# Neutrophil extracellular traps identify patients at risk of increased disease activity and cardiovascular comorbidity in systemic lupus erythematosus

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**Running head:** NETs in SLE

**ABSTRACT**

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

**Methods:** Levels of NETs (MPO-DNA complexes) were analyzed in plasma from four cross-sectional SLE cohorts (n=44-142), one longitudinal SLE cohort (n=47), and healthy individuals (n=100) using ELISA. Type I interferon (IFN) activity was determined using a cell reporter system.

**Results:** SLE patients had elevated levels of NETs 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 NETs were associated with *future* increase in SLEDAI within three months (OR=1.75, $p=0.01$), as well as an overall heightened SLEDAI over one year ($p<0.01$). Finally, levels of NETs were associated with arterial events (OR=5.0, $p=0.02$) and endothelial cell activation ($p<0.001$).

**Conclusion:** NET levels are elevated in SLE patients, 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, which may allow for early interventions.

## INTRODUCTION

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 (NETs). 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. (1-6) Though NET formation is beneficial from a host-pathogen perspective, impaired NET formation (or clearance) has been implicated in inflammation and autoimmunity, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). (2, 3, 7-9) In SLE, NETs have been suggested to be an important source of autoantigens, with SLE patients having high levels of antibodies binding to NETs, including anti-dsDNA and anti-histone antibodies. (10) The released NETs engage DNA receptors TLR9 and cGAS to induce type I interferons (IFNs), key cytokines in SLE pathogenesis. (2, 7, 11, 12) The relevance of NETs in SLE, however, is uncertain as evidence from lupus-prone mice are conflicting, with NET inhibition either ameliorating or worsening lupus-like disease. (13-17) Even in SLE patients, there are limited data to support a role of NETs in the disease pathogenesis. Early investigations have demonstrated the presence of an inflammatory neutrophil sub-population, low-density granulocytes (LDGs), enriched in SLE patients and capable of undergoing spontaneous NETosis *ex vivo*. (18) In addition, immune complexes (ICs), commonly found in SLE patients, induce NETs in an FcgRIIA- and TLR8-dependent manner *in vitro*. (2, 7, 19) Further, SLE patients have reduced capacity to degrade NETs, due 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 NETs in SLE patients. Prior work have found NET-

forming neutrophils to infiltrate both skin and kidney of SLE patients. (8) We recently made the novel observation of elevated levels of circulating NETs in SLE patients. (2) However, surprisingly, so far, nothing is known about the clinical implications of NETs in SLE, and whether levels of NETs could be useful in monitoring disease activity and/or identifying a more severe disease phenotype, including nephritis.

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

## MATERIALS AND METHODS

*Patient cohorts*

Four cross-sectional SLE cohorts were recruited at two separate sites; University of Washington, Seattle, USA (n=44, Cohort I) 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 cardiovascular morbidity and mortality. Cohort III and Cohort IV include the same patients at time-points 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), was recruited at Skane University Hospital, Lund, Sweden, consisting of patients followed longitudinally every 3 months for more than 2 years. To identify biomarkers of disease progression, one sample per patient was selected from the longitudinal cohort at a time-point of clinical remission (SLEDAI=0). The samples were selected as to enrich for worsening of disease (n=33) within three months of the clinical visit as compared to patients remaining in remission (n=14). On average, patients had been in remission for 6 months prior to analyzing the levels of NETs (Supplemental Table 2). Healthy controls (n=100) were recruited at Skane University Hospital, Lund, Sweden. 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 Supplemental Tables 1 and 2. The Swedish patient cohorts have been described in great detail previously. (21-27) Myocardial infarction, cerebrovascular incidents, angina pectoris, claudication intermittens, deep venous thrombosis or pulmonary embolisms were defined by the SLICC/ACR Damage Index. (28) Arterial events were defined as either myocardial infarction,

cerebrovascular incidents, angina pectoris or claudication 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 NETs, a 96-well microtiter plate (Corning) was coated with a mouse monoclonal anti-MPO antibody (4 μg/mL, Biorad, clone 4A4) overnight at 4°C, followed by blocking with 1% bovine serum albumin (BSA) in PBS for 2 hours at room temperature. After blocking, plasma samples (10%) were added and incubated overnight at 4°C. For detection, anti-dsDNA-HRP antibody (diluted 1/100, Roche Diagnostic) was added for 2 hours, room temperature. The reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB, 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 NETs were used as a standard curve with 1 U/mL equaling NETs released by 10,000 neutrophils.

