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Concise report

Neutrophil extracellular trap release is associated with antinuclear antibodies in systemic lupus erythematosus and anti-phospholipid syndrome

Maarten van der Linden[1,a], Lucas L. van den Hoogen[2,a], Geertje H. A. Westerlaken[1], Ruth D. E. Fritsch-Stork[2,3], Joël A. G. van Roon[2], Timothy R. D. J. Radstake[2,b] and Linde Meyaard[1,b]

Abstract

Objectives. Increased release of neutrophil extracellular traps (NETs) is implicated in the activation of plasmacytoid dendritic cells, vascular disease and thrombosis in SLE and APS. However, studies comparing NET release between patients with SLE and APS are lacking. Here we evaluated plasma-induced NET release in a large cohort of patients with SLE, SLE + APS and primary APS in relation to clinical and serological parameters.

Methods. Neutrophils from healthy controls were exposed to plasma of heterologous healthy controls ($n = 27$) or SLE ($n = 55$), SLE + APS ($n = 38$) or primary APS (PAPS) ($n = 28$) patients and NET release was quantified by immunofluorescence. In a subset of SLE patients, NET release was assessed in longitudinal samples before and after a change in treatment.

Results. Plasma-induced NET release was increased in SLE and APS patients, with the highest NET release found in patients with SLE (±APS). Plasma of 60% of SLE, 61% of SLE + APS and 45% of PAPS patients induced NET release. NET release did not correlate with disease activity in SLE or APS. However, increased levels of anti-nuclear and anti-dsDNA autoantibodies were associated with increased NET release in SLE and APS. Only in SLE patients, elevated NET release and an increased number of low-density granulocytes were associated with a high IFN signature.

Conclusion. Increased NET release is associated with autoimmunity and inflammation in SLE and APS. Inhibition of NET release thus could be of potential benefit in a subset of patients with SLE and APS.

Key words: anti-phospholipid syndrome, autoantibodies, interferon signature, neutrophil extracellular traps, systemic lupus erythematosus

CLINICAL SCIENCE

Rheumatology key messages

- Plasma of SLE, SLE + APS and primary APS patients induces neutrophil extracellular trap release.
- Plasma-induced neutrophil extracellular trap release is associated with increased anti-nuclear and anti-dsDNA antibodies.
- SLE, but not APS, plasma-induced neutrophil extracellular trap release is associated with the IFN signature.

[1]Laboratory of Translational Immunology, Department of Immunology, [2]Laboratory of Translational Immunology, Department of Rheumatology and Clinical Immunology, University Medical Centre Utrecht, Utrecht University, Utrecht, The Netherlands and [3]1st Medical Department and Ludwig Boltzmann Institute of Osteology at the Hanusch Hospital of WGKK and AUVA Trauma Centre Meidling, Hanusch Hospital and Sigmund Freud University, Vienna, Austria

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[a]Maarten van der Linden and Lucas L. van den Hoogen contributed equally to this study.

[b]Timothy R. D. J. Radstake and Linde Meyaard contributed equally to this study.

Correspondence to: Linde Meyaard, Lundlaan 6, 3584 EA, Utrecht, The Netherlands.
E-mail: l.meyaard@umcutrecht.nl

Introduction

SLE and APS are overlapping autoimmune diseases that can occur separately or in the same patient. In SLE, immune complexes (ICs) of autoantibodies are deposited into tissues, leading to inflammation in several organs, including the kidney, skin and joints. In APS, aPLs activate endothelial cells and trophoblasts resulting in thrombosis and pregnancy morbidity. APS is termed primary APS (PAPS) when no underlying disease such as SLE is present. In APS, most research has focused on the prothrombotic role of aPLs. However, research in recent years indicates an important role for immune cells in the pathogenesis of (P)APS, often in a similar fashion as in SLE, although studies that compare immunopathology between SLE, SLE + APS and PAPS patients are scarce [1].

