Low density granulocytes and neutrophil extracellular trap formation are increased in incomplete systemic lupus erythematosus

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Low density granulocytes and neutrophil extracellular trap formation are increased in incomplete systemic lupus erythematosus

Running head: LDGs and NETs in iSLE

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ABSTRACT

Objective
To investigate the proportion of low-density granulocytes (LDGs), circulating plasma neutrophil extracellular traps (NETs), and serum-induced NET formation in patients with incomplete systemic lupus erythematosus (iSLE) and systemic lupus erythematosus (SLE).

Methods
LDGs were measured cross-sectionally in 18 iSLE patients, 11 SLE patients and 14 healthy controls (HCs), whereas circulating NETs and serum-induced NET formation were assessed in 35 iSLE patients, 41 SLE patients and 16 HCs. LDGs (CD14^low CD15^+) were measured in PBMCs using flow cytometry and circulating plasma NETs were measured using anti-myeloperoxidase-DNA, anti-citrullinated histone H3 and anti-elastase-DNA complex ELISAs. Serum-induced NET formation was assessed by incubating healthy neutrophils with serum from iSLE patients, SLE patients or HCs and visualizing NETs with fluorescence microscopy.

Results
Proportions of LDGs and circulating plasma NETs were similarly elevated in iSLE and SLE patients compared to those in HCs. Furthermore, patients under hydroxychloroquine (HCQ) treatment had lower proportions of LDGs than those without. Serum from iSLE and SLE patients similarly induced NET formation in healthy neutrophils. In iSLE patients, myeloperoxidase-DNA complexes were correlated with proportions of age-associated B-cells, memory B-cells and negatively with naïve B-cells, while we did not find associations between measures of NETs or serum-induced NET formation and interferon score or clinical parameters.

Conclusion
These results show that neutrophil dysfunction, including higher proportions of LDGs, and increased NET formation, already occur in iSLE, similar to SLE, despite differences in disease
manifestations. Thereby, neutrophil dysfunction may contribute to sustained exposure to autoantigens and autoreactivity in early stages of SLE.

**Keywords:** Systemic lupus erythematosus, incomplete systemic lupus erythematosus, low density granulocytes, neutrophil extracellular traps, innate immune dysfunction.
KEY MESSAGES

1) Proportions of LDGs and circulating plasma NETs were similarly elevated in iSLE and SLE patients.

2) Hydroxychloroquine-use in iSLE patients was associated with lower proportions of LDGs.

3) Serum from iSLE and SLE patients led to increased NET formation in healthy neutrophils.
INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease with heterogeneous manifestations that predominantly affects women. Neutrophil dysfunction has been identified as a major factor contributing to autoreactivity and tissue damage in SLE (1–3). Neutrophils are essential in immune responses against pathogens (1,2) and their mechanisms of defense include phagocytosis, degranulation, production of pro-inflammatory cytokines such as interferons, and neutrophil extracellular trap (NET) formation (1,2). During NET formation, nuclear and granule components which include DNA, RNA, and granular proteins such as myeloperoxidase, are expelled extracellularly into net-like structures that entrap and kill pathogens effectively (1,2). In SLE, NETs are produced independently of pathogens and their clearance is impaired (4–6). Components in serum from SLE patients, such as autoantibodies, are associated with increased NET formation (7,8). NET formation leads to increased autoantigen exposure, interferon production, autoreactive B-cell activation, autoantibody production and tissue damage (1,4,6,9–11).

A subtype of circulating neutrophils called low density granulocytes (LDGs) shows increased ability for NETosis and produces more proinflammatory cytokines than normal density granulocytes (12–15). Circulating LDGs are elevated and have been linked to disease activity in SLE patients (15–17). Furthermore, LDGs expressing the maturation marker CD10 seem to possess higher ability for NETosis and phagocytosis compared to less mature CD10- LDGs (15).

Given the important role of LDGs and NETs in SLE and other autoimmune diseases, new therapies to target LDGs and NETs are currently being developed (1,2,18).