*Type I Interferon assay*

Type I IFN activity was measured as previously described assessing the capacity of circulating type I IFNs to signal through IFNAR. (10, 29, 30) Briefly, WISH cells were cultured with patient serum (50%) and analyzed for induction of six IFN-regulated genes (*LY6E, MX1, OAS1, ISG15, IFIT1,*

*EIF2AK2*) and three house-keeping 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 as compared to healthy controls.

*Immune complex assay*

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

*Low density granulocytes*

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

*Endothelial microparticles*

For detection of endothelial microparticles (EMPs), flow cytometry was performed directly on heparinized platelet-poor plasma. (31) MP gating was accomplished using 1 μm beads for setting upper limits in both forward and side scatter. EMPs 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's 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 cut-off for positivity, the 99$^{th}$ percentile of the healthy controls was used. GraphPad Prism and IBM SPSS were used for the analyses. All analyses were considered statistically significant at $p<0.05$.

**RESULTS**

*SLE patients have elevated levels of circulating NETs*

Levels of NETs (MPO-DNA complexes) were analyzed by an in-house ELISA in plasma samples from four cross-sectional SLE cohorts spanning patients from the US and Sweden with varying degree of disease activity. As illustrated in Figure 1A, we confirmed that SLE patients had elevated levels of NETs as compared to healthy individuals ($p<0.01$ for all analyses, adjusted for multiple comparisons). Of interest, levels of NETs 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 NETs were not related to treatment with hydroxychloroquine or immunosuppressive drugs, including prednisone, other than in cohort II where patients on prednisone had slightly elevated levels of NETs ($p=0.02$, data not shown).

SLE patients had increased frequency of an inflammatory neutrophil sub-population, LDGs ($p<0.0001$, Figure 1B). However, levels of LDGs did not correlate with levels of NETs ($r=0.04$, $p=0.63$, Figure 1C). Thus, it is unlikely that LDGs account for the overall increase in circulating NETs in SLE patients. Instead, levels of ICs correlated with levels of NETs in all four SLE cohorts (Figure 1D, and Supplemental Figures 1A-C), suggesting that IC-mediated NETosis occurs *in vivo* in SLE patients.

For one of the cohorts (II), SLE patients with elevated levels of NETs (NET High) had increased type I IFN score ($p=0.02$, Figure 1E). However, this was not reproducible in the other two cohorts (Supplemental Figures 1D-E). As such, NETs are likely not the sole inducer of type I IFNs in SLE.

*Levels of calprotectin, but not NETs, associate with current disease activity*

No correlation was observed between levels of NETs and SLEDAI in either of the four cohorts (Figures 2A-D), nor did levels of NETs 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 SLE patients. Though calprotectin is an important component of NETs, 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 NETs (data not shown), suggesting that the majority of calprotectin was not NET-derived.

*Levels of NETs are associated with higher SLEDAI within three months*

Levels of NETs 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 three months. The patients developing higher SLEDAI had a history of more active disease with less time in remission (p=0.03, Supplemental Table 1) and requiring more prednisone use (p=0.009, Supplemental Table 1) as compared to patients remaining in remission within three months. Interestingly, we found that patients who developed higher SLEDAI within three months had elevated levels of NETs 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 odds ratio of 1.75 (1.13-2.73, p=0.01, Table 1). Patients with elevated levels of NETs at baseline, i.e. in remission, had an overall more active disease phenotype with higher SLEDAI within three 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, NETs may be a marker of subclinical disease activity, identifying patients at risk of developing active disease.