There is growing interest in the role of neutrophils in rheumatic diseases [2]. Neutrophils act as a first line of defence against infectious invaders by, among other strategies, the release of neutrophil extracellular traps (NETs). NETs consist of decondensed chromatin decorated with neutrophil-derived proteases and antimicrobial peptides that trap and kill pathogens [3]. Neutrophils from SLE and PAPS patients are prone to release NETs spontaneously [4, 5]. In addition, healthy neutrophils release NETs when stimulated *in vitro* with autoantibodies present in sera of SLE or APS patients [5, 6]. Furthermore, DNase activity is decreased in SLE and APS, resulting in increased NET exposure [2, 7], and SLE and APS patients have increased numbers of circulating low-density granulocytes (LDGs) [8, 9], a subset of neutrophils prone to undergo NET formation. As a result, SLE and APS patients have elevated levels of NET remnants in the circulation [2, 7] and NETs are present in affected tissues such as the skin or kidney in SLE or in aPL-induced thrombi [9, 10].

Uncontrolled NET release triggers a pathological cascade of events relevant for the pathophysiology of SLE and APS. NETs induce tissue damage [2], activate the clotting system to promote thrombus formation [5], induce endothelial dysfunction [11] and represent a source of autoantigens [4]. Moreover, *in vitro*, NETs activate plasmacytoid dendritic cells to produce IFN-α [4, 9, 12], which might explain the IFN signature in SLE and APS patients [13].

Until now, NET release has only been studied in SLE and APS separately in small-scale studies. The different methodologies to induce and quantify NET release hamper the comparison across studies. Recently we developed a novel NET assay that specifically measures NET release, as it distinguishes NET release from other forms of neutrophil death while the automatic quantification avoids subjectivity [14]. Here we employed our assay to investigate plasma-induced NET release in a large cohort of SLE, SLE + APS and PAPS patients in relation to clinical and serological parameters, including the IFN signature.

Methods

A detailed description of the methods is available as supplementary data at *Rheumatology* online.

Study population

SLE, SLE + APS and PAPS patients and age- and sex-matched healthy controls (HCs) were recruited from our outpatient clinic or in-house healthy donor service. PAPS patients did not meet classification criteria for SLE nor had clinical evidence of SLE. None of the patients had evidence of an ongoing infection. Patients were stratified by a high or low IFN signature as previously described [13]. The University Medical Center Utrecht medical ethical committee approved this study and all study participants signed informed consent.

Quantification of NETs

HC neutrophils were cultured with 10% (heterologous) plasma of patients or controls for 4 h in a 96-well plate. NET release was quantified as previously described, with or without fixation after 4 h. Sytox Green images were used to quantify NET area [14] (Fig. 1A).

Statistical analysis

The NET area of 20 different microscopic fields per well was averaged. The mean of log-transformed NET areas per plasma donor of four independent experiments was reported as the mean NET area. The J-statistic of the Youden index of the receiver operating characteristics (ROC) curve of NET release in patients (SLE, SLE + APS and PAPS) as compared with HCs was used to define a cut-off to stratify patients into high or low NET inducers. Differences between groups were tested two-sided by analysis of variance (ANOVA) and Tukey's post-test or *t* test as appropriate ($\alpha = 0.05$) using SPSS (v22).

