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Neutrophil extracellular traps-associated markers are elevated in patients with systemic lupus erythematosus

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Abstract

Neutrophil extracellular traps (NETs) are the main source of autoantigens in systemic lupus erythematosus (SLE). The aim of this study was to evaluate the clinical importance of NETs-associated markers in SLE. We compared NETs-associated markers in SLE patients ($n=111$) with healthy controls ($n=50$). Moreover, in 35 patients with drug-naïve SLE ($n=35$), we investigated correlation between NETs-associated markers [DNase I concentration, myeloperoxidase (MPO) activity, anti-MPO antibodies, cell-free DNA (cfDNA), NETolytic activity] with serological parameters [anti-dsDNA antibodies, C3, C4 and B-cell activating factor (BAFF) levels] and disease activity measured by modified SLE Disease Activity Index (M-SLEDAI-2K). In comparison with healthy controls, SLE patients had higher cfDNA, MPO activity, anti-MPO antibodies ($p<0.001$), BAFF and DNase I concentration ($p<0.01$). Contrary, NETolytic activity was lower in SLE patients ($p<0.05$), despite higher concentration of DNase I. MPO activity and cfDNA levels showed correlation with DNase I concentration ($p<0.001$, $p<0.01$, respectively). BAFF levels correlated with cfDNA, DNase I concentration and MPO activity ($p<0.05$). Anti-dsDNA antibodies showed correlation with MPO activity ($p<0.01$), cfDNA and BAFF levels ($p<0.001$). Anti-dsDNA and C3 levels were independent predictors of M-SLEDAI-2K in multivariate analysis ($p<0.01$). We demonstrated that sera of SLE patients have decreased NETolytic activity, leading to increased levels of various NETs-associated markers, which correlate with anti-dsDNA antibodies in drug-naïve SLE. We showed that BAFF participates in a complex relationship between NETosis and anti-dsDNA antibodies production. These findings have important implications for a better understanding of SLE pathogenesis and development of therapy that inhibits NETs persistence and disease progression.

Keywords NET · SLE · DNase I · Anti-dsDNA · Cell-free DNA · BAFF

Introduction

Inadequate clearance of dead cells seems to be the main mechanism that triggers autoimmunity in systemic lupus erythematosus (SLE) patients [1]. It has been long believed that apoptotic cells are the main source of autoantigens until the discovery of neutrophil extracellular traps (NETs). NETs are structures composed of neutrophil DNA decorated with antimicrobial proteins which usually arise by expulsion of neutrophil DNA upon encountering different stimuli, usually cytokines and microbial products [2]. Beside expressing common nuclear antigens, during NETosis, some neutrophil proteins are modified by enzymatic activity [3] leading to the appearance of neoepitopes that can further stimulate immune response. In SLE patients, NETs activate plasmacytoid dendritic cells to produce large amounts of interferon α (IFNα) in DNA and Toll-like receptor 9-dependent manner

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[4]. NET-derived LL37-DNA complexes trigger polyclonal B-cell activation also via Toll-like receptor 9 and induce self-reactive memory B cells producing anti-LL37 antibodies in an antigen-dependent manner [5]. High mobility group B (HMGB-1) and heat shock proteins, also released during NETosis, contribute to the loss of autotolerance [6]. Bone marrow neutrophils in lupus patients are an important source of B-cell activating factor (BAFF) and type I interferons [7] that have a decisive role in B-cell development and maturation, as well as lupus development. Mice transgenic for BAFF develop autoimmune disease with high levels of circulating immune complexes, anti-dsDNA antibodies and glomerular deposits of immunoglobulins [8]. Considering that neutrophils of lupus-prone mice produce BAFF and activate CD4+ T- and B-cell response [9], BAFF may be a link between NETs and autoantibody production. Release of inflammatory NETs in conjunction with BAFF might support the survival and differentiation of autoreactive B cells. It has been recently shown that cocaine and levamisole can induce simultaneous release of inflammatory NETs and BAFF production [10].