*Levels of circulating NETs identify patients with nephritis*

We observed significantly higher levels of NETs in patients with a history of nephritis in patients with low disease activity (Cohorts I-III), but not in patients with high disease activity (Cohort IV, Figure 4A, and Supplemental Figures 2A-C). Further, SLE patients with anti-C1q autoantibodies,

commonly associated with nephritis, had higher levels of NETs in patients with low disease activity (Cohorts II-III), but not in patients with high disease activity (Cohort IV), compared to SLE patients without anti-C1q antibodies (Figure 4B and Supplemental Figure 2D-E). Using logistic regression analysis, we demonstrated that in patients with low disease activity (Cohorts II and III), but not in patients with active disease (Cohort IV), increased levels of NETs associated with history of nephritis, independent of presence of either anti-dsDNA or anti-C1q antibodies (Table 1). Thus, elevated levels of NETs, at time-point of low disease activity, identified patients with a lupus nephritis phenotype.

*Levels of circulating NETs associate with arterial thrombosis*

As illustrated in Table 2, levels of NETs were selectively associated with arterial events (OR=5.04, p=0.01), but not venous events (OR=0.42, p=0.43, Table 2), suggesting a distinct mechanism by which NETs affect only arterial vessels. There were too few events to further stratify the patients based on individual arterial events, e.g. myocardial infarction, stroke, angina pectoris and claudicatio intermittens. SLE patients with elevated levels of NETs (NET+), had increased presence of endothelial microparticles (EMP), relative to NET- patients ($p<0.001$, Figure 4C). Moreover, levels of EMPs correlated with levels of circulating NETs in SLE patients ($r=0.34$, $p<0.0001$, Figures 4D). Together, these data demonstrate a link between endothelial damage and NETs, which could be relevant to the pathogenesis of atherosclerosis and arterial thrombosis in SLE. Further studies are warranted to study potential predictive value of NETs in the development of cardiovascular disease.

**DISCUSSION**

Neutrophils have for 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 SLE patients, e.g. the lupus erythematosus (LE) cell. (41) More recently, we have come to recognize that the engulfed ICs are prominent inducers of NETosis and subsequent release of inflammatory and immunogenic material. (2, 7, 19) However, albeit important observations on the role of NETs in lupus-prone mice, (2, 16, 17, 39) the inflammatory properties of NETs *in vitro*, (2, 7, 12, 37) as well as the presence of NETs in SLE patients, (2, 8) the *clinical significance* of NET formation in human SLE has not been addressed. Further, evidence from lupus-prone mice are conflicting, with NET inhibition either ameliorating or worsening lupus-like disease. (13-17) Though NET components, such as cell-free DNA and MPO, have been observed in the circulation of SLE patients, surprisingly few studies have assessed levels of *actual* NETs in SLE patients. Recent studies by Villanueva and colleagues elegantly found NET-forming neutrophils in the skin and the kidneys of SLE patients. However, they did not assess levels of circulating NETs. (8) In our original study, we reported presence of NETs in a single SLE cohort. (2) Since then, a recent study has evaluated levels of NETs in serum samples, (42) 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 NETs in SLE.

In the current investigation, we assessed *true* levels of NETs in plasma samples from four well-characterized cross-sectional SLE cohorts, as well as one longitudinal cohort, in an effort to determine the clinical significance of NETs in SLE patients. Using four cohorts, we were able to confirm that levels of NETs were markedly elevated in SLE patients as compared to healthy individuals. Of note, similar to our prior observation on calprotectin, (24) levels of NETs were elevated in SLE patients even at time-point of low disease activity. The occurrence of neutrophil activation and cell death even at low disease activity 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 activity, (24) levels of NETs were surprisingly not associated with *concurrent* disease activity. Instead, levels of NETs associated with worsening of disease within three 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. As such, NET formation may be an ideal therapeutic target, inhibiting disease at an early stage. Though NET inhibition has shown effect in lupus-prone mice, (2, 16, 17, 39) the effect of NET inhibition in humans is still under investigation.

