

Role of Neutrophil Extracellular Traps Regarding Patients at Risk of Increased Disease Activity and Cardiovascular Comorbidity in Systemic Lupus Erythematosus

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ABSTRACT. **Objective.** Neutrophil extracellular traps (NET) are essential in host defense, but are also linked to inflammation and autoimmunity, including in systemic lupus erythematosus (SLE). We recently described that immune complexes (IC) induce NET formation, promoting SLE-like disease in mice. In the current study, we investigated, for the first time to our knowledge, the role of NET in human SLE and their association with disease activity and severity.

Methods. Levels of NET (myeloperoxidase-DNA complexes) were analyzed in plasma from 4 cross-sectional SLE cohorts ($n = 44\text{--}142$), 1 longitudinal SLE cohort ($n = 47$), and healthy individuals ($n = 100$) using ELISA. Type I interferon activity was determined using a cell reporter system.

Results. Patients with SLE had elevated levels of NET in circulation compared to healthy controls ($p < 0.01$). NET levels identified patients with a severe disease phenotype characterized by IC-driven nephritis ($p < 0.05$). Though not associated with current disease activity ($p = 0.20$), levels of NET were associated with future increase in the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) within 3 months (OR 1.75, $p = 0.01$), as well as an overall heightened SLEDAI over 1 year ($p < 0.01$). Finally, levels of NET were associated with arterial events (OR 5.0, $p = 0.02$) and endothelial cell activation ($p < 0.001$).

Conclusion. NET levels are elevated in patients with SLE, associated with IC-driven disease. NET levels provide significant clinical value in identifying patients at risk of active disease and/or severe disease, including nephritis and cardiovascular disease, and may allow for early interventions.

Key Indexing Terms: cardiovascular disease, nephritis, neutrophil extracellular trap, systemic lupus erythematosus

Neutrophils are essential cells of our innate immune system, partaking in host defense mechanisms through production of reactive oxygen species (ROS), phagocytosis, and formation of neutrophil extracellular traps (NET). NET formation, or NETosis, is a cell death process in which DNA is expelled together with cytosolic and granular content in a web-like structure to trap and eliminate extracellular pathogens^{1,2,3,4,5,6}. Though NET formation is beneficial from a host-pathogen perspective, impaired NET formation (or clearance) has been implicated in inflammation and autoimmunity, including in rheumatoid

arthritis and systemic lupus erythematosus (SLE)^{2,3,7,8,9}. In SLE, NET have been suggested as an important source of autoantigens, with SLE patients having high levels of antibodies binding to NET, including anti-dsDNA and antihistone antibodies¹⁰. The released NET engage DNA receptors Toll-like receptor (TLR) 9 and cyclic GMP-AMP synthase (cGAS) to induce type I interferons (IFN), key cytokines in SLE pathogenesis^{2,7,11,12}. The relevance of NET in SLE, however, is uncertain because evidence from SLE-prone mice are conflicting, with NET inhibition either ameliorating or worsening SLE-like disease^{13,14,15,16,17}. Even in patients with SLE, there are limited data to support a role of NET in the disease pathogenesis. Early investigations have demonstrated the presence of an inflammatory neutrophil subpopulation, low-density granulocytes (LDG), enriched in patients with SLE and capable of undergoing spontaneous NETosis *ex vivo*¹⁸. In addition, immune complexes (IC), commonly found in patients with SLE, induce NET in an Fc-gamma-Receptor IIa (FcgrIIa)- and TLR8-dependent manner *in vitro*^{2,7,19}. Further, patients with SLE have reduced capacity to degrade NET, owing to presence of autoantibodies, as well as low levels of DNases^{10,20}. Though several studies have been done in mice and *in vitro*, very little is known about NET in patients with SLE. Prior work has found NET-forming

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neutrophils to infiltrate both skin and kidney of patients with SLE⁸. We previously made the novel observation of elevated levels of circulating NET in patients with SLE². However, it is surprising that to date, nothing is known about the clinical implications of NET in SLE, and whether levels of NET could be useful in monitoring disease activity and/or identifying a more severe disease phenotype, including nephritis.

Here, we analyzed levels of NET in 5 cross-sectional and longitudinal SLE cohorts to determine the relationship between NET and clinical disease activity and severity in SLE. In brief, using 4 cross-sectional cohorts, we confirmed that levels of NET are elevated in SLE, likely driven by IC-mediated neutrophil activation. Surprisingly, levels of NET did not associate with disease activity at the time of blood draw. However, using a unique longitudinal cohort, we made the novel, and clinically significant, finding that levels of NET were associated with future worsening of disease within 3 months. Finally, NET identified patients with a severe clinical phenotype including lupus nephritis (LN) and cardiovascular disease (CVD). In all, levels of NET provide significant clinical value in identifying SLE patients at risk of flare and/or severe disease, including nephritis and CVD, which may allow for early interventions.

