

Anti-neutrophil extracellular trap antibodies in autoimmune rheumatic diseases: a suitable biomarker of thrombosis in systemic lupus erythematosus

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Abstract

Objective

This case-control study aimed to elucidate the prevalence and role as biomarker of anti-neutrophil extracellular trap (anti-NET) antibodies in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and systemic sclerosis (SSc).

Methods

Serum samples from SLE, RA, SSc and healthy donors (HD) were evaluated for anti-NETs IgG, using an ELISA home-made coated with phorbol myristate acetate (PMA)-induced NET. Based on a positivity threshold set at the 99th percentile for HD sera, we calculated the positive samples.

Results

We enrolled 349 patients with autoimmune rheumatic diseases (ARD), finding a prevalence of anti-NETs of about 40%. Of the 136 SLE patients 50 (36.8%) were anti-NETs positive, revealing an association between anti-NETs and antiphospholipid syndrome (APS) (OR 3.37 [95% CI 1.22–9.36], $p=0.02$) and with arterial thrombosis regardless of coexisting APS (OR 5.52 [95% CI 1.07–28.52], $p=0.032$). In RA patients 52 out 131 (39.7%) tested positive for anti-NETs and we found a significant association between testing positive for anti-NETs and the presence of ACPA ($p=0.049$). Of the 82 SSc patients enrolled 33 (40%) were anti-NETs positive, and we found a direct correlation with CCL-18 (r 0.289 [CI 95% 0.04–0.50], $p=0.02$), a biomarker of worst prognosis and mortality in ILD-SSc patients.

Conclusion

In SLE patients, anti-NETs could have a role as a biomarker of thrombosis, a major cause of mortality in SLE.

Key words

anti-NET, systemic lupus erythematosus, thrombosis

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Received on September 23, 2025; accepted
 in revised form on January 14, 2026.

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 EXPERIMENTAL RHEUMATOLOGY 2026.

Introduction

In 2004, Brinkmann *et al.* described for the first time the ability of neutrophils to kill pathogens by releasing chromatin enriched with cytotoxic proteins (S100 calcium-binding proteins A8, A9, A12; actin and α -actinin) and nuclear proteins (especially histones) into the extracellular environment, creating neutrophil extracellular traps (NETs) (1). The release of intracellular material during NET formation – NETosis – has a role in breaking immunological tolerance to self-components.

During NETosis, several proteins are exposed to the extracellular environment, many of which can act as autoantigens, potentially disrupting self-tolerance and contributing to the development of autoimmune diseases. Several proteins identified in NETs – through immunofluorescence or proteomic analysis – are known targets of autoantibodies (*e.g.* citrullinated proteins, MPO, PR3, histones, LL-37, C1q) or can bind immune complexes (*e.g.* HMGB1, LL-37) (2).

Also, NET proteins can undergo post-translational modifications (PTM), a process well known for its role in autoimmunity. In fact, citrullinated, methylated and acetylated histones have been identified in NETs (2), and NET-associated histone 4 is a target of antibodies in Rheumatoid Arthritis (RA) (3).

When NETs are formed in an uncontrolled manner or are not cleared properly, the immune system might recognise NETs and trigger an autoimmune reaction against NET components, giving rise to anti-NET antibodies. In fact, in patients with systemic lupus erythematosus (SLE), RA, ANCA-associated vasculitis and antiphospholipid syndrome (APS), a subgroup of neutrophils with a higher propensity for spontaneous NETosis – low-density granulocytes (LDGs) – has been found (4). In addition, studies on the pathogenesis of SLE revealed that the reduced clearance of NETs could be the result of different mechanisms:

- polymorphisms in the gene coding for DNase I that cause reduced DNase I synthesis or activity (5),
- presence of DNase I inhibitors such as globular actin (6) or anti-DNase I antibodies (7)

- antibodies or the C1q fraction of complement that bind NETs and prevent their degradation (8).

In the context of the SLE, NETs are also able to: activate plasmacytoid dendritic cells, leading to the release of type I interferon (IFN-I); activate the NLRP3 inflammasome, promoting the release of IL-1 and IL-18; and facilitate and induce the production of IFN- γ , TNF, and IL-10 (9). Furthermore, a recent study on a pristane-induced mouse model revealed that NETosis may be a primary event triggering the development of lupus (10). Because limited information is available regarding the prevalence and the clinical significance of anti-NET in RA, SLE and systemic sclerosis (SSc) patients (11, 12), this study aimed to assess the prevalence of autoantibodies against NETs in SLE, RA and SSc patients and their potential role as a clinically relevant biomarker.