Several candidates have been put forward as potential contributors to the elevated levels of NETs observed in SLE patients. Early work by Kaplan's group demonstrated a high prevalence of LDGs in SLE patients, with LDGs undergoing spontaneous NET formation upon *ex vivo* isolation. (18) Though we could verify an increased prevalence of LDGs in SLE, their levels did not

correspond to levels of circulating NETs. We have recently demonstrated that ICs have the capacity to induce NETs in an FcgRIIA-, TLR8- and mitochondrial ROS-dependent mechanism, (2, 19) generating mitochondrial-enriched NETs promoting type I IFN induction through the cGAS-STING pathway. (2) Consistent with these studies, we found a strong correlation between levels of NETs and those of ICs, suggesting that IC-mediated NETosis, and subsequent inflammation, may also occur in SLE patients. However, given the study design, causality can not be demonstrated other than through intervention studies, e.g. clinical trials. A recent clinical trial, assessing combined treatment with Rituximab and Belimumab, observed a lowered capacity of serum to induce NETs concomitant with reduction in autoantibody titers. (44) Though the authors did not report actual levels of circulating NETs 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 btk 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 SLE patients to develop a high SLEDAI, they were more prominent in SLE patients with a severe disease phenotype involving nephritis and cardiovascular disease, in particular arterial thrombosis. Of note, the association between NETs and nephritis was only observed in patients during low disease activity. Though we were unable to find any direct correlation between SLEDAI and levels of NETs, our results suggest that processes operating in active disease, such as immune complex-mediated neutrophil activation, may affect levels of NETs 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 lupus nephritis patients. (24) Consistent with a

role of NETs in lupus nephritis pathogenesis, infiltrating neutrophils undergoing NETosis have been found in the kidneys of SLE patients, (8) and serum levels of NETs are particularly high in lupus nephritis patients. (42) Of note, NET inhibition reduces nephritis development in lupus-prone mice, clearly suggesting an important role of NETs in this process. (2, 16)

Finally, we made the novel observation that levels of NETs were associated with cardiovascular disease, in particular arterial thrombosis. NETs have been recognized as pro-atherogenic and pro-thrombotic in their interaction with, and activation of, platelets and endothelium. (35, 43, 46-48) Further, released NETs, and NET-containing enzymes, e.g. MPO, oxidize HDL to accelerate atherosclerotic processes. (37) Though mice develop atherosclerosis and thrombosis in a NET-dependent manner, (16, 17, 46) our study is the first to identify an association between cardiovascular disease and NETs in human SLE. However, in particular when studying cardiovascular disease, we are limited by low number of events in our cohorts. Further studies are warranted to validate our findings, as well as determine whether levels of NETs may be predictive of organ damage, including nephritis and cardiovascular disease.

In all, using several well-characterized SLE cohorts, we have demonstrated the presence of *true* NETs in plasma from SLE patients. This observation has significant clinical implications and importance as we showed that presence of NETs identifies patients prone to having high disease activity, as well as to developing severe IC-mediated nephritis and CVD, two main morbidities of lupus. Our findings provide a model by which NETs are induced by circulating nucleic acid-containing ICs, deposit 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 SLE patients.