Results

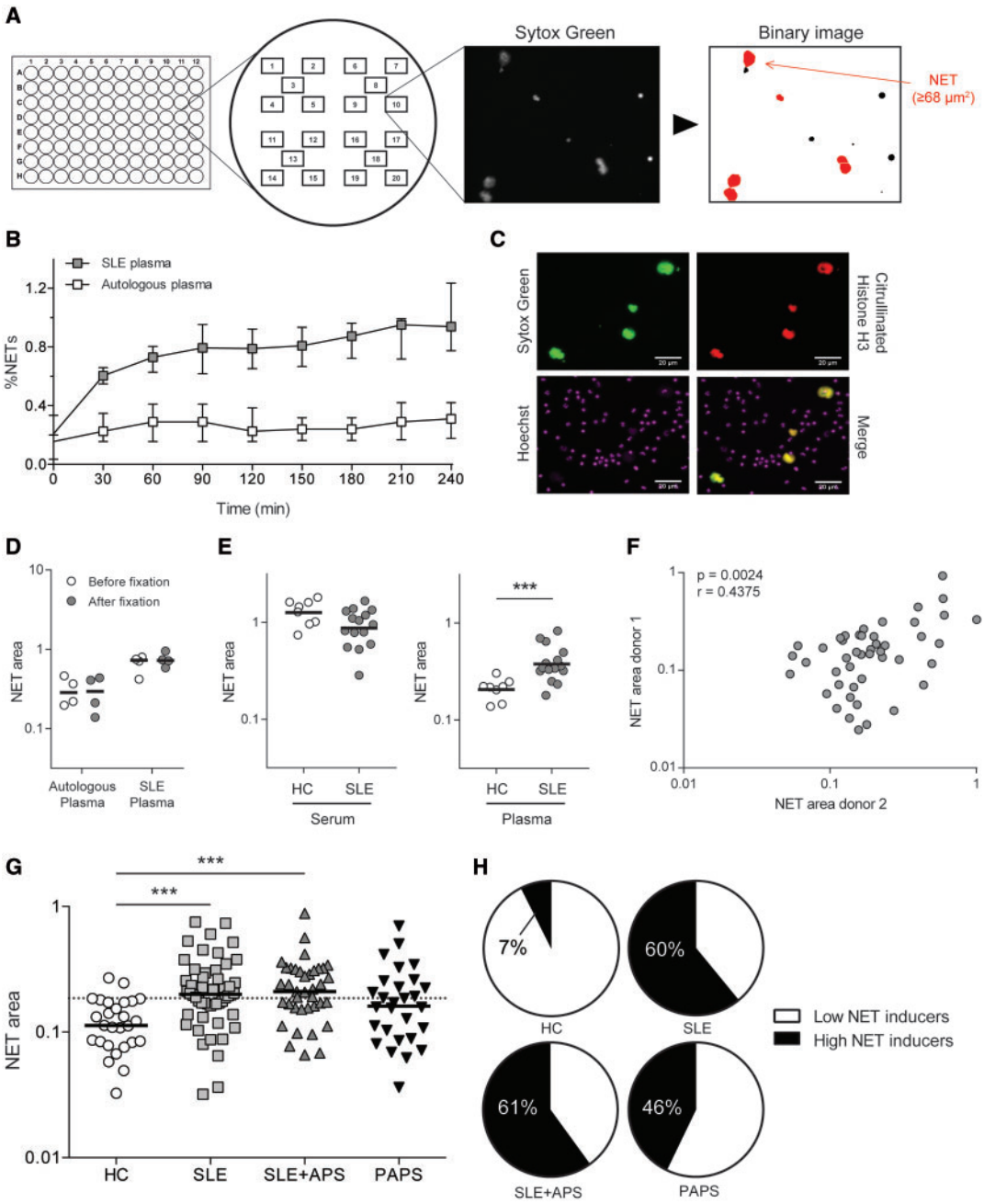
Validation of a high-throughput assay to measure plasma-induced NET release

Our live-imaging assay to monitor NET release over time revealed NET release within 30 min after exposure to SLE plasma (Fig. 1B; supplementary Video S1, available at *Rheumatology* online). The presence of citrullinated histone H3 in the extracellular DNA confirmed the formation of NETs (Fig. 1C). To allow the measurement of >100 samples without a time difference between the first and last sample, we introduced a fixation step after 240 min, which did not affect the quantification of NETs (Fig. 1D). Pilot experiments with plasma and serum samples from SLE patients ($n = 15$) and HCs ($n = 8$) showed increased NET induction by plasma from SLE patients as compared with HCs. Although the sera of both patients and controls had higher NET induction capacity than plasma, no difference was observed between SLE patients and HCs (Fig. 1E). In these pilot experiments we observed a moderate ($r = 0.4375$, $P = 0.002$) correlation of NET release between independent experiments using different neutrophil donors (Fig. 1F).

Increased plasma-induced NET release in SLE, SLE + APS and PAPS

We next used plasma samples of HCs ($n = 27$), SLE ($n = 55$), SLE + APS ($n = 38$) and PAPS ($n = 28$; supplementary