Material and methods

Patient population

The case-control study included consecutive patients with established RA, SLE and SSc diagnosed according to the latest American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria (13-15), attending outpatient clinics (Arthritis Centre, Lupus Clinic, Scleroderma Clinic) at Sapienza University of Rome between the years 2017 and 2023. Patients with ongoing infections and malignancy at the time of enrolment were excluded. The clinical data of the patients were reported in the electronic medical record. We enrolled healthy donors (HD) matched for sex and age with patients, and we excluded subjects with autoimmune-inflammatory disorders or with active infection or malignancy. Serum samples from patients and HD were collected and stored at -20°C until use. This study was approved by ethic committees of Sapienza University of Rome and participants gave written informed consent.

Human neutrophil purification and NETs isolation

Neutrophils were isolated from HD undergoing peripheral venous blood sampling. Blood treated with 2 nM ethey-

Competing interests: none declared.

lenediaminetetraacetic acid (EDTA) was stratified on Histopaque 1119/1077 and centrifuged. Neutrophils, separated by gradient, were collected, and washed with phosphate buffered saline (PBS) and resuspended in Roswell Park Memorial institute (RPMI) medium at a concentration of 3×10^6 neutrophils/mL. Neutrophils were incubated at 37°C , 5% CO_2 for 3h with phorbol myristate acetate (PMA) (100nM). The NETs were isolated and digested as described in our recent study in which we also performed protein analysis of PMA-induced NETs by mass spectrometry (16).

NETs were digested with micrococcal nuclease. The efficiency and consistency of digestion were verified by agarose gel electrophoresis. Following digestion, both DNA and protein concentrations were quantified, and were found to be within the expected ranges (DNA: 0.6–1.1 μg per million neutrophils; protein: 3–6 μg per million neutrophils). DNA and protein concentrations of the digested NET preparations were quantified using a NanoDrop spectrophotometer and was used to normalise the NET coating concentration at 5 $\mu\text{g}/\text{mL}$ across experiments.

Anti-NET IgG enzyme-linked immunosorbent assays (ELISA)

A high-binding 96-well EIA/RIA plate was coated overnight at 4°C with NETs diluted to a concentration of 5 $\mu\text{g}/\text{mL}$ in 0.05 M bicarbonate buffer. The plates were blocked with 4% bovine serum albumin (BSA) for 2 hours at room temperature. Then, serum samples were diluted 1:100, added to the plate and incubated for 2 hours. The plate was washed 3 times with wash buffer and then incubated for 90 minutes at 37°C with anti-human IgG-HRP (Invitrogen Goat anti-Human IgG Cross-Adsorbed Secondary Antibody, HRP no. 62-8420) diluted 1:10,000 in blocking buffer.

The plate was washed 3 more times with wash buffer and was incubated with HRP Substrate Solution (Luminata Forte ELISA Substrate) and optical density measured at 450 nm. Each sample was tested against a corresponding control in which no NETs antigen was plated. This created an individual back-

ground value for each sample, which was subtracted to obtain the result.

We performed serial dilutions of a positive control and confirmed a clear concentration-response relationship within the linear range of the instrument. The limit of detection (LOD), calculated as the mean blank OD plus three times the standard deviation, was exceeded by all positive samples. Intra-assay variability was low, with coefficients of variation of 2.0% for SLE, 1.6% for SSc, and 1.2% for RA.

Antibodies against carbamylated proteins (anti-CarP) ELISA

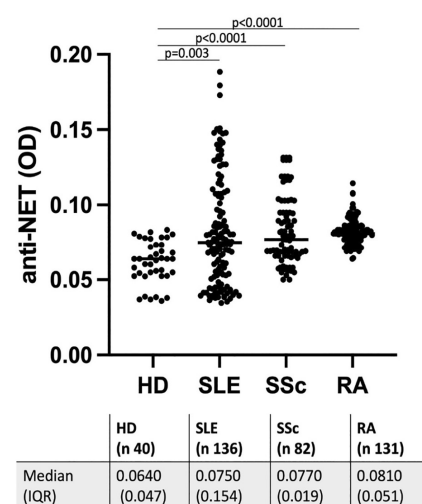
Anti-CarP antibodies were detected in RA sera by a modified solid phase 'home-made' ELISA as we described previously (17).