Innate immune dysfunction plays a key role in the initiation of SLE, which is supported by high expression of interferon-related genes in incomplete SLE (iSLE) (19,20). iSLE refers to an early stage of the disease, characterized by the presence of SLE-related symptoms, which are not
sufficient for classification. However, up to 57% of iSLE patients will progress to SLE (21). We hypothesize that neutrophil dysfunction already occurs in patients with iSLE and contributes to sustained autoantigen exposure in this phase. Therefore, the aim of this study was to examine whether LDGs, especially CD10+ LDGs and NETs, are increased in iSLE and SLE patients compared to healthy controls (HCs), and whether iSLE serum can induce NETosis in healthy neutrophils. Furthermore, we tested whether circulating NETs and serum-induced NET formation were correlated with interferon signature, B-cell subsets, and clinical parameters, including treatment.
MATERIALS AND METHODS

Study Population

Two cross-sectional data sets from the longitudinal iSLE cohort study were analysed. This cohort study was conducted at the University Medical Centre in Groningen, from 2016 onwards. It was approved by the local ethics committee (METc 2015/313) and conducted in line with the declaration of Helsinki. Patients gave written consent prior to participation. For this study, iSLE patients, SLE patients and HCs were included. All iSLE patients had an ANA titre of ≥1:80 and at least one additional 2012 SLICC criterium (22), but not enough criteria to classify as SLE. Some iSLE patients were treated with hydroxychloroquine (HCQ), but none used other immunosuppressive medication. SLE patients were eligible if they met the 2012 SLICC criteria (22). Disease related information and participants’ demographics were recorded after routine blood measurements and clinical assessment by a physician. Disease activity was assessed with the systemic lupus erythematosus disease activity index (SLEDAI) (23). Given that most originally enrolled SLE patients had low disease activity, we also included 8 SLE patients with a SLEDAI >4 (METc 2014). All patients gave written consent prior to participation and the study was approved by the ethics committee in Amsterdam (VU medical centre; METc 2014/081).

Low density granulocytes

LDGs (CD14low CD15+) were measured in freshly isolated PBMCs with flow cytometry in cross-sectional samples from a subset of HCs, iSLE, and SLE patients that were analysed between 2021 and 2022. Briefly, blood was drawn into heparin tubes (BD), PBMCs were isolated with Ficoll density centrifugation and incubated with fluorescence labelled, antibodies targeting CD10, CD14 and CD15 (Supplementary Table S1). After 15 min of incubation in the...
dark, FACS lysing solution (eBioscience, Invitrogen) was added according to manufacturer’s instructions and incubated for 10 minutes. Flow cytometry analysis was performed on a Novocye Quanteon Flow Cytometer within 5 hours after blood collection. Single stains were used to correct for spectral overlap and fluorescent minus one staining to set gates. Data was analysed with NovoExpress software from Agilent. LDGs were identified as CD14\textsuperscript{low} and CD15\textsuperscript{+} as previously described (12) and expressed as percentage of PBMCs. LDGs were further gated into CD10\textsuperscript{+} and CD10\textsuperscript{−} cells (see Supplementary Figure S1 for gating strategy). Absolute cell counts were given per µl of PBMCs. To calculate the LDG-to-lymphocyte ratio, we gated lymphocytes based on the forward and side scatter and divided the absolute number of LDGs by the absolute number of lymphocytes.

**Free circulating plasma NETs**

Free circulating plasma NETs were measured as MPO-DNA complexes, citrullinated histone H3 (cit-H3) DNA complexes and elastase-DNA complexes in plasma with ELISA. Plasma was isolated from whole blood by centrifugation and stored at -80°C until further processing.

For this experiment, we adapted the recently published protocol by Matta et al (24). Plates were coated with 50µl of coating buffer (15mM Na\textsubscript{2}CO\textsubscript{3}, 35mM NaHCO\textsubscript{3}, pH 9.6) containing 2.5µg/ml of anti-MPO antibody (Abcam, Ab 25989), 5 µg/ml of anti-cit-H3 antibody (Abcam 5103), or 10 µg/ml anti-neutrophil elastase (Calbiochem, # 481001) and incubated overnight at 4°C. The plate was then washed and blocked with 5% BSA and 5% Normal Rat Serum (Thermo Fisher Scientific). After washing with PBS, 25µl of plasma and 25µl of 1% BSA in PBS was added in duplicate for every participant. After one hour of incubation, the wells were washed with 200µl of wash buffer (1% BSA and 0,05% Tween20 in PBS). Next, 50µl of anti-DNA POD detection antibody (Roche) diluted 1:200 was added and left to incubate for one hour. Subsequently, 100 µl of 3,3’, 5,5; -tetramethylbenzidine (TMB, Sigma-Aldrich) was added. To
stop the reaction, 100 µl of 2N H₂SO₄ was added after 15 minutes. The plate was then scanned at 450-575 nm. As internal standard for MPO-DNA complexes, a serial dilution of NET-containing supernatant of phorbol-12-myristate-13-acetate (PMA)-stimulated neutrophils from a healthy donor was used and values were given as arbitrary units (AU). Samples with values below the lower limit of detection (<0.15) were coded as 0.1. Cit-H3-DNA and elastase-DNA complexes were reported as OD values.