MATERIALS AND METHODS

Patient cohorts. Four cross-sectional SLE cohorts were recruited at 2 separate sites: University of Washington, Seattle, USA (Cohort I, n = 44), through the Division of Rheumatology Biorepository; and Skane University Hospital, Lund, Sweden (Cohort II, n = 142; Cohort III, n = 92; and Cohort IV, n = 92). Cohort II is designed to focus on CV morbidity and mortality. Cohort III and Cohort IV include the same patients at timepoints of lower disease activity (Cohort III) and higher disease activity (Cohort IV) to assess the influence of disease activity on biomarker levels. A fifth cohort (n = 47), recruited at Skane University Hospital, consisted of patients followed longitudinally every 3 months for more than 2 years. To identify biomarkers of disease progression, 1 sample per patient was selected from the longitudinal cohort at a timepoint of clinical remission [Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) = 0]. The samples were selected to enrich for worsening of disease (n = 33) within 3 months of the clinical visit compared to patients remaining in remission (n = 14). On average, patients had been in remission for 6 months prior to analyzing the levels of NET (Supplementary Tables 1 and 2, available with the online version of this article). Healthy controls (n = 100) were recruited at Skane University Hospital.

The study was approved by the regional ethics boards (#3100, LU06014520, and LU 378-02). Informed written consent was obtained from all participants according to the Declaration of Helsinki. Patient characteristics are reported in Supplementary Tables 1 and 2. The Swedish patient cohorts have been described in great detail previously^{21–27}. Definitions were obtained from the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index²⁸ for myocardial infarction (MI), cerebrovascular incidents, angina pectoris, *Claudicatio intermittens*, deep venous thrombosis, and pulmonary embolisms. Arterial events were defined as either MI, cerebrovascular incidents, angina pectoris, or *Claudicatio intermittens*. Patients and the public were not involved in the design, conduct, or reporting of the research.

Neutrophil activation and cell death markers. Levels of calprotectin (S100A8/A9) were analyzed using a commercial ELISA kit according to the manufacturer's instructions (R&D Systems). The lower detection limit of the assay

is 94 pg/ml. For the detection of NET, a 96-well microtiter plate (Corning) was coated with a mouse monoclonal anti-myeloperoxidase (MPO) antibody (4 µg/ml, Biorad, clone 4A4) overnight at 4°C, followed by blocking with 1% bovine serum albumin in phosphate buffered saline for 2 h at room temperature. After blocking, plasma samples (10%) were added and incubated overnight at 4°C. For detection, anti-dsDNA-horseradish peroxidase antibody (diluted 1/100, Roche Diagnostics) was added for 2 h at room temperature. The reaction was developed with 3,3',5,5'-tetramethylbenzidine (BD Biosciences), and ended by the addition of 2 N sulfuric acid. Absorbance was measured at 450 nm by a plate reader (Synergy, BioTek). Isolated NET were used as a standard curve with 1 U/ml equaling NET released by 10,000 neutrophils.

Type I IFN assay. Type I IFN activity was measured as previously described, assessing the capacity of circulating type I IFN to signal through IFN-alpha-beta receptor^{10,29,30}. Briefly, WISH cells were cultured with patient serum (50%) and analyzed for induction of 6 IFN-regulated genes (*LY6E*, *MX1*, *OAS1*, *ISG15*, *IFIT1*, *EIF2AK2*) and 3 housekeeping genes (*GAPDH*, *PPIB*, *B2M*) using the Quantigene Plex 2.0 assay as described by the manufacturer (Panomics Inc.). Increased type I IFN activity was defined as a 2-fold increase in type I IFN-regulated genes compared to healthy controls.

IC assay. Levels of IC were analyzed using a commercial kit according to the manufacturer's instructions (Quidel).

Identification of LDG. Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep according to the manufacturer's protocol (Axis-Shield PoC). Within the PBMC, LDG were identified as CD14+CD15+CD16++ cells with high forward and side scatter properties using flow cytometry. The data are presented as percentage of LDG among total PBMC.

Endothelial microparticles (EMP). For detection of EMP, flow cytometry was performed directly on heparinized platelet-poor plasma³¹. MP gating was accomplished using 1 µm beads for setting upper limits in both forward and side scatter. EMP were identified as CD45-CD42a-CD146+ cells by flow cytometry as previously described^{31,32}.

Statistics. For non-paired sample sets with non-Gaussian distribution, Mann-Whitney U test and Spearman correlation test were used, as applicable. For paired sample sets, Wilcoxon matched-pairs signed-rank test was used. In some analyses, logistic regression analysis was used for dichotomized variables. As a cutoff for positivity, the 99th percentile of the healthy controls was used. GraphPad Prism (GraphPad Software) and IBM SPSS (IBM Corp.) were used for the analyses. All analyses were considered statistically significant at p < 0.05.