Statistical analysis

The distribution of continuous variables was analysed by Shapiro-Wilk test. Data are expressed as mean $\pm$ standard deviation or median (interquartile range - IQR) according to the variable distribution. χ^2 test or Fisher's exact test was used to compare categorical variables. To assess differences between continuous variables among the groups, Student's t-test or Mann-Whitney U-test were performed according to data distribution. We performed a univariate logistic regression to examine the relationship of anti-NET results with clinical manifestations and laboratory findings. In the multivariate logistic regression, we adjusted the results for demographic variables (age, sex) and disease duration. The Spearman rank correlation was performed. p -values < 0.05 were considered statistically significant. Data analysis was performed using SPSS v. 20 (SPSS Inc., Chicago, IL, USA).

Results

We enrolled 349 patients with autoimmune rheumatic diseases (ARD) with a mean age of 53 years (SD 14.6) at the time of sampling and with a female predominant ratio of approximately 6:1. Among all patients assessed (136 SLE, 131 RA and 82 SSc), anti-NETs OD were significantly higher than in 40 HD. Additionally, within the patient groups, the levels of anti-NETs were



Anti-NET IgG were measured in Systemic Lupus Erythematosus (SLE), Systemic Sclerosis (SSc), Rheumatoid Arthritis (RA) and healthy donor (HD). Levels of anti-NET IgG at 450-m optical density (OD) were compared between the groups.

Fig. 1. Levels of anti-NET in autoimmune rheumatic disease (ARD) patients and healthy donors (HD).

significantly higher in RA patients than in those with SLE ($p=0.029$) (Fig. 1).

Based on a positivity threshold set at the 99th percentile for HD samples, 136 ARD patients out of 349 (39%) tested positive for anti-NETs IgG.

Of the 136 SLE patients enrolled (Table I), 125 were females, with a mean age of 46 years (SD 12.6) and a median disease duration of 13 years (25th–75th percentile 8–23.5); 50 (36.8%) were anti-NET positive. Positivity for anti-NET was associated with APS (odds ratio (OR) 3.43 [CI 95% 1.25–9.42] $p=0.013$). Indeed, the levels of anti-NET in SLE patients with APS were higher than in SLE without APS (median 0.088 OD [IQR 0.11] vs. median 0.074 OD [IQR 0.15], respectively; $p=0.02$).

Anti-NET positivity was also associated with arterial thrombosis (Fig. 2), regardless of coexisting APS (OR 5.52 [95% CI 1.07–28.52], $p=0.032$). In adjusted models for age, sex, and disease duration we found that positive testing for anti-NET was significantly associated with APS (OR 3.37 [95% CI 1.22–9.36], $p=0.02$). However, anti-NET did not correlate with C3, C4, anti-dsDNA, antiphospholipid antibodies levels, and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI).

One hundred and thirty-one RA patients were enrolled (104 females), with a mean age of 55 years (SD 14)

Table I. Clinical and demographic features of systemic lupus erythematosus patients.

Clinical and demographic features	SLE n=136
Age (years), mean (SD)	46 (12.6)
Female, n (%)	125 (91.9)
Disease duration (years), median (25 th -75 th percentile)	13 (8-23.5)
SAPS, n (%)	19 (14)
SLEDAI mean (SD)	1.4 (2.1)
Neurological manifestations, n (%)	6 (4.4)
Cutaneous manifestations, n (%)	77 (56.6)
Renal manifestations, n (%)	35 (25.7)
Cytopenia, n (%)	54 (39.7)
Serositis, n (%)	19 (14)
Arthritis, n (%)	84 (61.8)
Thrombosis, n (%)	17 (12.5)

SD: standard deviation; SAPS: secondary antiphospholipid syndrome; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

Table II. Clinical and demographic features of rheumatoid arthritis patients.