Ex-vivo serum-induced NETosis

NETosis induction was assessed with fluorescence microscopy after incubating freshly isolated neutrophils from one healthy donor with baseline serum samples from HCs, iSLE, and SLE patients. This experiment was based on the protocol published by Arends et al. (25). Neutrophils from one healthy donor were isolated from whole blood, drawn into EDTA tubes, (BD) with Ficoll density centrifugation (25). Ten million neutrophils were labelled with red fluorescent cell linker (PKH26, Sigma-Aldrich). Next, 10µl serum from HCs, iSLE and SLE patients was added in triplicate to 37,500 neutrophils per well in a 96-well plate and left to incubate for 4 hours at 37°C. As negative and positive control, we added PBS and 20 nM PMA. To visualize NETs, SYTOX green impermeable DNA dye (Gibco) was added and cells were fixed with formaldehyde (25).

Fluorescence signals were measured with a Zeiss Cell Discoverer 7 microscope at a 10x magnification using an PlanApochromat 20x/0.35-0.8 lens with autocorrection for the plate material, and images were subsequently acquired with an Axiocam 506 b/w widefield camera. For every sample, a total of 16 images was taken and merged using the Zeiss ZEN Blue Microscopy software. The surface area of neutrophils and NETs was determined after visual correction for background signal and scattered light, using the open-source imaging software
FIJI. We then calculated the amount of NET formation by dividing the acquired surface area of NETs by the surface area of neutrophils.

**Proportions of B-cell subsets**

Proportions of B-cells were analysed with flow cytometry in PBMCs from baseline as previously reported (26). B-cells were identified as CD19$^+$ and further subdivided into the following subsets: naïve B-cells (CD27$^-$, CD38$^-$), transitional B-cells (CD27$^+$, CD38$^+$), plasma blasts/plasma cells (CD27$^+$, CD38$^+$, IgD$^+$), memory B-cells (CD27$^+$, CD38$^+$), switched memory B-cells (CD27$^+$, CD38$^+$, IgM$^+$), non-switched memory B-cells (CD27$^+$, CD38$^-$, IgM$^+$, IgD$^-$), and age-associated B-cells (CD21$^+$, CD11c$^+$). Proportions of B-cells were expressed as a percentage of PBMCs and switched memory B-cells as a percentage of all memory B-cells.

**Interferon score**

Expression of twelve commonly measured interferon-related genes (CXCL10, IFI44L, IFIT3, LY6E, MX1, SERPING1, IFITM1, IRF7, STAT1, C1QA, IFI16, IRF9), was measured with qPCR in whole blood samples drawn into PAXgene RNA tubes (Qiagen) as previously described (19).

Interferon scores were then calculated based on the log transformed relative expression according to the following formula: $\sum(\text{RE}_{\text{subject}} - \text{Mean}_{\text{HC}})/\text{SD}_{\text{HC}}$.

**Autoantibody positivity**

Autoantibodies were measured in serum by automated fluorescence enzyme immunoassay (FEIA-Elia) on a Phadia 250 instrument (ThermoFisher Scientific, Nieuwegein, The Netherlands). Anti-dsDNA levels were considered positive above 15 IU/ml, anti-SSA values above 10 AU/ml and anti-Sm above 5AU/ml, according to the manufacturer’s instruction.
**Statistical Analysis**

For continuous variables, median and interquartile range (IQR) were reported while count and percentage are shown for categorical variables. Differences between continuous variables between all three groups were assessed with Kruskal Wallis tests. Pairwise comparison, using Wilcoxon rank sum tests, were performed in case of significance. Fisher’s exact test was used for comparing categorical variables. For correlation analysis, Spearman rank tests were applied. A two-sided p-value <0.05 was considered statistically significantly.

Study data were collected and managed using REDCap electronic data capture tools (27). For data analysis and graphics, we used R version 4.0.5 (28).
RESULTS

Cohort characteristics

Characteristics from 14 HCs, 18 iSLE and 11 SLE patients, for which LDGs were measured, are depicted in Table 1. Characteristics from 16 HCs, 35 iSLE and 41 SLE patients, that were analysed for free circulating NETs and serum-induced NETosis, are depicted in Table 2. Most iSLE and SLE patients were female and White European and had low disease activity (median SLEDAI of 1 for iSLE and 2 for SLE patients). HCQ was used by 61% of iSLE and 82% of SLE patients assessed for LDGs and by 23% of iSLE and 83% of SLE patients assessed for circulating NETs and NET formation. The individual SLICC criteria and SLEDAI items at time of blood collection are shown in Supplementary Table S2 and Supplementary Table S3.