Special population of proinflammatory neutrophils, low density granulocytes (LDG) were isolated from blood of some SLE patients [11]. LDG secrete IFNα and are more prone to produce NETs [12]. NETs are degraded by enzyme DNase I which activity is often decreased in SLE patients, leading to NETs persistence and induction of immune response [13]. Although DNase I was first discovered in gastrointestinal tract, later it has been shown that DNase I exerts its main function in serum, degrading chromatin material remaining after apoptosis and necrosis of self-cells [14, 15]. Several studies revealed connection between low DNase I activity and SLE. Various DNase I gene polymorphisms are associated with SLE [16]. SLE patients have lower DNase I activity in comparison with healthy controls [17], as well as higher concentration of DNase I inhibitor. DNase I knockout mice have glomerular immune deposits, produce anti-DNA antibodies and develop clinical features of SLE [18]. In (NZBxNZW)F1 lupus-prone mice, renal loss of DNase I precedes proliferative glomerulonephritis [19]. Similar loss of renal DNase I activity was also observed in patients with SLE [20]. Serum ability to degrade NETs is mainly dependent on DNase I activity, and patients with low NETolytic activity have severe forms of lupus nephritis [13]. Elevated cell-free DNA (cfDNA) levels were found in lupus sera and may be associated with active lupus nephritis [21]. High levels of cfDNA in SLE may be related to impaired NETs degradation and clearance. Myeloperoxidase (MPO) is a major neutrophil protein having an essential role in chromatin decondensation [3]. Increased MPO activity was also observed in lupus sera, [22] and it may be related to NETs degradation and activity of DNase I.

Although NETosis plays a decisive role in SLE pathogenesis, the clinical significance of various NETs markers is still relatively unknown. It is well known that various SLE biomarkers are neutrophil-related, including histones undergoing post-translation modification during NETosis, neutrophil granular proteins and BAFF. The relationship between NETs-associated biomarkers and BAFF was not investigated in SLE. Also, the laboratory standardization of decreased NET degradation is needed and we investigated clinical usefulness of various NETs-associated biomarkers, relatively easy to perform in everyday laboratory practice. Therefore, we postulated hypothesis that patients with SLE have elevated NETs-associated markers and that their concentrations correlate with serological and clinical parameters of SLE activity.

Patients and methods

Patients

Our study included 111 SLE patients diagnosed and treated at the Clinical Centre of Serbia (Clinic of Dermatovenereology, Clinic of Allergy and Immunology) and Institute of Rheumatology, from January 2016 to April 2018. All procedures involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration. The Study has been approved by the Ethics Committee of the Clinical Centre of Serbia, Belgrade, number 2565/2, on 12th December 2013. This approval includes signed patient consent. Informed consent was obtained from all individual participants included in the study. The inclusion criteria for study were: all patients fulfilled at least four revised American College of Rheumatology (ACR) criteria for SLE [23], had positive antinuclear antibodies (ANA) and were 18 years or older. The exclusion criteria were: patients receiving cyclophosphamide and/or methylprednisolone pulses in the last 3 months, history of malignancies in the last 5 years, diabetes, and history of any uncontrolled disease that according to investigator judgment may influence results. Additionally, a group of drug-naïve SLE patients ($n=35$) was selected. In this group of patients, we investigated correlation between NETs markers [DNase I concentration, myeloperoxidase (MPO) activity, anti-MPO antibodies, cell-free DNA (cfDNA), NETolytic activity] with serological parameters [B-cell activating factor (BAFF), anti-dsDNA, C3 and C4 levels] and disease activity measured by modified SLEDAI-2K (M-SLEDAI-2K), which does not include complement and anti-dsDNA descriptors [24]. Control group consisted of 50 age-matched healthy blood donors (39 women, 11 men), whose mean age was 42.5 ± 17.4 years.

Serological parameters

Complement components

C3 and C4 complement components were determined by nephelometry (Orion Diagnostica, Espoo, Finland). Reference values for C3 were 0.8–2.1 g/L, and for C4 0.1–0.4 g/L.