Clinical and demographic features	RA n=131
Age (years), mean SD	55 (14)
Female, n (%)	104 (79.4)
Disease duration (years), median (25 th -75 th percentile)	5 (2-15)
Rheumatoid factor, n (%)	72 (55)
ACPA, n (%)	78 (59.5)
aCarP, n (%)	16 (12.2)
DAS28 _{ESR} at enrolment, median (25 th -75 th percentile)	3.6 (2.6-4.8)
Interstitial lung disease, n (%)	25 (19)

SD: standard deviation; ACPA: anti-citrullinated peptide antibodies; aCarP: anti-carbamylated proteins; DAS28_{ESR}: Disease Activity Score 28 calculated with erythrocyte sedimentation rate.

Table III. Clinical and demographic features of systemic sclerosis patients.

Clinical and demographic features	SSc n=82
Age (years), mean (SD)	61.8 (13.4)
Female, n (%)	73 (89)
Disease duration (years), median (25 th -75 th percentile)	10 (4-14)
dcSSc, n (%)	35 (42.7)
lcSSc, n (%)	47 (57.3)
Digital ulcers, n (%)	24 (29.3)
Puffy fingers, n (%)	28 (34.1)
Sclerodactyly, n (%)	42 (51.2)
Interstitial lung disease, n (%)	55 (67)
GERD, n (%)	41 (50)
Scleroderma pattern early, n (%)	11 (13.4)
Scleroderma pattern active, n (%)	22 (26.8)
Scleroderma pattern late, n (%)	30 (36.6)

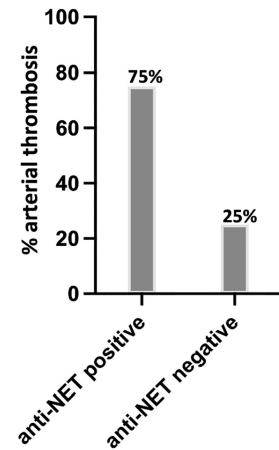
SD: standard deviation; dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc; GERD: gastroesophageal reflux disease.

and a median disease duration of 5 years (25th-75th percentile 2–15) (Table II). Anti-NETs resulted positive in 52 out 131 (39.7%) RA patients.

We tested RF, ACPA and anti-CarP in all RA samples, revealing positivity in 72 (55%), 78 (59.5%) and 16 (12.2%) patients, respectively. A great majority of patients (88.6 %) with positive anti-NET test were ACPA-positive. Consist-

ently, our results revealed a significant association between testing positive for anti-NET and the presence of ACPA ($p=0.049$). A significant difference in anti-NET OD was seen between seronegative (33/131) and seropositive patients (median 0.079 OD [IQR 0.04] vs. median 0.084 OD [IQR 0.05]; $p=0.04$, respectively).

No correlation was found between

**Fig. 2.** Percentage of anti-NET positivity in patients with systemic lupus erythematosus and arterial thrombosis.

anti-NET and RF, ACPA and anti-CarP serum levels and the Disease Activity Score calculated with erythrocyte sedimentation rate.

Of the 82 SSc patients enrolled (73 females), with a mean age of 61.8 years (SD 13.4) and a median disease duration of 10 years (25th-75th percentile 4–14) (Table III), 33 (40%) were anti-NET positive. To better understand the clinical significance of anti-NETs, we explored their association with clinical manifestations and capillaroscopy features. Our investigation revealed an inverse association between anti-NET and gastroesophageal reflux disease (GERD) ($p=0.01$). In patients with SSc we found an inverse correlation with Interstitial Lung Disease (ILD) duration ($r -0.362$ [CI 95% $-0.60 - -0.07$], $p=0.02$), therefore we assayed Chemokine (C-C motif) ligand 18 (CCL-18) in SSc sera, a biomarker associated to a worse prognosis and mortality in ILD-SSc patients (18), founding a correlation with anti-NET ($r 0.289$ [CI 95% $0.0 - 0.50$], $p=0.02$).

Discussion

In our study we found a high prevalence, almost 40%, of anti-NET antibodies in patients with ARD. Several studies provided evidence that NETosis could have a role in the pathogenesis of autoimmune diseases. Indeed, NETs can induce the release of interferon-alpha ($INF-\alpha$) from dendritic cells (19), activate macrophages to release proinflammatory cytokines (20), activate en-

endothelial cells and induce TF expression (16) and, notably, NET proteins can be targets of autoantibodies. We identified 56 proteins and the 3 highest-ranking terms in the biological processes are related to peroxidation, cell detoxification and glycolysis (16). Reactome and KEGG pathways with the most significant enrichment seen in pathways associated with the glycolysis, cell death, neutrophil degranulation and innate immunity. Processes that have a key role in NETosis, autoimmunity and thrombosis (16).