Low density granulocytes

The proportion of LDGs (CD14\textsuperscript{low} CD15\textsuperscript{+}) was elevated in iSLE and SLE patients compared to HCs (p = 0.001 and p = 0.025) while there was no difference in proportion of LDGs between iSLE and SLE patients (p = 0.84; Figure 1A; Table 1). The same pattern was observed when looking at the absolute LDG count per µl of PBMCs Table 1).

Most LDGs were CD10\textsuperscript{+} in all groups. However, the median proportion of CD10\textsuperscript{+} LDGs was higher in iSLE patients (93%) than in HCs (82%) (p = 0.005; Figure 1B). There was no difference in the proportion of CD10\textsuperscript{+} LDGs between HCs and SLE patients or iSLE and SLE patients (HCs vs. SLE p = 0.58; iSLE vs SLE p = 0.18). In SLE patients, the proportion of LDGs correlated strongly with the proportion of CD10\textsuperscript{+} LDGs (r=0.82, p = 0.0018), indicating that patients with higher overall LDG proportions, had more CD10\textsuperscript{+} LDGs. The same trend was observed in iSLE patients but was not significant (r=0.43, p =0.076).
Low density granulocytes and clinical characteristics.

We limited our analyses to selected clinical characteristics given the relatively low number of patients per group. iSLE patients treated with HCQ had lower proportions of LDGs than patients who did not receive HCQ (p = 0.035; Figure 1C). We did not observe differences in LDG levels between iSLE and SLE patients with a history of cutaneous lupus or arthritis (Supplementary Figure S2A-B).

In addition, we assessed the LDG-to-lymphocyte ratio which has been proposed as a sensitive measure of disease activity in SLE (13). Both iSLE and SLE patients exhibited significantly elevated LDG-to-lymphocyte ratios compared to HCs (HCs vs. iSLE p = 0.015; HC vs. SLE p = 0.025, Supplementary Figure 2C).

Circulating NETs and serum-induced NETosis

MPO-DNA and elastase-DNA complexes were similarly elevated in iSLE and SLE patients compared to HCs (MPO-DNA HCs vs. iSLE p = 0.014, HCs vs. SLE p = 0.019 Figure 2A; elastase-DNA HCs vs. iSLE p = 0.004, HCs vs. SLE p = 0.009, Figure 2C). Levels of cit-H3-DNA complexes were increased in iSLE patients compared to both HCs and SLE, while there was no difference in cit-H3-DNA complexes between HCs and SLE patients (HCs vs. iSLE p = p < 0.0001, iSLE vs. SLE p <0.001 Figure 2B). There were no differences in levels of MPO-DNA, cit-H3-DNA, elastase-DNA complexes or serum-induced NET formation between SLE patients with or without a history of lupus nephritis (Supplementary Figure S3A-D). As disease activity was low in all but two SLE patients, we measured MPO-DNA complexes in eight additional samples of patients with high disease activity (SLEDAI >4) and stratified the data accordingly. SLE patients with active disease (n = 10) had more MPO-DNA complexes compared to iSLE and SLE patients with low disease activity (Supplementary Figure S3E).
Serum from iSLE and SLE patients led to more NET formation than serum from HCs (HCs vs. iSLE p = 0.006; HCs vs. SLE p < 0.001, Figure 3). There was no difference in NET formation between neutrophils stimulated with iSLE or SLE serum (p = 0.44).

**Correlation between circulating NETs, serum-induced NETosis and clinical and immunological parameters**

Serum-induced NETosis was correlated with MPO-DNA and cit-H3-DNA complexes in iSLE patients only (r = 0.4, p = 0.041; r = 0.39, p = 0.047). MPO-DNA complexes only, were negatively correlated with lymphocytes and C4 levels in SLE patients but not in iSLE patients (r = -0.45, p = 0.0041; r = -0.32, p = 0.04; Supplementary Figure S4). We did not find associations between MPO-DNA, cit-H3-DNA, elastase-DNA or serum-induced NET formation and age, interferon score, SLEDAI disease activity, classification criteria or anti-dsDNA antibodies in iSLE or SLE patients. There was no difference in free plasma NET measures or serum NET formation when stratifying iSLE and SLE patients for smoking or use of HCQ (Supplementary Figure S5).