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**FIGURE LEGENDS**

**Figure 1. Neutrophil extracellular traps (NETs) in SLE patients.** A) Levels of NETs were analyzed in healthy controls (HC, n=100) as well as four 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 99$^{th}$ percentile of the HC, used to define patients with elevated levels of NETs. B) Percentage of low-density granulocytes (LDGs) in HC and SLE patients as determined by flow cytometry. C) Correlation between levels of NETs and LDGs. D) Correlation between levels of NETs and levels of immune complexes (ICs) in SLE cohort II. E) Type I IFN activity in SLE cohort II with or without elevated levels of NETs. Statistical analyses were done with Mann-Whitney U test, Wilcoxon, and Spearman's correlation with * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

**Figure 2. Levels of NETs are not associated with disease activity in SLE.** A-D) Correlation between SLEDAI and levels of NETs in SLE cohort I-IV, respectively. E) Correlation between levels of calprotectin and SLEDAI in Cohort II. Statistical analyses were done with Mann-Whitney U test and Spearman's correlation with * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

**Figure 3. Levels of NETs can predict disease activity in SLE patients.** A) Levels of NETs were analyzed at time-point of remission in Cohort V, with patients stratified based on their clinical status three months later, e.g. remaining in remission or developing increased disease activity. The dotted line represents the 99$^{th}$ percentile of the healthy controls. B-C) Patients where stratified based on low or high levels of NETs. B) SLEDAI at the three-month follow-up visit based on NET stratification. C) Average SLEDAI for the one-year follow-up period based on NET stratification. Statistical analyses were done with Mann-Whitney U test with ** $p<0.01$, and *** $p<0.001$.

**Figure 4. Levels of NETs are associated with history of nephritis and endothelial activation.** Levels of NETs were analyzed in SLE patients and related to A) history of nephritis and B) history of anti-C1q antibodies in Cohort II. C) SLE patients were stratified based on NET levels, and endothelial-derived microparticles (EMPs) analyzed by flow cytometry in SLE II. D) Correlation between levels of NETs and EMPs in SLE II. Statistical analyses were done with Mann-Whitney U test, and Spearman's correlation with * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

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

| Marker | Events | OR[1] | p-value | OR[2] | p-value[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

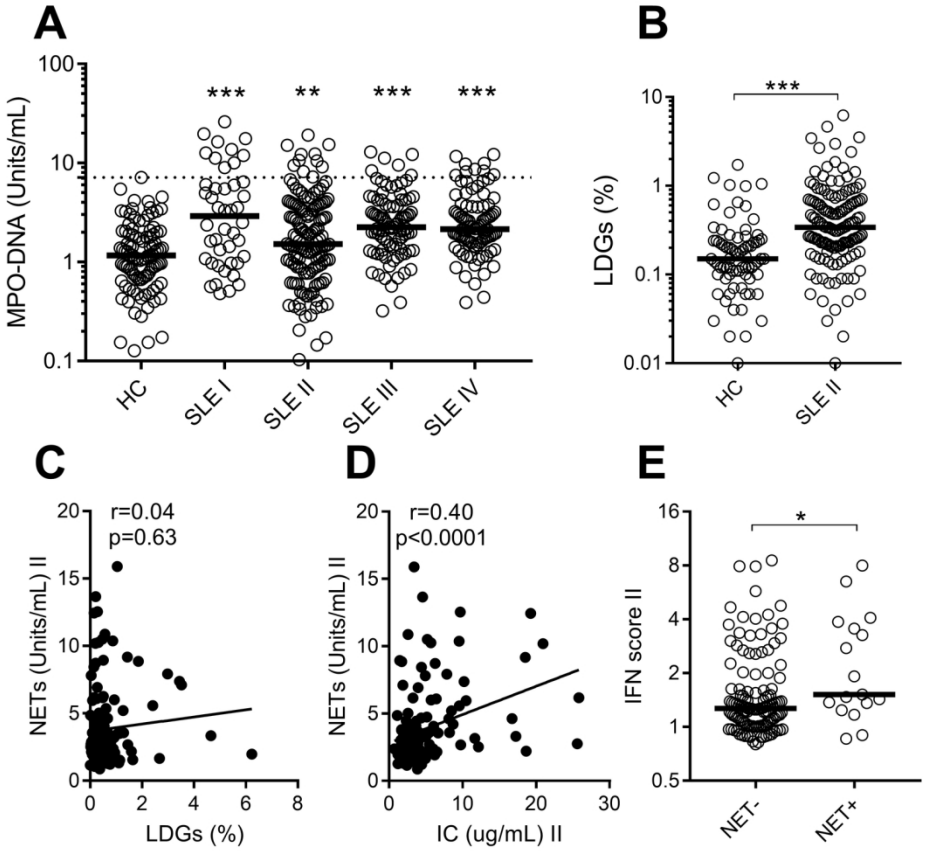
[2]Adjusted for anti-C1q and anti-dsDNA antibodies at time-point of blood draw