Autoantibodies

Titre of ANA was detected by indirect immunofluorescence test (IIF) on HEp-2 cells (EUROIMMUN AG, Lübeck, Germany). Anti-dsDNA antibodies were determined by *Crithidia luciliae*-based IIF-CLIFT (EUROIMMUN AG, Lübeck, Germany). Fluorescence of kinetoplast at serum dilution of 1:10 or greater was considered positive. Anti-dsDNA antibodies were also determined by commercial ELISA with recombinant antigen (ORGENTEC Diagnostica, Germany). Values ≥ 50 IU/mL were considered positive. Anti-MPO antibodies were determined by commercial ELISA (ORGENTEC Diagnostica, Germany) with human recombinant MPO (native conformation) as antigen. Cut-off value was 5 U/mL.

DNase I concentration

DNase I concentration was measured by commercial ELISA test (Human deoxyribonuclease I, DNase I ELISA Kit, Cusabio). The test is based on quantitative sandwich enzyme-linked assay. DNase I concentration of the samples was determined by comparison to a standard curve generated using recombinant DNase I and expressed as ng/mL.

Parameters of netosis

cfDNA

Concentration of cfDNA was determined by commercial Quant-iT™ PicoGreen® dsDNA Assay Kit, Thermo Fisher Scientific. Briefly, the samples were dissolved in Tris–EDTA buffer containing fluorescent dye PicoGreen which binds DNA in equimolar amounts. After incubation, emitted fluorescence was read at 520 nm (excitation at 480 nm). DNA concentration was calculated from standard curve. Standard solutions of DNA were prepared using commercial solution K562 DNA (Promega) with 10 ng/μL as starting concentration.

MPO activity

MPO activity was determined using 3, 3′, 5, 5′-tetramethybenzidine (TMB) as a chromogenic substrate, based on a previously described method [25] with some modifications. Briefly, 10 μL of serum sample was mixed with 80 μL 0.75 mM hydrogen peroxide and 110 μL TMB (2.9 mM TMB in 14.5% dimethyl sulfoxide). Sodium phosphate buffer pH 5.4 was added to the reaction mixture in final concentration of 150 mM. After 5 min at 37 °C, reaction was stopped with 50 μL of 2 M sulphuric acid. Intensity of developed yellow color was read at 450 nm, and intensity was directly proportional to MPO activity. MPO activity of the sera was calculated from standard curve made by serial dilution of commercially available MPO enzyme (Sigma) and was expressed in relative units (RU/mL).

NETolytic activity

NETolytic activity was determined by measurement of cfDNA release from human NETs after partial lysis with diluted sera, according to previously published method, with some modifications [26]. Isolated human neutrophils were stimulated to produce NETs with 20 nM phorbol myristate acetate (Sigma) in RMPI-1640 medium (Gibco). After NETs induction, they were incubated with micrococcal DNAse (Sigma) of defined concentration (served as control) or with diluted 10% sera during 1 h, at 37 °C. Reaction was stopped by adding EDTA (final concentration 5 mM), and released cfDNA was measured by commercial Quant-iT™ PicoGreen® dsDNA Assay Kit, Thermo Fisher Scientific as previously described. The amount of released DNA was directly proportional to the NETolytic activity.

Serum BAFF

BAFF was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK). Cytokine concentrations of the samples were determined by comparison to a standard curve generated using recombinant cytokines and expressed as pg/mL.

Statistical analysis

Distribution of the continuous variables was assessed using the Kolmogorov–Smirnov test. Depending on the normality, continuous data were presented as mean ± SD or median (interquartile range—IQR). Categorical data are summarized as numbers (percentages). Comparison of continuous features of patients was done using the Student's *t* test or Mann–Whitney *U* test. Comparison of serological parameters between three groups (healthy controls, SLE de novo and treated SLE) was done using Kruskal–Wallis test. Post hoc analysis was applied with Bonferroni correction for all multiple comparisons. Pearson's Chi-square test or Fisher exact test was used for comparison of categorical characteristics between the two groups. Spearman's correlation was used to estimate association between serological parameters.