Fig. 1 NET release in response to plasma of SLE, SLE + APS and PAPS patients



(**A**) Experimental approach to measure NET release at a fixed time point. Twenty fields of view were captured per condition and Sytox Green images were used to analyse NET area. (**B**) Live imaging showed enhanced NET release of HC neutrophils when exposed to SLE plasma compared with autologous plasma. Data points represent median ± inter-quartile range of four independent experiments with four plasma samples each. (**C**) Citrullinated histone H3 staining confirmed the presence of actual NETs and (**D**) fixation after 4 h did not affect NET quantification. In a pilot experiment, serum and plasma from HCs (n = 8) and SLE (n = 15) patients were used to induce NET release in HC neutrophils. The NET area of independent experiments with neutrophils from three different HC donors is presented. (**E**) Elevated NET release was shown in neutrophils exposed to plasma, not serum, from SLE patients compared with those exposed to plasma or serum from heterologous HCs. (**F**) Plasma-induced NET release correlated between independent experiments. (**G**) HC neutrophils exposed to heterologous plasma from SLE (n = 55), SLE + APS (n = 38) and PAPS (n = 28) patients displayed increased NET release compared with those exposed to plasma from HCs (n = 27). The NET area of independent experiments with neutrophils from four different HC donors is presented. (**H**) Prevalence of high NET release in patients with SLE, SLE + APS or PAPS. The data in (D), (E) and (G) are presented as means, $^{***}P < 0.001$. The images in (C) are representative of at least three experiments with neutrophils from different donors.

Table S1, available at *Rheumatology* online) patients to induce NET release in neutrophils of four HC donors in four independent experiments. Confirming our pilot experiments, the mean NET release of four independent experiments was higher using plasma from SLE and SLE + APS patients as compared with HC plasma ($P < 0.001$), with a similar trend when using PAPS plasma ($P = 0.14$, Fisher's least significant difference $P = 0.03$; Fig. 1G; supplementary Fig. S1, available at *Rheumatology* online). NET release did not differ among SLE, SLE + APS or PAPS patients (ANOVA $P = 0.19$). Setting a threshold by ROC curve analysis (supplementary Fig. S2, available at *Rheumatology* online), plasma samples from 33/55 (60%) of SLE, 23/38 (61%) of SLE + APS and 13/28 (46%) of PAPS patients had high NET release (Fig. 1H), as compared with 2/27 (7%) of HC plasma samples. Thus our data show that plasma from the majority of SLE and APS patients induces NET release.

NET release did not correlate with clinical measures of disease activity, including Safety of Estrogen in Lupus Erythematosus National Assessment–SLEDAI for SLE (±APS) patients ($P = 0.57$; supplementary Fig. S2A, available at *Rheumatology* online) and the adjusted global APS score (aGAPSS) for (P)APS patients ($P = 0.88$; supplementary Fig. S2B, available at *Rheumatology* online). Furthermore, there were no significant differences among clinical phenotypes including (active) LN in SLE patients or APS patients with or without arterial or venous thrombosis or pregnancy morbidity. Also, NET release did not differ between patients treated with or without prednisolone, AZA, aspirin or other immunosuppressants ($P > 0.05$, data not shown).

Plasma-induced NET release is associated with ANA and anti-dsDNA antibodies

In vitro studies implicate NET release as a source of autoantigens eliciting the production of autoantibodies against nuclear components in SLE [5]. In line with these observations, SLE patients with high NET release had increased levels of anti-dsDNA antibodies compared with patients whose plasma induced low NET release ($P = 0.008$; Fig. 2A). Likewise, PAPS patients with high NET release had elevated ANA staining intensities ($P < 0.05$; Fig. 2B). In longitudinal samples, collected from SLE patients before and after a change in immunosuppressive therapy, a decline in anti-dsDNA antibodies ($P = 0.02$; Fig. 2C) was paralleled by a decline in NET release ($P = 0.03$; Fig. 2D), whereas patients with stable or increasing anti-dsDNA antibodies (Fig. 2E) between two time points did not have a decrease in plasma-induced NET release ($P = 0.48$; Fig. 2F). Among SLE or APS patients, no specific association between the presence or absence of anti-β_2 glycoprotein I and anti-RNP antibodies was observed.

NET kinetics is similar in SLE and PAPS

We previously showed that the kinetics of NET release differs between stimuli [14]. We observed no difference in the kinetics of plasma-induced NET release among high-inducing plasma samples of SLE or PAPS patients (Fig. 2G). In comparison to patient plasma, exposure of neutrophils to ICs induced abundant NETs, ~30% compared with 3% in patient plasma samples. Diphenyleneiodonium (DPI) inhibits, among others, NADPH oxidase, and NET release in response to ICs was inhibited by 60–70% in the presence of DPI while PAPS and SLE plasma-induced NET release was not inhibited in the presence of DPI (Fig. 2H).