**Table 2. NET levels are associated with cardiovascular disease**

| Event | Sens. | Spec. | OR | p-value | OR[1] | p-value[1] |
|---|---|---|---|---|---|---|
| Arterial event | 27.3% | 90.8% | 3.72 (1.21-11.44) | 0.02 | 5.04 (1.31-19.37) | 0.02 |
| Venous event | 6.3% | 87.3% | 0.46 (0.06-3.71) | 0.47 | 0.37 (0.04-3.21) | 0.36 |

[1]adjusted for age, gender, dyslipidemia, hypertension, and smoking

[2]Arterial event includes myocardial infarction, stroke, claudicatio intermittens and angina pectoris.

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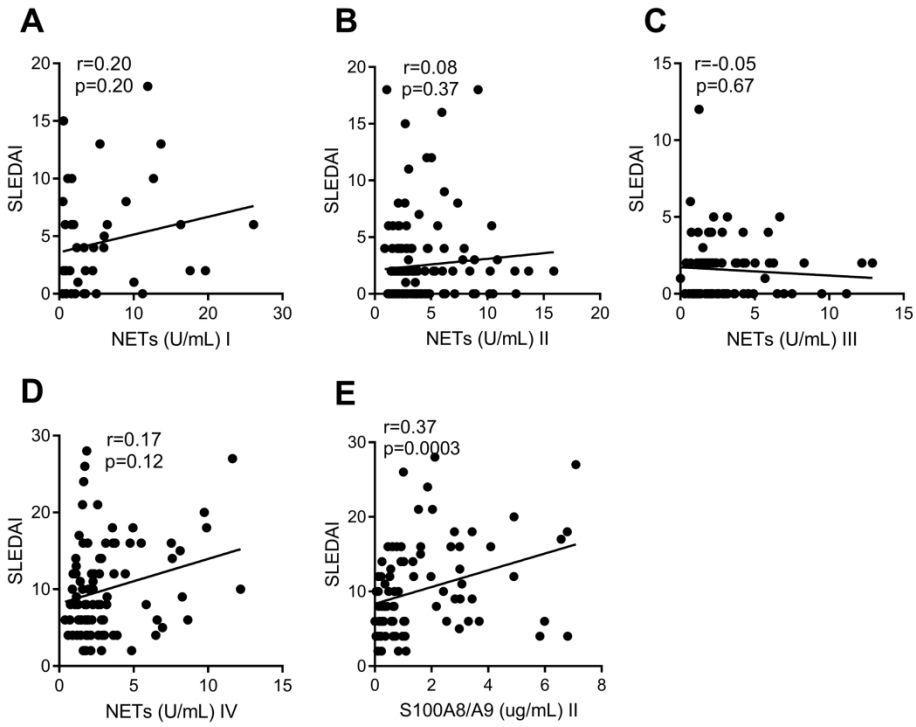