To estimate which of the serological parameters independently correlated with M-SLEDAI-2K score univariate and multivariate linear regression analyses were performed. Variables that were associated with M-SLEDAI-2K in univariate linear regression at the significant level < 0.1 were entered into the multivariate linear regression model and stepwise multivariate regression was applied. Multicollinearity was checked using values of tolerance and variance inflator factor (VIF). The level of significance for independent variables remaining in the final model was set to 0.05. To assess independent predictors of certain disease manifestation, logistic regression analysis was applied (Hosmer–Lemeshow method) [27]. All statistical tests were two-sided and performed at the 5% significance level. The statistical analysis was performed using IBM SPSS Statistics version 20.0 software (IBM Corp., Armonk, NY, USA).

Results

Main demographic and clinical data of our study group are summarized in Table 1. SLE patients had significantly higher levels of cfDNA and higher MPO activity than healthy controls (Table 2). Levels of BAFF and anti-MPO antibodies were also significantly higher in patients with SLE. Contrary, NETolytic activity was lower in SLE patients than in healthy controls, despite higher DNase I concentration (Table 2).

Drug-naïve SLE patients had also significantly higher levels of DNase I, anti-MPO, cfDNA, anti-dsDNA (determined by ELISA) and MPO activity than healthy controls (Table 3). Moreover, drug-naïve SLE patients (n-35) had significantly higher MPO activity (1710 ± 374.5 vs 1180 ± 248.5 RU/mL) and cfDNA levels (2.3 ± 0.9 vs 1.9 ± 0.7 ng/mL ng/mL) in comparison with SLE patients (n-76) tested during the follow-up ($p < 0.05$). MPO activity showed correlation with DNase I concentration and NETolytic activity, while cfDNA levels correlated with DNase I concentration, anti-MPO and BAFF levels (Table 4).

Correlations between standard serological parameters (C3, C4, anti-dsDNA) and parameters of netosis were examined. Anti-dsDNA antibodies (determined by ELISA) showed correlation with all tested parameters: DNAse I ($\rho = 0.258$, $p < 0.05$), MPO activity ($\rho = 0.303$, $p < 0.01$), anti-MPO antibodies ($\rho = 0.519$, $p < 0.001$), cfDNA ($\rho = 0.492$, $p < 0.001$) and BAFF levels ($\rho = 0.425$, $p < 0.001$), except for NETolytic activity ($p > 0.05$).

We further tested independent predictors of M-SLEDAI-2K score and disease manifestations in 35 patients with drug-naïve SLE. In linear regression model, 5 of 10 tested variables appeared significant in univariate analysis: BAFF levels, MPO activity, DNase I concentration, anti-dsDNA (determined by CLIFT) and C3 levels, and these were further tested. Only two were shown to be independent predictors of M-SLEDAI-2K score in multivariate analysis: anti-dsDNA determined by CLIFT ($p = 0.002$) and C3 ($p = 0.005$) (Table 5a). Moreover, we tested if NETs-associated markers or standard serological tests are predictor of specific disease manifestation. Of 10 variables tested in logistic regression analysis, none was shown to be a predictor of skin involvement, arthritis, renal disease and central nervous system involvement, except that anti-dsDNA antibodies determined by standard ELISA were predictor of cytopenia (OR 1.049, $p = 0.027$) (Table 5b).

Table 1 Main demographic, clinical and immunoserological data of systemic lupus erythematosus patients

	$n = 111$
Age, years, mean ± SD (range)	43.6 ± 14.3 (19–77)
Gender: F/M	99/12
Mean duration of disease (years) mean (range)	4.5 (0–12.1)
New onset SLE (%)	35 (31.5)
Gender: F/M	29/6
Age, years, mean ± SD (range)	39.3 ± 13.6
Therapy option	111
Corticosteroids (%)	69 (62.2)
Antimalarials (%)	65 (58.5)
Mycophenolate mofetil (%)	16 (14.4)
Azathioprine (%)	21 (18.9)
Methotrexate (%)	3 (2.7)
Cyclosporine A (%)	8 (7.2)
Immunological findings	
ANA (%)	111 (100)
Presence of auto-antibodies against dsDNA	
Standard ELISA (%)	86 (77.4)
CLIFT (%)	82 (73.8)
Low levels of complement components	
C3 (%)	25 (22.5)
C4 (%)	57 (51.4)
Visceral involvement in drug naïve SLE	35
Cutaneous (%)	13 (37.0)
Articular (%)	21 (60.0)
Renal (%)	17 (48.6)
Neuropsychiatric (%)	2 (5.7)
Serositis (%)	11 (31.4)
Hematologic (%)	16 (45.7)