RESULTS

Patients with SLE have elevated levels of circulating NET. Levels of NET (MPO-DNA complexes) were analyzed by an in-house ELISA in plasma samples from 4 cross-sectional SLE cohorts spanning patients from the United States and Sweden with varying degrees of disease activity. As illustrated in Figure 1A, we confirmed that patients with SLE had elevated levels of NET as compared to healthy individuals (p < 0.01 for all analyses, adjusted for multiple comparisons). Of interest, levels of NET were increased even in patients with low to modest disease activity (p < 0.001; Figure 1A, Cohorts I–III), illustrating that neutrophil activation and cell death may occur even in the absence of manifest clinical disease. Levels of NET were not related to treatment with hydroxychloroquine or immunosuppressive drugs, including prednisone, other than in cohort II, where patients

taking prednisone had slightly elevated levels of NET ($p = 0.02$; data not shown).

Patients with SLE had increased frequency of an inflammatory neutrophil subpopulation, LDG ($p < 0.0001$; Figure 1B). However, levels of LDG did not correlate with levels of NET ($r = 0.04$, $p = 0.63$; Figure 1C). Thus, it is unlikely that LDG account for the overall increase in circulating NET in patients with SLE. Instead, levels of IC correlated with levels of NET in all 4 SLE cohorts (Figure 1D, and Supplementary Figures 1A-C, available with the online version of this article), suggesting that IC-mediated NETosis occurs *in vivo* in patients with SLE.

For one of the cohorts (II), SLE patients with elevated levels of NET (NET+) had increased type I IFN score ($p = 0.02$; Figure 1E). However, this was not reproducible in the other 2 cohorts (Supplementary Figures 1D-E, available with the online version of this supplement). Thus NET are likely not the sole inducer of type I IFN in SLE.

Levels of calprotectin, but not NET, associate with current disease activity. No correlation was observed between levels of NET

and SLEDAI in either of the 4 cohorts (Figure 2A-D), nor did levels of NET associate with any individual disease manifestations in these cohorts (data not shown). In stark contrast, levels of calprotectin (S100A8/A9), a neutrophil activation marker, correlated with SLEDAI in Cohort II ($r = 0.37$, $p = 0.0003$; Figure 2E). Thus, neutrophil activation, but not neutrophil cell death (NETosis), is associated with current disease activity in patients with SLE. Though calprotectin is an important component of NET, calprotectin can be released by several cells and also in NET-independent processes. In our cohorts, levels of calprotectin did not correlate with levels of NET (data not shown), suggesting that the majority of calprotectin was not NET-derived.

Levels of NET are associated with higher SLEDAI within 3 months. Levels of NET were analyzed in 47 patients in clinical remission, with 14 patients remaining in remission and 33 patients having a higher SLEDAI score with additional serological and/or clinical manifestations within 3 months. The patients developing higher SLEDAI had a history of more active disease with less