NET release and low-density granulocytes are associated with the IFN signature in SLE

In vitro experiments implicate NETs and LDGs as a trigger for IFN-α production by plasmacytoid dendritic cells [4, 9], although no studies have explored NET release in relation to the presence or absence of the IFN signature. SLE patients with a high IFN signature (IFN-high) had higher NET release than patients with a low IFN signature (IFN-low) ($P < 0.01$; Fig. 2I). Corroborating this finding we observed that IFN-high SLE patients had increased numbers of circulating LDGs ($P < 0.01$; Fig. 2J). Interestingly, these associations were not seen in APS patients, neither in SLE + APS nor in PAPS ($P > 0.05$).

Discussion

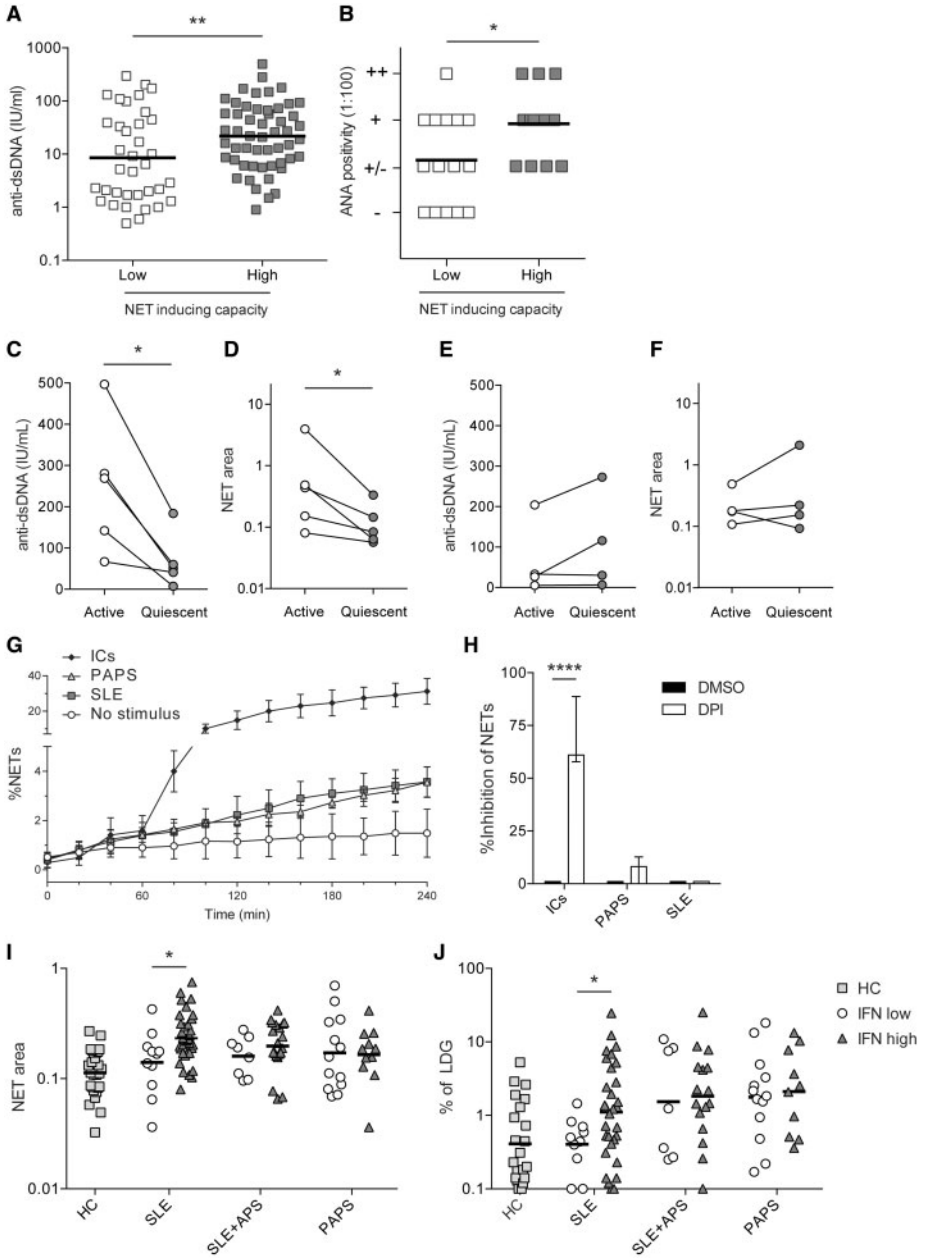
Using a novel high-throughput assay we show that plasma of SLE, SLE + APS and PAPS patients induces NET release, which is associated with ANAs in PAPS and anti-dsDNA autoantibodies and the IFN signature in SLE patients. This study highlights the potential role of NET release in relation to autoimmunity and inflammation in SLE and APS and compares NET release in a large cohort of SLE and APS patients.