SD standard deviation, *CLIFT Crithidia luciliae* immunofluorescent test, *SLE* systemic lupus erythematosus, *F* female, *M* male

Discussion

In this study, we demonstrated that sera of SLE patients have decreased NETolytic activity, leading to increased levels of various NETs-associated markers (DNAse I, MPO activity,

Table 2 Neutrophil extracellular traps-associated markers, standard serological parameters and B-cell activating factor levels in systemic lupus erythematosus patients and healthy controls

	SLE $n=111$	Healthy controls $n=50$	p value
anti-dsDNA ELISA (U/mL)	84.7 ± 68.1	5.6 ± 3.3	**< 0.001**
anti-dsDNA IIF,1/titre, median (range)	160 (10–1280)	neg	**< 0.001**
C3 (g/L)	0.9 ± 0.2	1.2 ± 0.1	**< 0.05**
C4 (g/L)	0.2 ± 0.1	0.3 ± 0.1	**< 0.05**
BAFF (pg/mL)	1818.5 ± 2642.5	891.6 ± 184.4	**< 0.01**
DNase I concentration (ng/mL)	10.4 ± 6.9	5.8 ± 5.7	**< 0.01**
MPO activity (RU/mL)[a]	1354.8 ± 521.3	560.3 ± 182.5	**< 0.001**
anti-MPO (U/mL)	13.8 ± 43.4	0.9 ± 0.3	**< 0.001**
cfDNA (ng/mL)	2.3 ± 0.9	1.0 ± 0.3	**< 0.001**
NETolytic activity (RU/mL)[a]	4.3 ± 1.7	6.8 ± 1.8	**< 0.05**

Values are expressed as mean ± SD or median, bolded values are statistically significant

DNA deoxyribonucleic acid, *BAFF* B-cell activating factor, *MPO* myeloperoxidase, *cfDNA* cell-free deoxyribonucleic acid

[a]Relative units

Table 3 Comparison of serological and neutrophil extracellular traps-associated markers between healthy controls and 35 drug- naïve systemic lupus erythematosus patients

	Healthy controls $n=50$	SLE drug-naïve $n=35$	p value
anti-dsDNA ELISA (U/mL)	5.6 ± 3.3	67.3 (85.4)	**< 0.001**
BAFF (pg/mL)	891.6 ± 184.4	1126.8 (2587.0)	**> 0.05**
DNase I concentration (ng/mL)	5.8 ± 5.7	13.2 (14.7)	**<0.05**
MPO activity (RU/mL)[a]	560.3 ± 182.5	1710 ± 374.5	**< 0.001**
anti-MPO (U/mL)	0.9 ± 0.3	2.9 ± 0.6	**< 0.001**
cfDNA (ng/mL)	1.0 ± 0.3	2.5 ± 1.0	**< 0.001**
NETolytic activity (RU/mL)[a]	6.8 ± 1.8	5.3 ± 1.5	**> 0.05**

BAFF B-cell activating factor, *MPO* myeloperoxidase, *cfDNA* cell-free deoxyribonucleic acid

[a]Relative units, results represent MV ± SD or median, bolded values are statistically significant

Table 4 Correlation between studied parameters in 35 drug-naïve systemic lupus erythematosus patients

