analyses were performed using STATISTICA Version 13.3 (StatSoft STATISTICA™, Poland) program. Categorical variables were presented as numbers and percentages and were analyzed by Pearson's χ^2 or two-tailed Fisher's exact test. Continuous variables were expressed as mean±standard deviation (SD) or median and interquartile range (IQR). Normality was analyzed by the Shapiro-Wilk test. The Student's test was used for normally distributed continuous variables. Differences between groups were compared using the Mann-Whitney U test for non-normally distributed continuous variables. Associations between nonparametric variables were assessed by Spearman's tests. The study was powered to have a 90% chance of detecting a 10% difference in citH3 levels, using a p-value of 0.05. Based on our previous report [9], to demonstrate such a difference or greater, at least 9 patients were required in each group. P-values of <0.05 were considered statistically significant.

3. Results

3.1. Characteristics of the studied groups

Baseline characteristics of AS patients are shown in Table 1. There were no differences between AS patients and healthy controls with regard to age, gender, or CRP (all p>0.05).

3.2. NETosis

In AS patients, the ELISAs results showed 174% higher serum citH3 concentrations (2.25 [1.8-2.9] vs. 0.82 [0.5-1.7] ng/mL, p<0.001) and 456% higher levels of circulating nucleosomes (3.67 [1.15-6.35] vs. 0.66 [0.021-1.94] µg/mL, p<0.001) compared to controls. The concentrations of serum MPO (0.52 [0.25-1.09] vs. 0.22 [0.13-0.43] ng/mL, p=0.021) and DNASE1 (0.69 [0.55-2.08] vs. 0.58 [0.45-0.88] ng/mL, p=0.039) were 136% and 19% higher, respectively. Serum citH3 concentrations strongly correlated with nucleosome levels both in AS patients (r=0.75, p=0.005) and controls (r=0.76, p<0.001) but not with MPO or DNASE1 levels (all p>0.05). We found no associations between MPO or DNASE1 in either the AS group or the control group (all p>0.05). There was a strong association between AS severity, measured as AVA, and serum citH3 concentrations (r= -0.75, p=0.003). The results of ELISA assays are presented in Fig. 1. Immunofluorescence staining confirmed that ionomycin-stimulated neutrophils from healthy donors generate NETs. The median percentage of NET-releasing neutrophils was 49 [43-58] (Fig. 2).

Table 1. Baseline characteristics of patients with aortic stenosis (AS) and healthy controls

Variable	Patients with AS (n=13)	Controls (n=9)	
Age, years	66 [60-70]	59 [58-65]	0.148
Male, n (%)	10 (76.9)	7 (77.8)	0.991
Body mass index, kg m^{-2}	31.2 ±5.4	24 ±6.5	0.002
Risk factors, n (%)			
Arterial hypertension	12 (92.3)	0	ND
Current smoking	1 (7.7)	0	ND
Medications, n (%)			
Beta-blockers	11 (84.6)	0	ND
Acetylsalicylic acid	7 (53.8)	0	ND
Angiotensin converting enzyme inhibitors	9 (69.2)	0	ND
Statins	10 (76.9)	0	ND
Echocardiographic parameters			
Aortic valve area, cm^2	0.86 [0.8-0.9]	ND	
Mean gradient, mmHg	48 ±10.0	ND	
Maximum gradient, mmHg	80 ±15.4	ND	
Peak transvalvular velocity, m/s	4.5 ± 0.4	ND	
Left ventricular ejection fraction, %	52.3 ±12.5	ND	
Laboratory investigation			
White blood cells, 10^3/µl	6.2 ±1.4	6.5 ±1.7	0.655
Neutrophils, 10^3/µl	3.5 [2.9-4.0]	3.2 [2.3-3.6]	0.189
Creatinine, µmol/L	96 [86-100]	67 [48-76]	0.003
C-reactive protein, mg/L	1.5 [1.0-2.5]	0.99 [0.99-1.46]	0.015
Glucose, mmol/L	5.2 [5.0-5.6]	5.0 [4.7-5.4]	0.115
Total cholesterol, mmol/L	4.2±1.1	4.9±0.9	0.131
Low density lipoprotein cholesterol, mmol/L	2.9±1.0	3.0±1.1	0.827
High density lipoprotein cholesterol, mmol/L	1.1±0.3	1.4±0.3	0.032
Triglycerides, mmol/L	1.7±0.7	1.5±0.7	0.518