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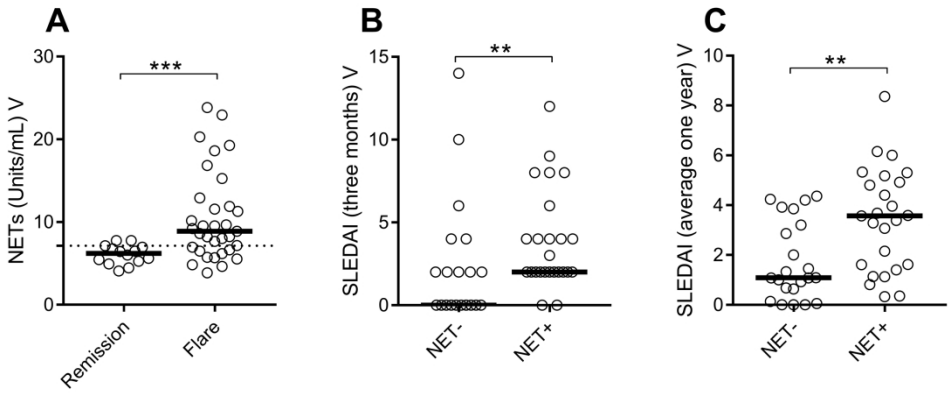


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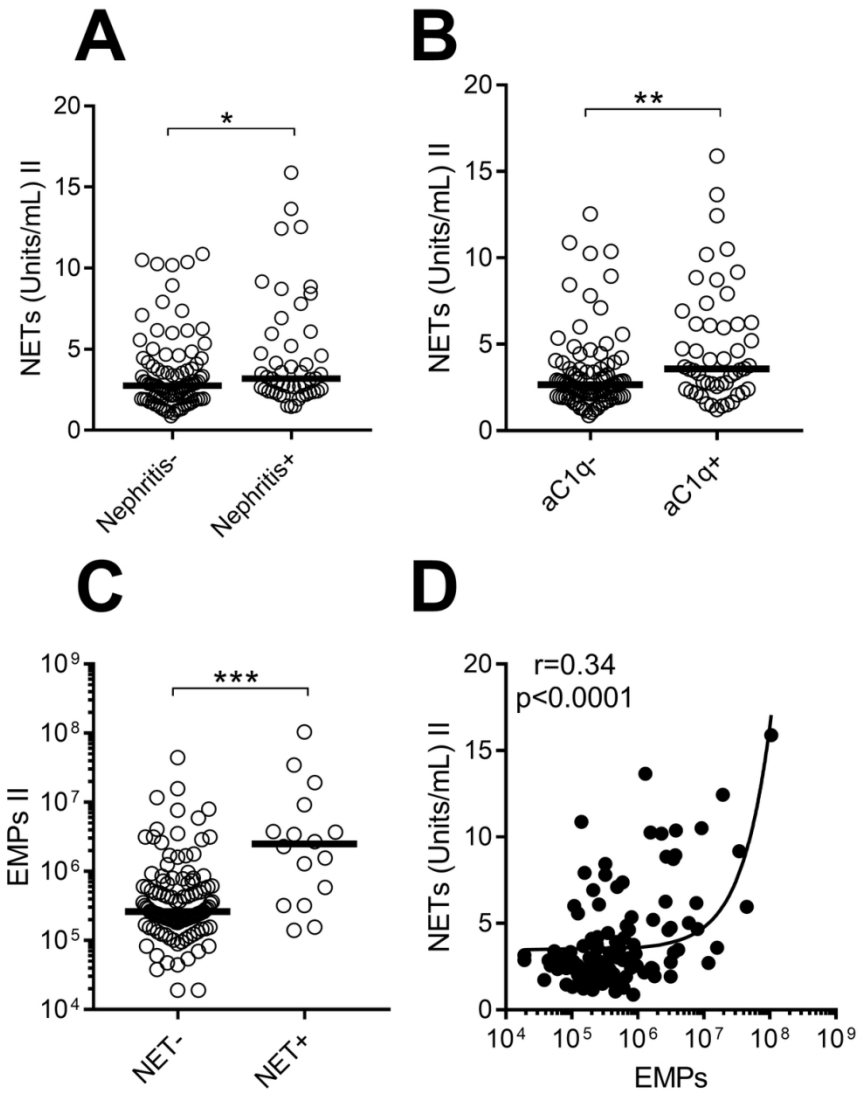


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118x140mm (300 x 300 DPI)