	BAFF	DNase I[a]	MPO[b]	Anti-MPO	cfDNA	NETolytic activity
BAFF (p)		**< 0.05**	**< 0.05**	> 0.05	**< 0.05**	> 0.05
DNase I[a] (p)			**< 0.001**	> 0.05	**< 0.01**	> 0.05
MPO[b] (p)				**< 0.01**	> 0.05	**< 0.05**
anti-MPO (p)					**0.001**	> 0.05
cfDNA (p)						> 0.05

BAFF B-cell activating factor, *MPO* myeloperoxidase, *cfDNA* cell-free deoxyribonucleic acid

[a]Concentration, bolded values are statistically significant

[b]Activity

anti-MPO antibodies and cfDNA), which correlate with anti-dsDNA antibodies in drug-naïve SLE patients. We also showed that BAFF participates in a complex relationship between NETosis and anti-dsDNA antibodies production. Using M-SLEDAI-2K, standard serological parameters (anti-dsDNA and C3 levels) are predictors of activity in drug-naïve SLE. Besides apoptotic cells, NETs are the major source of antigens in SLE, triggering autoimmune response. Decreased ability of SLE sera to degrade NETs was shown in several studies [13, 26], being most prominent in patients with the most severe disease [26].

Initially, it was assumed that cfDNA originates from apoptotic and necrotic cells, but later it became clear that living cells can also release DNA into serum and that concentration is mainly dependent on clearance mechanisms [28]. NETs, as structures composed almost exclusively of

Table 5 (a) Linear regression analysis of neutrophil extracellular traps-associated markers and standard serological parameters as predictors of systemic lupus erythematosus disease activity measured by modified-SLEDAI-2K in with 35 drug-naive systemic lupus erythematosus patients; (b) Logistic regression analysis of studied parameters as predictors of cytopenia in 35 drug naïve systemic lupus erythematosus patients

	Univariate		Multivariate	
	B [95% CI]	*p* value	*B* [95% CI]	*p* value
(a)				
BAFF	0.001 [0.00–0.002]	**0.015**		
Anti-dsDNA ELISA	0.011 [−0.019–0.038]	0.486		
Anti-MPO	−0.002 [−0.038–0.032]	0.916		
MPO activity	0.002 [0.00–0.003]	**0.046**		
DNase I concentration	0.338 [0.04–0.58]	**0.026**		
Anti-dsDNA, CLIFT	0.005 [0.00–0.01]	**0.003**	0.005 [0.002–0.008]	**0.002**
C3	−6.395 [−8.06–(−1.01)]	**0.013**	−4.907 [−9.808–(−0.006)]	**0.005**
C4	−17.904 [−32.20–(−1.04)]	0.122		
cfDNA	−1.218 [−3.14–0.84]	0.304		
NETolytic activity	0.283 [−0.96–1.49]	0.670		
	Univariate		Multivariate	
	OR [95% CI]	*p* value	OR [95% CI]	*p* value
(b)				
BAFF	1.001 (1.000–1.002)	0.218		
Anti-dsDNA ELISA	1.022 (1.002–1.043)	**0.029**	1.049 (1.021–1.135)	**0.027**
Anti-MPO	1.009 (0.991–1.028)	0.323		
DNase I	1.043 (0.931–1.169)	0.466		
MPO	1.000 (1.000–1.001)	0.650		
Anti-dsDNA CLIFT	1.000 (0.999–1.001)	0.622		
C3	1.515 (0.382–6.008)	0.554		
C4	1.905 (0.011–3.791)	0.805		
cfDNA	1.261 (0.611–2.605)	0.531		
NETolytic activity	0.599 (0.355–1.011)	**0.055**		