MPO-DNA complexes in iSLE patients, were positively associated with the proportion of memory B-cells (r = 0.46, p = 0.019), proportion of age-associated B-cells (r = 0.49, p = 0.012) and negatively associated with the proportion of naïve B-cells (r = -0.4, p = 0.046; Supplementary Figure S4). There was no correlation between cit-H3-DNA, elastase-DNA complexes and B-cell subsets. Serum-induced NET formation in iSLE was correlated with switched memory B-cells (r = 0.39, p = 0.049). We did not observe correlations between measures of free plasma NETs or serum-induced NETosis and B-cell subsets in SLE patients.
DISCUSSION

In this cross-sectional study, the proportion of LDGs was similarly increased in iSLE and SLE patients compared to that in HCs. In addition, circulating plasma NETs were elevated to a similar extent in iSLE and SLE patients, and serum from iSLE patients, like SLE serum, induced more NETosis in healthy neutrophils than serum from HCs. Furthermore, there was no relation between circulating NETs, serum-induced NET formation, and interferon score in iSLE or SLE patients. These results imply that neutrophil dysfunction and impaired clearance of NETs already occur in early stages of SLE and might thereby contribute to sustained autoreactivity.

Several studies showed LDGs to be increased in SLE which is in line with our results (12–14,29). To our knowledge, this is the first study to show that LDGs are already elevated in iSLE to a similar degree as in established SLE. Mistry et al. described two different subsets of LDGs: CD10⁺ intermediate mature and CD10⁻ immature LDGs that show phenotypical and functional differences (15). In our study, iSLE patients exhibited higher levels of CD10⁺ LDGs than HCs. In comparison to CD10⁻ LDGs, CD10⁺ LDGs have been shown to express more IFN type-I related genes, have enhanced abilities for NETosis and correlated with vascular damage, all characteristics of SLE (15). Which factors contribute to increased CD10⁺ LDGs in iSLE and SLE can only be speculated since the origin of these cells is still unknown (2). The fact that we did not find a difference in proportions of CD10⁺ LDGs between SLE patients and HCs, might be related to the use of immunosuppressive medication. Unlike previously described (12,13), the proportion of LDGs was not related to disease activity in our study, which might be explained by the overall low disease activity in iSLE and SLE patients. Rahman et al. recently showed that the LDG-to-lymphocyte ratio might serve as a highly sensitive measure of disease activity in SLE (13). Concordantly, the LDG-to-lymphocyte ratio
was elevated in iSLE and SLE patients compared to HCs. Interestingly, iSLE and SLE patients treated with HCQ had lower proportions of LDGs than patients who did not receive HCQ. HCQ has been shown to inhibit NETosis in vitro (30) and in a mouse study (31) and might lower LDGs levels by reducing interferon load (2); however its direct effects have not yet been described. In contrast to our findings, two previous studies did not report relations between HCQ use and proportion of LDGs in SLE patients (12,13). However, it should be noted that iSLE patients generally had milder disease and did not receive immunosuppressive medications in this study.

LDGs have a higher ability to go into NETosis compared to normal density neutrophils (1,15,29). The higher proportion of LDGs observed in iSLE patients in this study, might then partly account for the higher levels of free circulating plasma NETs. Remarkably, circulating NETs, assessed as MPO-DNA, cit-H3-DNA and elastase-DNA complexes were similarly elevated in iSLE and SLE patients with overall quiescent disease, highlighting the immunological similarities between the two groups. Next to increased production of NETs via LDGs, impaired clearance of NETs has been described in SLE (2,5,32) and might also contribute to the observed NET burden in iSLE patients. In addition, pro-inflammatory components in serum from SLE patients have been shown to induce NET formation in healthy neutrophils (7,8). In this study, we showed that also serum from iSLE patients induced heightened NETosis in healthy neutrophils similar to serum from SLE patients. It has been described that damage-associated proteins like high-mobility group box 1 (HMBG1), autoantibodies and immune complexes in serum activate NET formation partly via Fcγ receptors (1,7,8,33). Also, iSLE patients exhibit autoantibodies like anti-dsDNA, anti-SSA and antiphospholipid antibodies but to a lesser extent than in established SLE (19). Here, we did not find relations between free-circulating NETs, serum-induced NETosis and autoantibodies
in both iSLE and SLE, but our relatively small sample size should be considered when interpreting these results.