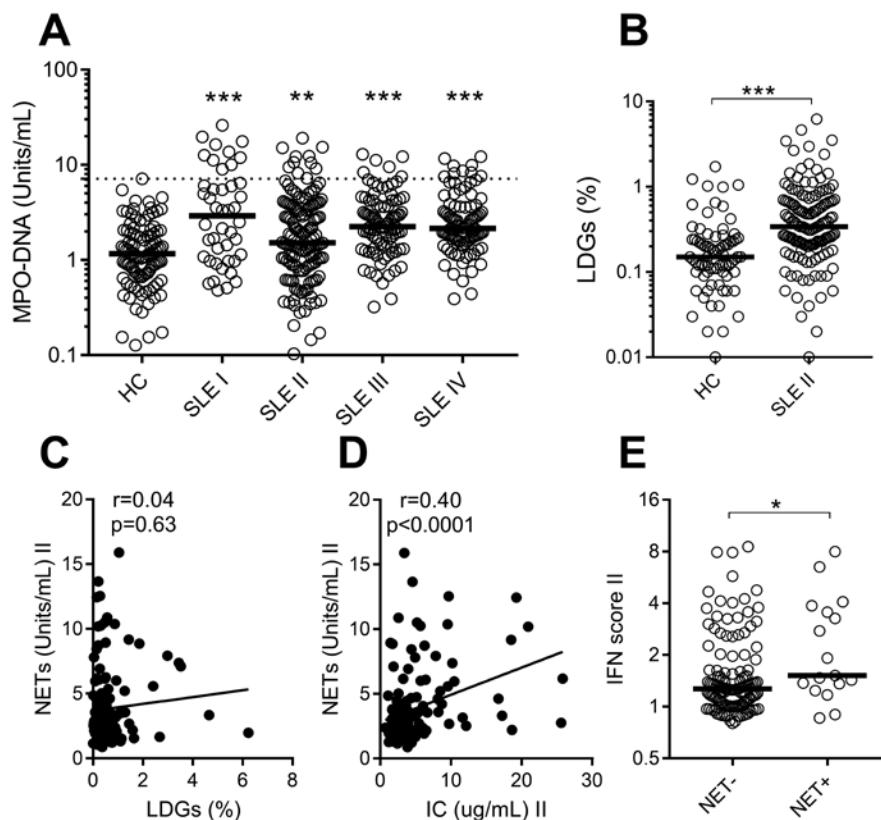


Figure 1. Neutrophil extracellular traps (NET) in patients with SLE. A. Levels of NET were analyzed in healthy controls (HC; $n = 100$) as well as 4 SLE cohorts (SLE I, $n = 44$; SLE II, $n = 142$; SLE III, $n = 92$; and SLE IV, $n = 92$) using an in-house MPO-DNA ELISA. The bar represents the median value, and the dotted line represent the 99th percentile of the HC, used to define patients with elevated levels of NET. B. Percentage of low-density granulocytes (LDG) in HC and patients with SLE as determined by flow cytometry. C. Correlation between levels of NET and LDG. D. Correlation between levels of NET and levels of immune complexes (IC) in SLE cohort II. E. Type I IFN activity in SLE cohort II with or without elevated levels of NET. Statistical analyses were done with Mann-Whitney U test, Wilcoxon, and Spearman correlation with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. SLE: systemic lupus erythematosus; MPO: myeloperoxidase; IFN: interferon.