Data presented as numbers (percentages), mean ± SD or median [interquartile range]
ND: non determined

By flow cytometry, AS patients had about twice the percentage of NET-releasing neutrophils compared to controls (12.9 [11.3-20.1]% vs. 6.4 [2.1-10.3]%, p=0.003, Fig.3a-f.).

The percentage of NET-releasing neutrophils weakly correlated with age in healthy subjects (r=0.27, p=0.019), but not in AS patients (p>0.05). In both groups we found no other associations of the percentage of NET-releasing neutrophils with demographic parameters (all p>0.05).

In AS patients the percentage of NET-releasing neutrophils assessed by flow cytometry correlated positively with serum concentrations of citH3 (r=0.86, p<0.001) and nucleosomes

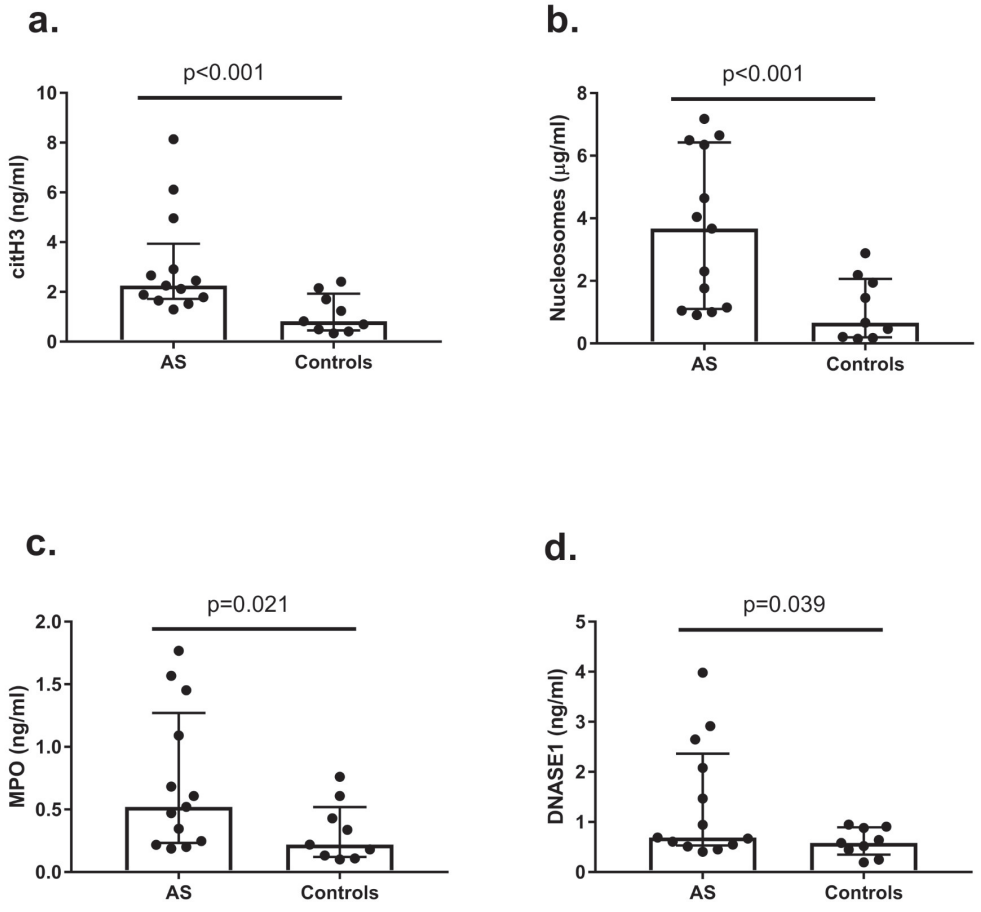

Figure 1. Serum levels of NET-specific biomarkers in AS patients and healthy controls
Single dot represents individual result. Horizontal central lines express median values. Boxes express interquartile (Q1-Q3) range