Not only do autoantibodies stimulate NET formation, but NETs can themselves activate plasmacytoid dendritic cells, autoreactive B-cells and complement which leads to production of interferon and autoantibodies in SLE (5,10,11,34). In contrast to previous reports, we did not find correlations between circulating NETs or serum-induced NET formation and interferon score (6,8,9). This might be related to heterogeneity in cohorts, differences in interferon genes analysed and overall low disease activity in our cohort (19).

In iSLE patients only, MPO-DNA complexes were positively correlated with proportions of age-associated B-cells, memory B-cells and negatively with naïve B-cells. We previously showed age-associated B-cells to be elevated in iSLE and SLE (26) and these B-cells can produce autoantibodies and inflammatory cytokines (35). One characteristic of age-associated B-cells is the expression of the transcription factor T-bet that is associated with autoreactivity. Recently, it has been shown that NETs from lupus nephritis patients can stimulate T-bet expression and might thereby promote autoreactivity (10). Moreover, memory B-cells have been shown to be elevated while naïve B-cells are reduced in SLE, which is in line with the observed correlations with circulating MPO-DNA (36). The fact that this was not observed in SLE could be related to the use of immunosuppressive medication. MPO-DNA complexes were negatively correlated with C4 in SLE patients which confirms earlier observations (5,37) and implies that NETs can activate the complement system. This was not observed for iSLE patients in this study, possible due to fewer iSLE patients with reduced C4 levels.

Recently, Slief et al. showed that SLE and iSLE patients share the same genetic risk for SLE despite differences in phenotype (37). However, not all patients with iSLE will progress to established SLE and which environmental or immunological factors influence this progression is still unknown. It has been speculated that sustained interferon production in early stages of
SLE occurs first and drives adaptive autoreactivity including production of autoantibodies (3). It is possible that also neutrophil dysfunction exerts similar and synergistic effects, and this should be investigated in future studies. It should also be further investigated which components in serum and plasma exert NET formation and whether these are similar between iSLE and SLE.

To date, there are no specific treatments available for patients with iSLE. Frequently, iSLE patients, especially with skin or joint involvement, are treated with HCQ which reduces NET formation (30,31) and interferon load (38). Whether HCQ can reduce progression from iSLE to SLE is currently being investigated in the SMILE trial (39). Based on the here presented results, future treatments targeting neutrophil dysfunction might also be promising in iSLE.

A strength of this study is that we analysed three different aspects of neutrophil dysfunction in a well-characterized cohort. Limitations of this study include the relatively small sample size, which hinders stratification and correlation analysis. Although NET anti-MPO-DNA, cit-H-DNA and elastase DNA ELISAs have been conducted with great care and diligence, these methods are experimental. For serum-induced NETosis, we used semi-automated single-layer fluorescence microscopy, which requires manual adjustment of some images.

In conclusion, this study shows that neutrophil dysfunction including higher proportions of LDGs, and increased NET formation occurs in iSLE, similar to SLE, despite differences in disease manifestations. Neutrophil dysfunction thereby contributes to sustained autoreactivity and exposure to autoantigens in early stages of SLE and might induce adaptive autoreactivity.
REFERENCES


AUTHORSHIP CRITERIA

Svenja Henning: Substantial contribution to study conception and design, acquisition of data, analysis and interpretation of data, drafting the article, and approval of the final version for publication.

Tobias Reimers: Substantial contribution to study conception and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, and approval of the final version for publication.

Wayel Abdulahad: Substantial contribution to study conception and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, and approval of the final version for publication.

Juan J. Fierro: Substantial contribution to analysis and interpretation of data, critical revision of the manuscript for important intellectual content and approval of the final version for publication.

Berber Doornbos-van der Meer: Substantial contribution to analysis and interpretation of data, critical revision of the manuscript for important intellectual content and approval of the final version for publication.

Hendrika Bootsma: Substantial contribution to analysis and interpretation of data, critical revision of the manuscript for important intellectual content and approval of the final version for publication.

Barbara Horvath: Substantial contribution to analysis and interpretation of data, critical revision of the manuscript for important intellectual content and approval of the final version for publication.