Recently, antibodies anti-AIM2 and anti-IFI16 binding NETs have been described in sera and renal biopsy of SLE patients (10). Also, histone 4 detected in NETs resulted as a target of antibodies in RA patients (5).

However, little is known about the prevalence and clinical significance of anti-NET in SLE. In particular, before this study, the prevalence of anti-NET in SLE had been tested only in small sample sizes (11, 12), and no data were available regarding their role as a biomarker. In our cohort of SLE patients, anti-NET positivity was associated with APS and arterial thrombosis, therefore representing a candidate biomarker of thrombotic events.

According to our results, anti-NET would appear to be an independent biomarker for arterial thrombosis. To this regard, a previous large study on aPL-positive patients, that excluded SLE patients, revealed an association between anti-NET IgG and brain white matter lesions, suggesting a possible role of anti-NET in endothelial dysfunction and thrombogenesis (21).

At present, it is not known whether anti-NETs play a pathogenetic role in thrombogenesis, our results and those of Zuo and colleagues (21) seem to support such hypothesis. Nevertheless, it is well known that NETosis has a role in thrombogenesis. Indeed, NETs are enriched in TF, chromatin activates coagulation factor XII and histones activate platelets. In addition, the neutrophil serine proteases of NETs degrade the tissue factor pathway inhibitor (TFPI), which is crucial for the inhibition of thrombogenesis (22). Furthermore, we demonstrated the ability of NETs to ac-

tivate the endothelium and induce TF expression (16).

SLE patients have a 2–10-fold increased risk of developing arterial thrombotic event, such as stroke and myocardial infarction, compared to the general population (23), therefore, a biomarker that can identify these patients may be helpful.

Considering that in SLE patients, LDGs are associated with vascular damage (24, 25), and an increased NET release by LDGs may contribute to the high cardiovascular morbidity in SLE, we might speculate that anti-NET can play a role in the pathogenesis of arterial thrombotic complications.

In this study, we showed that a substantial fraction of patients with RA are anti-NET positive, as demonstrated in a previous study (11). In RA patients, ACPA targets include autoantigens (such as filaggrin, fibrinogen, vimentin, collagen II and histones) that undergo citrullination mediated by the calcium-dependent enzyme peptidyl arginine deiminase (PAD) (26).

Based on the premise that PAD4 activation and citrullination play a crucial role in NETosis, indeed 15 of the 54 most common NET proteins identified by a proteomic approach presented citrullinated residues (27), moreover, citrullinated vimentin, histone 1, histone 3, and histone 4 were also detected on NETs using immunofluorescence (28). NET could potentially serve as a source of citrullinated proteins and promote the formation of ACPA and have a role in the pathogenesis of RA. It is not surprising that our results revealed that almost all anti-NET positive RA patients were ACPA positive, and that anti-NET are associated with ACPA.

Since anti-NET levels showed no correlation with ACPA levels and were detected in seronegative patients, this suggests that although anti-NET may be partially related to ACPA, they may also be directed towards other targets.

In our study we also found a high prevalence of anti-NET antibodies in patients with SSc. Of note, we observed an inverse correlation between anti-NET levels and ILD disease duration and a direct correlation between anti-NET and CCL-18 levels. CCL-18 is a rec-

ognised biomarker for ILD in SSc patients, associated to a worse prognosis and mortality (18). It is secreted by M2 macrophages, and elevated levels of this biomarker were identified in both the serum and bronchoalveolar lavage (BAL) of SSc patients (29). An analysis conducted on participants in the Scleroderma Lung Study II trial revealed that higher baseline values of CCL-18 were linked to a deterioration in respiratory function test results, despite treatment with mycophenolate (30).

Currently, the role of NETosis in the pathogenesis of SSc has been little explored (31, 32), but these results may open the path for investigating a possible role of anti-NETs in SSc-ILD, but further targeted studies are certainly needed.

In conclusion, anti-NET antibodies are remarkably prevalent in patients with ARD. In SLE patients, anti-NETs could have a role as a biomarker of thrombosis, a major cause of mortality in SLE.

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