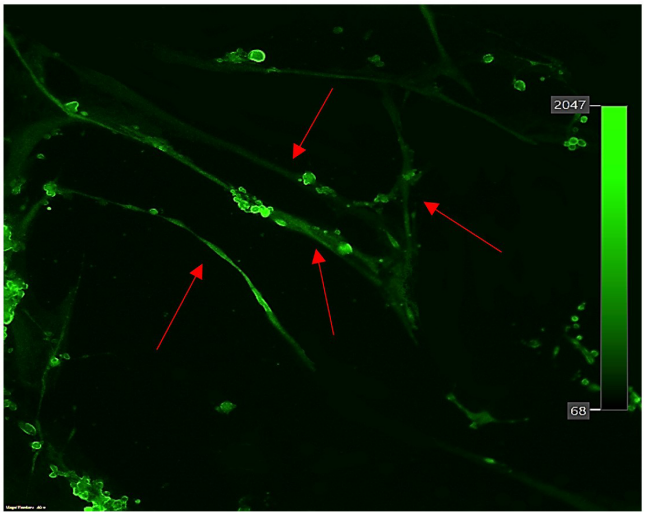

Figure 2. Representative microphotograph of NETosis generated after ionomycin- stimulation of isolated neutrophils from healthy volunteers
Immunolabelling for citH3 (green). The red arrows indicate the NET-releasing neutrophils. Original magnification 40×

(r=0.58, p=0.039). There was no association between the percentage of NET-releasing neutrophils and nucleosome concentrations (p=0.425) as well as the AS severity expressed as transvalvular gradients or AVA (all p>0.05).

4. Discussion

To our knowledge, this study is the first to show that AS patients are characterized by increased potential of neutrophils to form NETs, which corresponds to increased serum citH3 and nucleosome levels. We used flow cytometry, a method that allows for simultaneous analysis of a large number of neutrophils.

We have adapted the protocol of Gavillet et al. [11], who proposed the principles of NETs cytometric analysis. In sepsis, they examined spontaneous NETosis in the circulation without prior stimulation, due to the high baseline percentage of NET-releasing neutrophils. In contrast, to characterize NETosis in healthy controls, they preceded cytometric acquisition with cell stimulation (PMA or ionomycin). In the current study we followed this approach because both controls and AS patients are