Karina de Leeuw: Substantial contribution to study conception and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, and approval of the final version for publication.
Johanna Westra: Substantial contribution to study conception and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content and approval of the final version for publication.
DISCLOSURE OF INTERESTS

The authors do not report conflicts of interest.
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DATA AVAILABILITY

The data underlying this article will be shared on reasonable request to the corresponding author.
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### Table 1. Patient characteristics, LDG analysis

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<td>1 (6%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>African European</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1 (7%)</td>
<td>1 (6%)</td>
<td>2 (20%)</td>
<td></td>
</tr>
<tr>
<td>Current smoking, n</td>
<td>0 (0%)</td>
<td>4 (22%)</td>
<td>1 (10%)</td>
<td>0.479b</td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median SLEDAI score</td>
<td>NA</td>
<td>1 (0, 2)</td>
<td>2 (2, 4)</td>
<td>0.063c</td>
</tr>
<tr>
<td>Classification criteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median ACR criteria</td>
<td>NA</td>
<td>3 (2, 3)</td>
<td>4 (4, 5)</td>
<td></td>
</tr>
<tr>
<td>(IQR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median SLICC criteria</td>
<td>NA</td>
<td>3 (2, 3)</td>
<td>6 (6, 7)</td>
<td></td>
</tr>
<tr>
<td>(IQR)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Median EULAR ACR</td>
<td>NA</td>
<td>6.50 (4, 10)</td>
<td>17.00 (14, 25)</td>
<td></td>
</tr>
<tr>
<td>criteria (IQR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autoantibody positivity,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>NA</td>
<td>8 (44%)</td>
<td>7 (64%)</td>
<td></td>
</tr>
<tr>
<td>Anti-SSA</td>
<td>NA</td>
<td>8 (44%)</td>
<td>5 (45%)</td>
<td></td>
</tr>
</tbody>
</table>
### Anti-Smith

<table>
<thead>
<tr>
<th>Medication use, n (%)</th>
<th>0</th>
<th>4 (36%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxychloroquine</td>
<td>0 (0%)</td>
<td>11 (61%)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Mycophenolate Mofetil</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

### LDGs

- **Median proportion of LDGs from PBMCs (%):**
  - Hydroxychloroquine: 0.52 (0.34, 1.42 (0.78, 0.82 (0.66, 0.004)<sup>a</sup>)
  - Prednisolone: 0.86, 3.39, 3.87
- **Median absolute number of LDGs per µl of PBMCs:**
  - Hydroxychloroquine: 10.9 (6.74, 22.4 (11.52, 20.7 (15.50, 0.023)<sup>a</sup>)
  - Prednisolone: 17.05, 42.90, 67.90

### CD10+ LDGs

- **Median proportion of CD10+ LDGs (%):**
  - Hydroxychloroquine: 81.88 (73.95, 93.30 (87.41, 85.19 (87.41, 0.027)<sup>a</sup>)
  - Prednisolone: 90.68, 96.30, 96.30
- **Median absolute number of CD10+ LDGs per µl of PBMCs:**
  - Hydroxychloroquine: 9.00 (4.93, 23.80 (13.95, 18.50 (12.40, 0.003)<sup>a</sup>)
  - Prednisolone: 10.28, 41.82, 61.05

**HC:** healthy controls; **iSLE:** incomplete systemic lupus erythematosus; **SLE:** systemic lupus erythematosus; **IQR:** interquartile range; **SLICC:** systemic lupus international collaborating clinics; **LDG:** low density granulocytes; **PBMCs:** peripheral blood mononuclear cells; **NA:** not applicable

<sup>a</sup>Kruskal-Wallis rank sum test; <sup>b</sup>Fisher’s Exact Test for Count Data; <sup>c</sup>Wilcoxon rank sum test, HC group excluded; <sup>d</sup>Fisher’s Exact Test for Count Data, HC group excluded.
### Table 2. Patient characteristics, NET analysis

<table>
<thead>
<tr>
<th></th>
<th>HC (N=16)</th>
<th>iSLE (N=35)</th>
<th>SLE (N=41)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age in years (IQR)</td>
<td>47 (28, 59)</td>
<td>39 (28, 50)</td>
<td>43 (28, 51)</td>
<td>0.542&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>14 (88%)</td>
<td>31 (89%)</td>
<td>33 (80%)</td>
<td>0.642&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.926&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>White European</td>
<td>16 (100%)</td>
<td>31 (89%)</td>
<td>36 (88%)</td>
<td></td>
</tr>
<tr>
<td>Asian European</td>
<td>0 (0%)</td>
<td>1 (3%)</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>African European</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>0 (0%)</td>
<td>3 (9%)</td>
<td>3 (7%)</td>
<td></td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>0 (0%)</td>
<td>14 (40%)</td>
<td>8 (20%)</td>
<td>0.025&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Median SLEDAI score</td>
<td>NA</td>
<td>1 (0, 2)</td>
<td>2 (2, 4)</td>
<td>0.002&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Classification criteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median ACR criteria (IQR)</td>
<td>NA</td>
<td>2 (2, 3)</td>
<td>4 (4, 5)</td>
<td></td>
</tr>
<tr>
<td>Median SLICC criteria (IQR)</td>
<td>NA</td>
<td>3 (2, 3)</td>
<td>6 (5, 7)</td>
<td></td>
</tr>
<tr>
<td>Median EULAR ACR criteria (IQR)</td>
<td>NA</td>
<td>6 (4,8)</td>
<td>18 (14, 23)</td>
<td></td>
</tr>
<tr>
<td>Autoantibody positivity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>NA</td>
<td>6 (17%)</td>
<td>29 (71%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-SSA</td>
<td>Anti-Smith</td>
<td>Hydroxychloroquine</td>
</tr>
<tr>
<td>----------------</td>
<td>--------</td>
<td>---------</td>
<td>------------</td>
<td>--------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Medication use, n (%)</td>
<td></td>
<td></td>
<td>15 (43%)</td>
<td>14 (34%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14 (34%)</td>
<td>7 (17%)</td>
</tr>
</tbody>
</table>