BAFF B-cell activating factor, *DNA* deoxyribonucleic acid, *MPO* myeloperoxidase, *CLIFT* *Crithidia luciliae* immunofluorescent test, *SLE* systemic lupus erythematosus, *cfDNA* cell-free deoxyribonucleic acid, bolded values are statistically significant

nucleic acids and proteins, may be an important source of circulating cfDNA and their concentrations in serum may represent NETs turnover [21]. In our group of SLE patients, we found almost doubled concentration of cfDNA in comparison to healthy controls. Zhang et al. also found increased cfDNA levels in SLE patients and this result was attributed to poor NETs degradation [21]. In the same study, cfDNA correlated with appearance of lupus nephritis, proteinuria and LDG, but they did not observe correlation with DNase I activity. In our patients, cfDNA showed significant correlation with anti-dsDNA antibodies determined by ELISA and DNase I concentration. Considering that DNase I hydrolytically degrades DNA molecules, it might be expected that increased DNase I concentration will result in a decrease of cfDNA, which was not the case. Actually, NETs may be large agglomerates and DNA present inside NETs is surrounded by many proteins that reduce solubility. Partial NETs degradation leads to formation of shorter DNA chains and releases more DNA molecules into a solution that actually contributes to increased cfDNA concentration. DNase I concentration in physiological conditions is not sufficient for complete NETs degradation [29], further supporting our hypothesis.

Correlation between cfDNA and anti-dsDNA antibodies is expected considering that antibodies to dsDNA may be induced by NETs [30] and increased concentrations of cfDNA and anti-dsDNA antibodies may reflect the same phenomenon-increased NETs production. Despite the fact that pure cfDNA is not immunogenic, DNA present in immune complexes induces immune response and may be very important in SLE pathogenesis [4, 31]. Several research groups, including ours, previously reported lower DNase I activity in SLE patients than in healthy controls [17, 32, 33]. Contrary, in the present study we have shown, for the first

time, that concentration of DNase I was higher in patients with SLE than in healthy controls. This discrepancy may be explained by the fact that in all previous studies, activity of DNase I was measured; while, we were the first to measure its concentration in sera. Activity of enzyme is dependent on its concentration, but also, the presence of activators and inhibitors influence the catalytic activity of DNase I. Actin is a well-known inhibitor of DNase I. Moreover, two thirds of SLE patients have antibodies directed to DNase I that block its activity [34]. Recently, it has been shown that an unidentified inhibitor of DNase I is present in lupus sera [35]. Higher concentration of DNase I in our group of patients is in line with increased DNase I gene expression observed in SLE patients [36]. Although concentration of DNase I was higher, NETolytic activity (mainly dependent on activity of DNase I) was lower in our SLE patients, which further supports the presence of an inhibitor which lower DNase I activity. Hakkim et al. found that in group of 61 SLE patients 36% sera had low NETolytic activity [13] and that most of these patients had lupus nephritis. Adding DNase I to these sera did not recover NETolytic activity in most cases, confirming the presence of DNase I inhibitor(s). Similar results were obtained in a group of 94 patients where 29% had low NETolytic serum activity. Interestingly, NETolytic activity was low during disease activation, while only 12% of patients had persistent low activity even during disease remission [26].

MPO is an integral part of NETs [37]. In our study, MPO activity was significantly higher in SLE patients, which may reflect increased NETs burden. In the literature, results related to MPO concentration in SLE are conflicting. While Morgan et al. found decreased MPO levels in patients with SLE [22], more recent publication reported increased levels [38]. We have found that anti-MPO antibodies levels were in correlation with MPO activity in SLE patients. About 20% of SLE patients have anti-MPO antibodies [39]. Low levels of DNase I activity and appearance of anti-MPO antibodies were observed in propylthiouracil-induced lupus-like syndrome [40]. It has been demonstrated that in microscopic polyangiitis, anti-MPO antibodies arise due to poor NETs degradation. On the other hand, anti-MPO antibodies may trigger NETosis, forming in this way a vicious circle [41] that contributes to persistence of inflammatory process.

Anti-dsDNA antibodies showed significant correlation with NETs-associated markers (cfDNA, DNase I, MPO activity and anti-MPO antibodies), as well BAFF levels, in our group of 35 drug-naïve SLE patients. Correlation between anti-dsDNA antibodies and NETs levels was also found in pediatric SLE [42]. Plasma of lupus patients with anti-dsDNA antibodies induced increased levels of NETs [43].