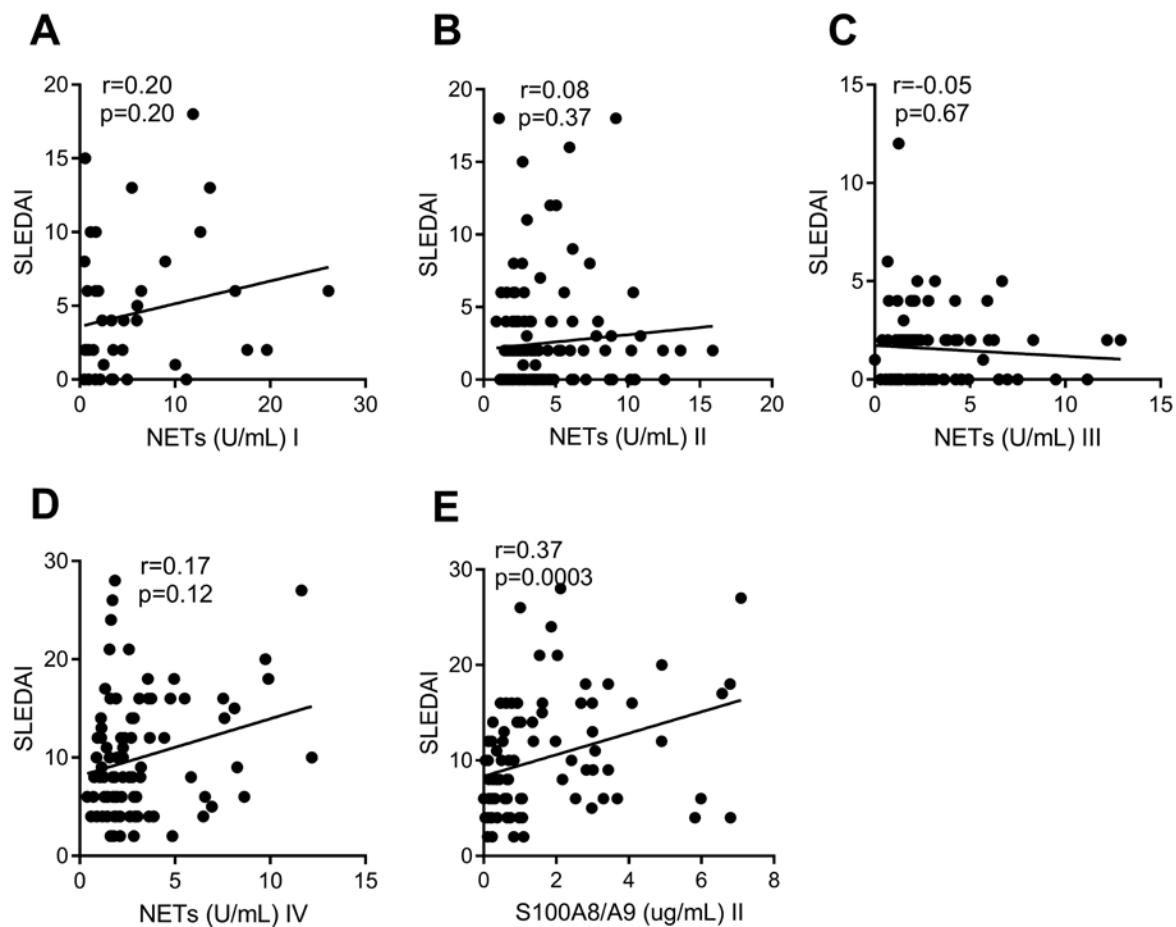


Figure 2. Levels of neutrophil extracellular traps (NET) are not associated with disease activity in SLE. A–D. Correlation between SLEDAI and levels of NET in SLE Cohorts I–IV, respectively. E. Correlation between levels of calprotectin and SLEDAI in Cohort II. Statistical analyses were done with Spearman correlation. SLE: systemic lupus erythematosus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

time in remission ($p = 0.03$; Supplementary Table 1, available with the online version of this article) and requiring more prednisone use ($p = 0.009$; Supplementary Table 1) compared to patients remaining in remission within 3 months. Interestingly, we found that patients who developed higher SLEDAI within 3 months had elevated levels of NET at baseline (Figure 3A). Using logistic regression analysis, we demonstrated that every 1 U/ml increase in baseline NET levels predicted higher future SLEDAI, with an OR of 1.75 (95% CI 1.13–2.73, $p = 0.01$; Table 1). Patients with elevated levels of NET at baseline (i.e., in remission) had an overall more active disease phenotype with higher SLEDAI within 3 months ($p = 0.006$; Figure 3B), as well as higher average SLEDAI over the following year ($p = 0.002$; Figure 3C). There were too few events for subgrouping of individual clinical manifestations. Overall, NET may be a marker of subclinical disease activity, identifying patients at risk of developing active disease.

Levels of circulating NET identify patients with nephritis. We observed significantly higher levels of NET in patients

with a history of nephritis among patients with low disease activity (LDA; Cohorts I–III), but not among patients with high disease activity (HDA; Cohort IV; Figure 4A, and Supplementary Figure 2A–C, available with the online version of this article). Further, SLE patients with anti-C1q autoantibodies, commonly associated with nephritis, had higher levels of NET in patients with LDA (Cohorts II–III), but not in patients with HDA (Cohort IV), compared to SLE patients without anti-C1q antibodies (Figure 4B, and Supplementary Figure 2D–E). Using logistic regression analysis, we demonstrated that in patients with LDA (Cohorts II and III), but not in patients with active disease (Cohort IV), increased levels of NET associated with history of nephritis, independent of the presence of either anti-dsDNA or anti-C1q antibodies (Table 1). Thus, elevated levels of NET, at timepoint of LDA, identified patients with an LN phenotype.

Levels of circulating NET associate with arterial thrombosis. As illustrated in Table 2, levels of NET were selectively associated with arterial events (OR 5.04, $p = 0.02$), but not venous events