**Measures of NETs**

<table>
<thead>
<tr>
<th>Metrics</th>
<th>Median (IQR) [n missing]</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO-DNA complex</td>
<td>0.19 (0.1, 0.45 (0.22), 0.39 (0.24-0.38))</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cit-H3-DNA-complex</td>
<td>0.16 (0.12, 0.44 (0.30), 0.20 (0.08))</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Elastase-DNA-complex</td>
<td>0.17 (0.16, 0.26 (0.2), 0.21 (0.19))</td>
<td>0.005</td>
</tr>
<tr>
<td>Serum-induced NET formation</td>
<td>4.52 (3.27, 7.13 (4.96), 8.07 (5.99))</td>
<td>0.003</td>
</tr>
</tbody>
</table>

HC: healthy controls; iSLE: incomplete systemic lupus erythematosus; SLE: systemic lupus erythematosus; IQR: interquartile range, SLEDAI: systemic lupus erythematosus disease activity index; ACR: American College of Rheumatology; SLICC: systemic lupus international collaborating clinics; NA: not applicable
NET: neutrophil extracellular trap. ¥ Autoantibody positivity here refers to increased values that have been recorded at least once.

\(^{a}\) Kruskal-Wallis rank sum test; \(^{b}\) Fisher’s Exact Test for Count Data; \(^{c}\) Wilcoxon rank sum test, HC group excluded; \(^{d}\) Fisher’s Exact Test for Count Data, HC group excluded.
Figure 1. Proportions of low density granulocytes in iSLE and SLE
Low density granulocytes (LDGs) are shown as a percentage of PBMCs (A) for HCs, iSLE and SLE patients. Proportions of CD10+ LDGs of total LDGs are shown per group (B). LDGs are depicted for iSLE and SLE patients and stratified by hydroxychloroquine use (C).

HC: healthy controls; iSLE: incomplete systemic lupus erythematosus; SLE: systemic lupus erythematosus; LDG: low density granulocyte; PBMC: peripheral blood mononuclear cells; *: p ≤ 0.05; **: p ≤ 0.01; ns: not significant

404x404mm (38 x 38 DPI)
Figure 2. Measures of free circulating plasma NETs

MPO-DNA complexes (A), cit-H3-DNA complexes (B) and elastase-DNA (C) complexes were measured in HCs, iSLE and SLE patients.

HC: healthy controls; iSLE: incomplete systemic lupus erythematosus; SLE: systemic lupus erythematosus; NET: neutrophil extracellular traps; MPO: myeloperoxidase; cit-H3: citrullinated histone H3; OD: optical density *: p ≤ 0.05; **: p ≤ 0.01; ns: not significant.

160x80mm (220 x 220 DPI)
Figure 3. Serum-induced NET formation visualised with fluorescence microscopy
Representative fluorescence microscopy images of NET formation in healthy control neutrophils incubated with PBS (negative control) (A), serum from an iSLE patient (B), serum from a SLE patient (C) or PMA (positive control) (D). Fluorescently labelled neutrophils are shown in red, and extracellular DNA is depicted in green. Quantification for serum-induced NET formation is shown for HC, iSLE and SLE patients (E). iSLE: incomplete systemic lupus erythematosus; SLE: systemic lupus erythematosus; NET: neutrophil extracellular traps; PBS: phosphate buffer saline; PMA: phorbol-12-myristate-13-acetate; OD: optical density; *: p ≤ 0.05; **: p ≤ 0.001; ns: not significant.

160x87mm (220 x 220 DPI)