Previous studies have shown induction of NET release using serum of SLE or APS patients [6, 15]. In small pilot studies, when comparing serum with plasma, serum induced a higher release of NETs than plasma, both in patients and HCs, and no difference between HCs and patients was observed. The generation of serum leads to platelet activation, which is a strong NET inducer [16] and is a likely cause for the higher NET release induced by serum samples. As a result, to avoid potential effects of platelet activation, we used patient plasma to trigger NET release in further experiments. Importantly, although similar trends were observed when using different neutrophil donors, the amount of NETs formed differed between neutrophil donors and therefore our results stress the need to use different neutrophil donors when studying NET release [16].

Besides the amount of NETs, the kinetics of NET release differ between stimuli [14]. We observed a rapid release of NETs (within 30 min) upon exposure to patient plasma in both SLE and PAPS patients. As the composition of NETs differs between stimuli [17], we speculate that the content of NETs could differ between SLE and APS, since NET release was differentially associated with the IFN signature in SLE and APS.

NET release in the context of SLE and APS has been mainly studied using purified antibodies or cytokines to trigger NET release, including anti-β_2 glycoprotein I, anti-RNP, anti-human neutrophil protein, anti-LL37, anti-MMP9 antibodies and IL-18 and hyperacetylated microparticles [4–6, 11, 12, 18–20]. As multiple factors present in patient plasma may induce NET release, it is

FIG. 2 Plasma-induced NET release is associated with ANA levels in plasma of SLE and PAPS



(**A**) Plasma of SLE patients classified as high NET inducers contained elevated levels of anti-dsDNA antibodies compared with those that are classified as low NET inducers. (**B**) Plasma of PAPS patients classified as high NET inducers contained increased ANA staining intensities compared with those that are classified as low NET inducers (−: negative; ±: weak; +: positive; ++: strongly positive). Longitudinal samples were collected from SLE patients at the time of active disease as well as subsequent quiescent disease. The (**C**) anti-dsDNA antibody level and (**D**) NET area were increased in SLE patients (n = 5) with active disease compared with those with quiescent disease. (**E, F**) Patients (n = 4) with stable or increasing anti-dsDNA antibodies between two time points did not have a decrease in plasma-induced NET release. (**G**) NET release in response to IC was high compared with SLE and APS plasma-induced NET release. Data points represent mean (S.D.) of independent experiments with neutrophils of three HC donors and four plasma samples per group. (**H**) DPI and NADPH oxidase inhibitor suppressed IC-induced NET release while SLE and PAPS plasma-induced NET release was independent of NADPH oxidase. The percentage of inhibition of NET release was calculated based on the area under the curve relative to neutrophils exposed to IC, PAPS or SLE plasma in the presence of DMSO. (**I**) Plasma from SLE patients classified as IFN-high displayed elevated levels of NET release compared with those that were classified as IFN-low. (**J**) An increased amount of LDGs were present in SLE IFN-high patients compared with SLE IFN-low patients. Differences in LDG amounts between IFN-high and IFN-low patients were not seen in SLE + APS and PAPS patients. The data in (A), (B), (H), (I) and (J) are presented as means, $^{*}P < 0.05$ and $^{**}P < 0.005$.

unknown which stimulus is responsible for NET release in our assay; however, the association of autoantibodies and the IFN signature with NET release suggest their involvement, and this is clearly different in plasma from healthy individuals. Nevertheless, NET release by purified factors should be interpreted with caution since the concentration and composition of these factors in patients' plasma might be different. Indeed, in our study, NET release in response to ICs was much higher than in response to patient plasma. Moreover, immune complex–induced NET release is dependent on NAPDH oxidase, in contrast to plasma-induced NET release, although it is unknown whether the magnitude of NET release *in vitro* can be directly translated to *in vivo* situations.