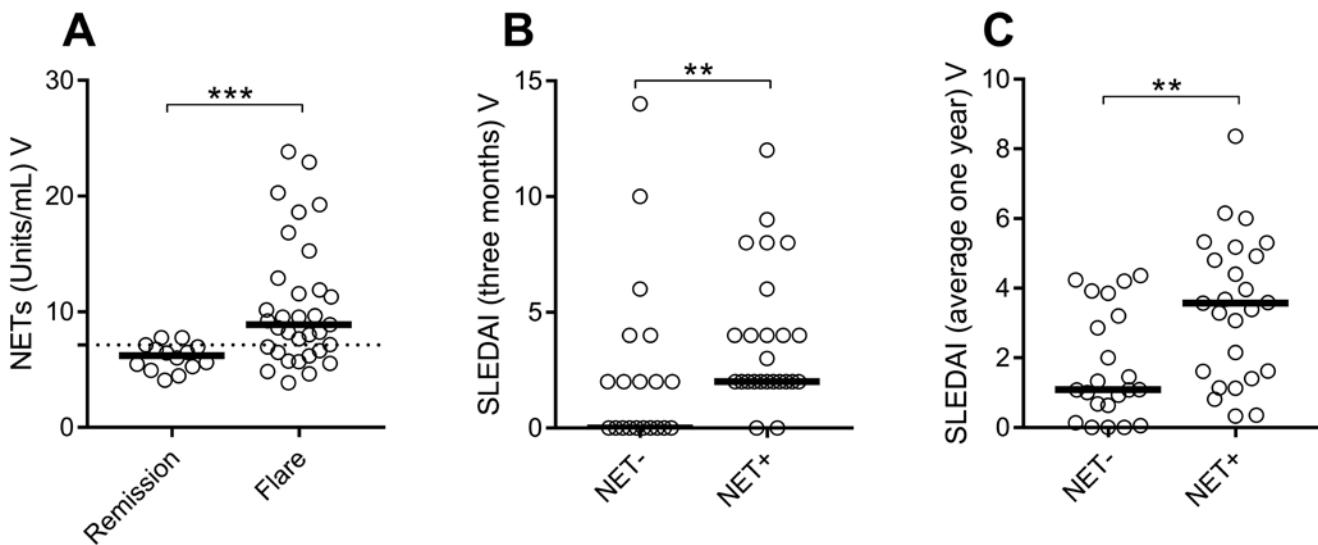


Figure 3. Levels of neutrophil extracellular traps (NET) can predict disease activity in patients with SLE. A. Levels of NET were analyzed at the time of remission in Cohort V, with patients stratified based on their clinical status 3 months later (e.g., remaining in remission or developing increased disease activity). The dotted line represents the 99th percentile of the healthy controls. B–C. Patients were stratified based on low or high levels of NET. B. SLEDAI at the 3-month followup visit based on NET stratification. C. Average SLEDAI for the 1-year followup period based on NET stratification. Statistical analyses were done with Mann-Whitney U test with $^{**}p < 0.01$, and $^{***}p < 0.001$. SLE: systemic lupus erythematosus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

Table 1. NET levels are associated with future disease activity and history of nephritis.

Marker	OR (95% CI) ¹	p	OR (95% CI) ²	p ²
Active disease	1.75 (1.13–2.73)	0.01	N/A	N/A
Nephritis cohort II	1.15 (1.02–1.31)	0.03	1.15 (1.01–1.31)	0.04
Nephritis cohort III	1.25 (1.03–1.51)	0.02	1.25 (1.02–1.52)	0.03
Nephritis cohort IV	1.11 (0.94–1.32)	0.21	1.01 (0.83–1.23)	0.93

¹ OR per 1 U/ml increase in NET levels. ² Adjusted for anti-C1q and anti-dsDNA antibodies at timepoint of blood draw. NET: neutrophil extracellular traps; N/A: not available.

(OR 0.46, $p = 0.47$; Table 2), suggesting a distinct mechanism by which NET affect only arterial vessels. There were too few events to further stratify the patients based on individual arterial events (e.g., MI, stroke, angina pectoris, and *Claudicatio intermittens*). SLE patients with elevated levels of NET (NET+) had increased presence of EMP, relative to NET patients ($p < 0.001$; Figure 4C). Moreover, levels of EMP correlated with levels of circulating NET in patients with SLE ($r = 0.34$, $p < 0.0001$; Figure 4D). Together, these data demonstrate a link between endothelial damage and NET, which could be relevant to the pathogenesis of atherosclerosis and arterial thrombosis in SLE. Further studies are warranted to study potential predictive value of NET in the development of CVD^{33–40}.

DISCUSSION

Neutrophils have long been thought to play a role in SLE pathogenesis, with early observations from the 1940s demonstrating neutrophils engulfing large amounts of antibody-coated nuclear

material in the bone marrow of patients with SLE (e.g., the lupus erythematosus cell)⁴¹. More recently, we have come to recognize that the engulfed IC are prominent inducers of NETosis and subsequent release of inflammatory and immunogenic material^{2,7,19}. However, although there have been important observations on the role of NET in SLE-prone mice^{2,16,17,39}, the inflammatory properties of NET *in vitro*^{2,7,12,37}, and the presence of NET in patients with SLE^{2,8}, the clinical significance of NET formation in human SLE has not been addressed. Further, evidence from SLE-prone mice are conflicting, with NET inhibition either ameliorating or worsening SLE-like disease^{13,14,15,16,17}. Though NET components, such as cell-free DNA and MPO, have been observed in the circulation of patients with SLE, surprisingly few studies have assessed levels of actual NET in patients with SLE. Previous studies by Villanueva and colleagues found NET-forming neutrophils in the skin and the kidneys of patients with SLE. However, they did not assess levels of circulating NET⁸. In our original study, we reported presence of NET in a single SLE cohort². Since then, a recent study has evaluated