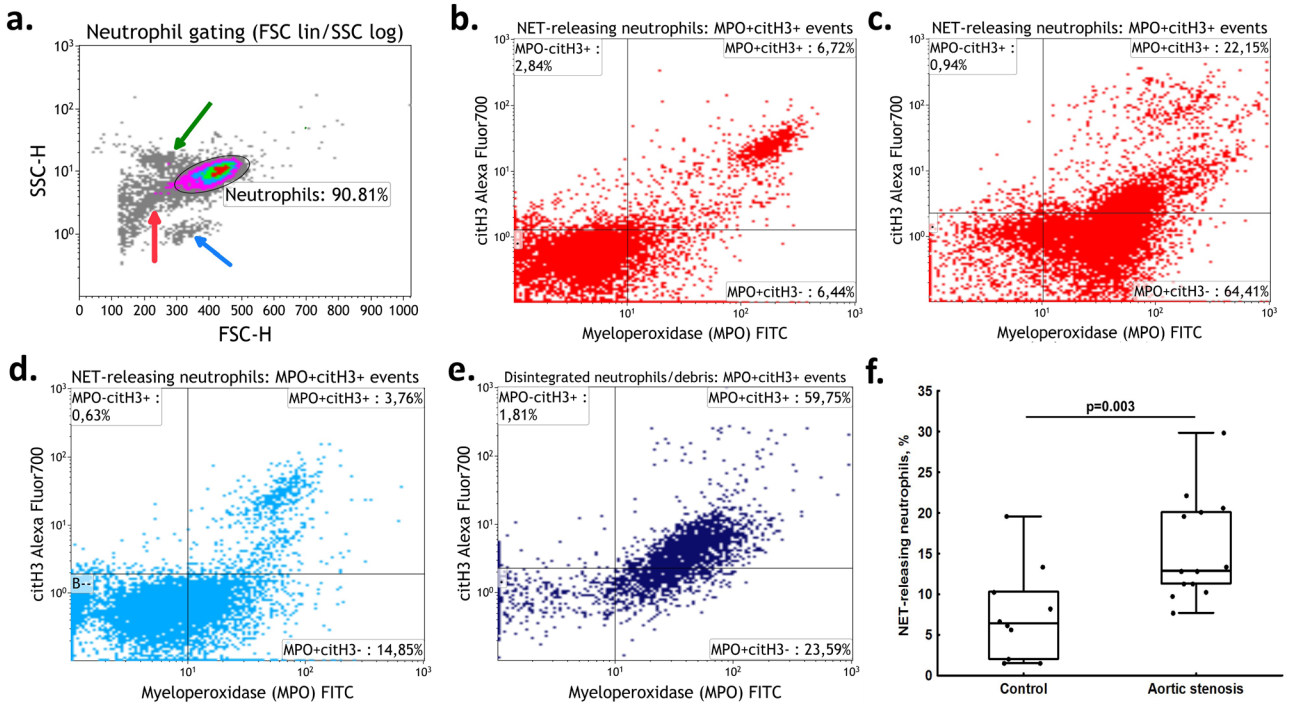

Figure 3. NETosis examination in flow cytometry
a. Healthy person from the control group, ionomycin-stimulated neutrophils. Density plot, FSC lin /SSC log (SSC data presented in logarithmic scale) display. Neutrophils gated as elliptic gate. Arrows indicate: eosinophils (green arrow), disintegrated neutrophils (debris, red arrow) and mononuclears (blue arrow).
b. The same sample as a. Gated neutrophils displayed as MPO FITC / citH3 Alexa Fluor700 dot plot. Markers inserted according to the control sample (stained with secondary antibodies, ionomycin-stimulated). NET-releasing neutrophils are MPO+citH3+ events in the upper right quadrant (6.7%).
c. In comparison to the control, ionomycin stimulated neutrophils in AS patient. High percentage of NET-releasing neutrophils (22.1%).
d. NET-releasing neutrophils in unstimulated (ionomycin-free) sample (baseline NETs formation in 3.7% neutrophils) from AS patient.
e. The cellular debris (disintegrated neutrophils) gated. They contain the major part of positive MPO+citH3+ staining in the sample but they are not NET-releasing neutrophils.
f. The percentage of NET-releasing (MPO+citH3+) neutrophils in peripheral blood of AS patients compared to healthy controls. Single dot represents individual result. Horizontal central lines express median values. Boxes express interquartile (Q1-Q3) range.

characterized by a relatively moderate NETosis. The percentage of NET-releasing neutrophils obtained in our study by flow cytometry was consistent with previous reports [12, 13].