In the group of 35 drug-naïve SLE patients, BAFF, MPO activity, DNase I, C3 and anti-dsDNA antibodies determined by CLIFT were significant predictors of M-SLEDAI-2K score in univariate analysis, but in multivariate analysis only anti-dsDNA and C3 remained significant. These findings are in accordance with the well-known view that reduced NET degradation precedes the subsequent production of anti-dsDNA, the deposition of immune complexes and inflammation which later causes an increase of SLE activity [6]. It is important to define temporal associations of NETs-associated markers with clinical and laboratory markers of SLE activity.

We further investigated if any of the tested parameter could be a predictor of specific disease manifestation. Of all tested parameters, only anti-dsDNA antibodies were significant predictor of cytopenia in multivariate analysis, while no relation with other disease manifestations (serositis, nephritis, central nervous system involvement) was found. Anti-dsDNA antibodies were predictor of lymphopenia in large cohort of patients with recent onset of rheumatic symptoms even regardless of SLE diagnosis [44].

Besides their role in SLE pathogenesis, NETs have important role in the development of premature atherosclerosis [45], its complications being the main reason for mortality in long-standing SLE. Neutrophils are major players in the host innate immune response and NETs are triggers of the onset, progression and possibly may mediate resolution of inflammation in SLE. Previously, it has been shown that large NETs agglomerates may actually degrade inflammatory cytokines and have decisive role in termination of inflammatory response in gout model [46].

The strength of our study comes from the findings that sera of SLE patients, in comparison with healthy controls, have decreased NETolytic activity that leads to increased levels of various NETs-associated markers. Anti-dsDNA antibodies showed significant correlation with NETs-associated markers (cfDNA, DNase I concentration, MPO activity and anti-MPO antibodies), as well as BAFF levels, in our group of 35 drug-naïve SLE patients. Our results also confirmed that BAFF participates in a complex relationship between NETosis and anti-dsDNA antibodies production. Previously, we have shown a reduced activity of DNase I in SLE patients [17]. In the present study, we found increased DNase I concentration that has not been found so far and indirectly confirms the presence of DNase I inhibitor(s). However, our finding should be interpreted cautiously, considering relatively low number of drug-naïve patients. Also, larger prospective longitudinal studies that assess NET-associated biomarkers, DNase I inhibitor(s) and SLE disease activity are needed. Also, it is important to define temporal associations of specific NETs-associated markers with clinical and laboratory markers of SLE activity.

Considering multitude roles of NETs in SLE, application of NETs inhibitors could be a novel approach to treat patients [47]. Promising results have already been obtained

in animal models [48]. Even already available treatment such as rituximab and belimumab may affect NETs formation. In a small trial including 16 refractory severe SLE patients combined therapy with rituximab and belimumab decreased NETs formation, ANA levels and lead to improvement in majority of cases [49]. Both conventional agents such as antimalarials and vitamin D, and novel agents, such as DNase I, antioxidants, terminal complement inhibitors, protein arginine deiminase 4 inhibitors, and anti-IFNα therapies, may target NETs in SLE. However, questions remain whether these therapies may be beneficial in only some phenotypes of SLE patients. [49]. As these new therapies develop in future, measurement of NETs-associated markers may be helpful in clinical–translational researches.

In conclusion, we demonstrated that sera of SLE patients have decreased NETolytic activity, leading to increased levels of various NETs-associated markers which correlate with anti-dsDNA antibodies in drug-naïve SLE patients. Our results also confirmed that BAFF participates in a complex relationship between NETs and anti-dsDNA antibodies production. These findings have important implications for a better understanding of SLE pathogenesis and development of therapy that inhibits NETs persistence. Measurement of NETs-associated markers, relatively easy to perform in everyday laboratory practice, may be helpful to define subsets of SLE patients that might benefit from therapies which target NETs.

Author contributions IJ and BBN designed study, performed clinical assessment, critically analyzed data and prepared the manuscript. OD performed the statistical analysis and prepared the manuscript for publication. MN analyzed data and prepared the manuscript. MV and IJ performed laboratory work and collected the data. AN and DR contributed to data analysis and critical revisions. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The Authors declare that there is no conflict of interest.

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