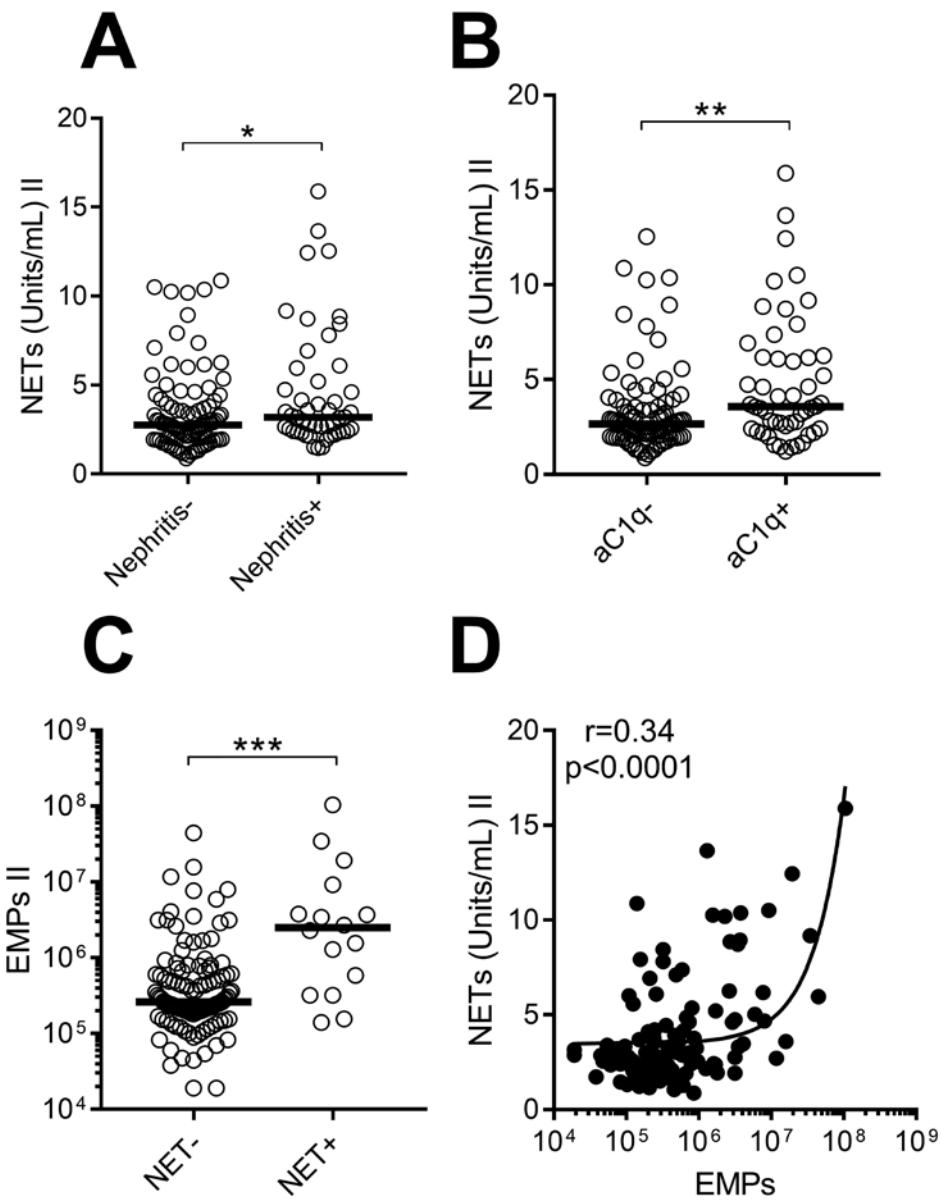


Figure 4. Levels of neutrophil extracellular traps (NET) are associated with history of nephritis and endothelial activation. Levels of NET were analyzed in patients with SLE and related to (A) history of nephritis, and (B) history of anti-C1q antibodies in Cohort II. (C) Patients with SLE were stratified based on NET levels, and endothelial-derived microparticles (EMP) analyzed by flow cytometry in SLE II. (D) Correlation between levels of NET and EMP in SLE II. Statistical analyses were done with Mann-Whitney U test, and Spearman correlation with $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. SLE: systemic lupus erythematosus; aC1q: anti-C1q autoantibodies.

Table 2. NET levels are associated with cardiovascular disease.

Event	Sens, %	Spec, %	OR	P	OR ¹	P ¹
Arterial ²	27.3	90.8	3.72 (1.21–11.44)	0.02	5.04 (1.31–19.37)	0.02
Venous	6.3	87.3	0.46 (0.06–3.71)	0.47	0.37 (0.04–3.21)	0.36

¹ Adjusted for age, sex, dyslipidemia, hypertension, and smoking. ² Arterial event includes myocardial infarction, stroke, *Claudicatio intermittens*, and angina pectoris. NET: neutrophil extracellular traps; Sens: sensitivity; Spec: specificity.

levels of NETs in serum samples⁴², without taking into consideration the propensity of neutrophils to undergo NETosis upon coagulation^{33,43}, which could influence the validity of the findings. None of the studies to date investigated the clinical relevance of NET in SLE.