We observed a 101% increase in NET-releasing neutrophils in AS patients, compared to the control group. The NETosis was relatively low when related to the parallel results of cytochemical staining presented here. This observation was a consequence of precise single-neutrophil flow cytometry gating that excluded aggregates and cell remnants (debris), which we applied as shown by Zharkova et al. [14] and based on our previous reports [15, 16]. A large proportion of stimulated neutrophils seem to disintegrate and fall outside the gate (as cell debris) after NETosis, which may be a result of long incubation times. On the other hand, too short incubation time may underestimate the percentage of NET-releasing neutrophils [12, 17]. Taken together, flow cytometric assessment of NETs has some methodological limitations by lowering the percentage of neutrophils that actually undergo NETosis. The doubts concern gating – in some studies neutrophil remnants are included into NETosis analysis [18, 19] – and data presentation – some authors used changes in mean fluorescence intensity (MFI) of NETosis markers [20, 21].

Summing up, the neutrophil gate strategy (parameters set: FSC lin/SSC log) presented in the current study seems to provide the optimal neutrophil visualization by differentiating them from non-neutrophil events and cell debris.

As an alternative, image flow cytometry (IFC) was applied [22]. IFC allows for visualization of whole cells and combines the analytical power of fluorescent microscopy with the statistical robustness of conventional flow cytometry. However, it is a time-consuming protocol due to IFC acquisition and analysis [21].

Thus, our study suggests that serum ELISA assays detecting NET markers seem to be more appropriate to assess NETosis in AS patients and most likely in other cardiovascular patients. Moreover, ELISA is a relatively simple technique: it is quick to perform and sufficiently discriminates differences between healthy controls and AS patients, who have moderate NETosis. However, an ELISA assay has some limitations as well. It does not allow for the observation of NETs at the very moment of their formation on the surface of neutrophils. The DNA-derived marker

such as modified histones, as well as a protein-derived marker (MPO) are presented as two separate results and do not always correlate with each other. Despite these limitations, ELISAs used for NET detection are widely regarded as a reliable and objective technique [23].

Our study showed for the first time the association between the potential of neutrophils to release NETs and the levels of serum citH3 and nucleosomes in AS patients, which agrees with the report by Lee et al. [24], who demonstrated that the percentage of MPO+citH3+ neutrophils correlated positively with serum double-strand DNA concentrations.

The current study could be relevant given growing interest in NETosis as a potential treatment target with anti-inflammatory and anti-thrombotic potential, which might be useful in the management of AS and possibly other cardiovascular diseases [25].

This study has several limitations. The number of study participants was small. However, the AS group was homogenous and matched with the control group. The use of CD15 and CD66 [3, 14] as neutrophil markers could be considered for flow cytometry; however, additional staining may exacerbate cell disintegration. The association of NET markers with the AS severity does not necessarily indicate a cause-and-effect relationship, and they should be interpreted with caution. The current results cannot be easily extrapolated to patients with mild-to-moderate AS or those with common comorbidities, in particular diabetes or coronary artery disease, known to increase NETosis[26].

5. Conclusions

In patients with severe AS, neutrophils have increased potential to release NETs compared to healthy subjects, which is associated with serum concentrations of NETosis markers. Our understanding is that flow cytometry and ELISA support each other in characterization of NETosis in human subjects. However, in AS patients we recommend using ELISA to assess NETosis in vivo, since this technique is simpler, burdened with fewer disadvantages and may give more reliable results compared to flow cytometry.

Authors' contribution

PK designed the experiments, performed experiments, acquired and analyzed data, drafted and revised the manuscript. MK performed experiments, acquired and analyzed data, drafted and revised the manuscript. MZ contributed to the experimental design and interpretation of data and drafted the manuscript. AU analyzed the data and critically revised the manuscript. JN designed the experiments, acquired and analyzed data, revised the manuscript. All of the authors read and approved the final manuscript.

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Conflicts of interest

The authors declare that they have no competing interests.

Ethics approval

Ethics committees (Krakow Medical District Chamber, Poland, approval number: 8/KBL/OIL/2019 for patients, and the Bioethics Committee of the Jagiellonian University, approval number: 1072.6120.186.2020 for healthy participants) approved the study, and all participants gave written informed consent in accordance with the Declaration of Helsinki.

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