Enhanced NET release is considered a major pathogenic factor linked to tissue damage, the IFN signature and other disease manifestations in both SLE and APS [4, 5, 10]. Consistent with this, we report increased NET release in patients with elevated autoantibodies or the IFN signature in SLE and APS. Treatment options that mitigate NET release could therefore be of added clinical value. Inhibition of NET release ameliorates mouse models of SLE and APS [10, 21]. Several small inhibitory molecules reduce NET release *in vivo* [22], while HCQ, a treatment for SLE, inhibits NET release *in vitro* [2]. We previously reported that triggering signal inhibitory receptor on leucocytes-1 attenuates SLE plasma and autoantibody-induced NET release [6]. Our current results indicate that only a subset of patients [~60% of SLE(±APS) and ~45% of PAPS patients] would benefit from inhibiting NET release.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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Clinical vignette

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Gout misdiagnosis due to dual-energy computed tomography artefact

A dual-energy CT (DECT) gout protocol (Fig. 1A) was performed for suspicion of gout of the left knee arthritis in 74-year-old woman with no monosodium urate crystals on initial SF analysis. DECT reconstructions revealed uric acid deposition automatically coloured in green (Fig. 1B, arrows) at the posterior surface of the femoral condyles. However, the localization of crystals deposition was unusual and the arthritis was not improved by colchicine. A new DECT was performed with flexion of the contralateral prosthetic knee (Fig. 1C) and did not show any green pixilation suggestive of gout, thus confirming a metal artefact due to the presence of the knee prosthesis (Fig. 1D). DECT is a valuable, non-invasive imaging modality for the diagnosis of gout when urate crystals confirmation in SF is not possible [1]. However, DECT artefacts may lead to a false-positive diagnosis [2], mainly due to skin or beam-hardening artefacts such as in this first described case of DECT metal artefact due to contralateral knee prosthesis.

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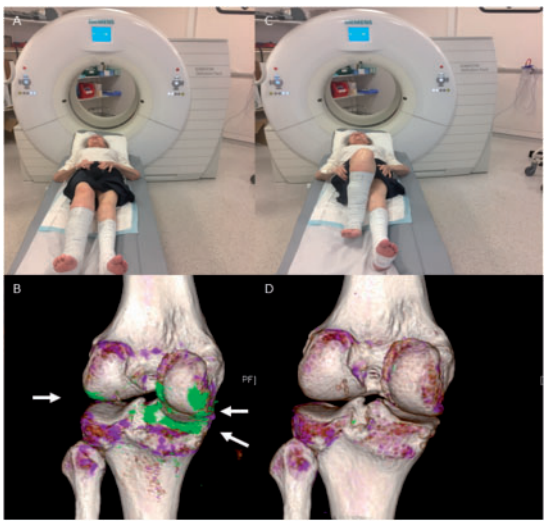
André Ramon[1], Pierre Pottecher[2,3] and Paul Ornetti[1,4]

[1]Department of Rheumatology, [2]Department of Radiology, Section of Musculoskeletal Imaging and Intervention, Dijon University Hospital, [3]LE2I UMR CNRS 6306, Arts et Métiers, University of Burgundy and [4]INSERM 1093 CAPS, University of Burgundy, Dijon, France

Correspondence to: Paul Ornetti, Department of Rheumatology, 14 rue Gaffarel, CHU Dijon, 21000 Dijon, France.
E-mail: paul.ornetti@chu-dijon.fr

Fig. 1 DECT scan of the left knee according to positioning of the right prosthetic knee



Coronal DECT reconstructions of a 74-year-old woman with left knee arthritis: beam-hardening artefact with green pixilation suggestive of gout (arrows) at the posterior surface of the left femoral condyles disappears when the contralateral prosthetic knee is flexed.

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