In the current investigation, we assessed true levels of NET in plasma samples from 4 well-characterized cross-sectional SLE cohorts, as well as 1 longitudinal cohort, in an effort to determine the clinical significance of NET in patients with SLE. Using 4 cohorts, we were able to confirm that levels of NET were markedly elevated in patients with SLE as compared to healthy individuals. Of note, similar to our prior observation on calprotectin²⁴, levels of NET were elevated in patients with SLE even at the time of LDA. The occurrence of neutrophil activation and cell death even at LDA is intriguing, and suggests that current treatment strategies, while reducing clinical symptoms, may not be sufficient to prevent low-grade chronic inflammation and subsequent organ damage.

In contrast to calprotectin, which is associated with disease activity²⁴, levels of NET were surprisingly not associated with concurrent disease activity. Instead, levels of NET associated with worsening of disease within 3 months. This observation is significant, as it could provide opportunities for preventive treatment and/or closer monitoring of patients at high risk of flare. It also provides insight into the pathogenesis of SLE, suggesting that NET formation may be an early event, occurring prior to apparent clinical disease. Therefore NET formation may be an ideal therapeutic target, inhibiting disease at an early stage. Though NET inhibition has shown effects in SLE-prone mice^{2,16,17,39}, its effect in humans is still under investigation.

Several candidates have been put forward as potential contributors to the elevated levels of NET observed in patients with SLE. Early work by Denny's group demonstrated a high prevalence of LDG in SLE patients, with LDG undergoing spontaneous NET formation upon *ex vivo* isolation¹⁸. Though we could verify an increased prevalence of LDG in SLE, their levels did not correspond to levels of circulating NET. We have more recently demonstrated that IC have the capacity to induce NET in an FcgRIIA-, TLR8-, and mitochondrial ROS-dependent mechanism^{2,19}, generating mitochondrial-enriched NET promoting type I IFN induction through the cGAS-stimulator of IFN genes (STING) pathway². Consistent with these studies, we found a strong correlation between levels of NET and those of IC, suggesting that IC-mediated NETosis, and subsequent inflammation, may also occur in patients with SLE. However, given the study design, causality cannot be demonstrated other than through intervention studies (e.g., clinical trials). One clinical trial, assessing combined treatment with rituximab and belimumab, observed a lowered capacity of serum to induce NET concomitant with reduction in autoantibody titers⁴⁴. Though the authors reported not actual levels of circulating NET in the patients but rather serum-mediated NET induction *in vitro*, the study supports the model of IC-driven NETosis in SLE. It will be important to determine the effect of other therapies, including

Bruton tyrosine kinase inhibitors (known to be involved in both TLR and FcgRIIA signaling)⁴⁵, on NET levels in future clinical trials.

Not only did NET levels reflect a propensity of patients with SLE to develop a high SLEDAI, they were more prominent in SLE patients with a severe disease phenotype involving nephritis and CVD, in particular arterial thrombosis. Of note, the association between NET and nephritis was observed in patients only during LDA. Though we were unable to find any direct correlation between SLEDAI and levels of NET, our results suggest that processes operating in active disease, such as IC-mediated neutrophil activation, may affect levels of NET and mask the association between baseline NET levels and nephritis. The association with nephritis is of further interest given our prior findings on calprotectin as a useful marker to monitor treatment efficiency in patients with LN²⁴. Consistent with a role of NET in LN pathogenesis, infiltrating neutrophils undergoing NETosis have been found in the kidneys of patients with SLE⁸, and serum levels of NET are particularly high in patients with LN⁴². Of note, NET inhibition reduces nephritis development in SLE-prone mice, clearly suggesting an important role of NET in this process^{2,16}.

Finally, we made the novel observation that levels of NET were associated with CVD, in particular arterial thrombosis. NET have been recognized as pro-atherogenic and prothrombotic in their interaction with, and activation of, platelets and endothelium^{35,43,46,47,48}. Further, released NET and NET-containing enzymes (e.g., MPO) oxidize high-density lipoprotein to accelerate atherosclerotic processes³⁷. Though mice develop atherosclerosis and thrombosis in a NET-dependent manner^{16,17,46}, to our knowledge our study is the first to identify an association between CVD and NET in human SLE. However, in particular when studying CVD, we are limited by a low number of events in our cohorts. Further studies are warranted to validate our findings, as well as to determine whether levels of NET may be predictive of organ damage, including nephritis and CVD.

Using several well-characterized SLE cohorts, we have demonstrated the presence of true NET in plasma from patients with SLE. This observation has significant clinical implications and importance because we showed that the presence of NET identifies patients prone to having HDA, as well as to developing severe IC-mediated nephritis and CVD, 2 main morbidities of SLE. Our findings provide a model by which NET are induced by circulating nucleic acid-containing IC, deposited in target organs, including the kidney, and induce organ damage. These observations may allow for early interventions and preventive treatment to reduce overall morbidity and mortality in patients with SLE.

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ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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