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First visualization of circulating neutrophil extracellular traps using cell fluorescence during human septic shock-induced disseminated intravascular coagulation

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ABSTRACT

Disseminated intravascular coagulation (DIC) is a severe complication of septic shock. Polymorphonuclear neutrophils (PMNs) may play a key role in septic shock-induced DIC *via* the release of neutrophils extracellular traps (NETs). NETs capture invading pathogens, but also act as a pro-coagulant surface at the interface between immunity and thrombosis. During septic shock-induced DIC, neutrophil activation may result in excessive NET formation. Herein, we originally report the presence of circulating NETs in human blood during septic shock-induced DIC.

To investigate NET formation during shock-induced DIC neutrophils were isolated from patients in septic shock associated with ($n = 3$) or without ($n = 3$) DIC. Neutrophils from healthy donors ($n = 3$) were stimulated *in vitro* with ionomycin as NET formation positive controls. PMNs smears were stained with mouse anti-human FITC anti-myeloperoxidase antibody and the blue-fluorescent DAPI nucleic acid stain. NETs were identified as elongated extracellular DNA fibers associated to myeloperoxidase detected by immunofluorescence.

NETs were unambiguously observed in PMNs from septic shock patients with DIC but not from patients without DIC. NETs features in DIC+ patients were undistinguishable from those observed in ionomycin-induced PMNs from healthy donors. Fluorescence images of NETs were associated to extracellular cytoplasmic expansions.

Our data report for the first time the direct visualization of circulating NETs in patients with septic shock-induced DIC. The *in vivo* relevance of previously reported indirect markers of NETosis (neutrophil side fluorescence) is confirmed.

1. Introduction

Septic shock is the most severe form of infection, defined as a subset of sepsis in which circulatory, cellular and metabolic abnormalities are profound [1], and responsible for multiple organ failure and a high mortality-rate [2]. Septic shock is characterized by a broad inflammatory response and coagulation activation that both contribute to bacterial containment, but also leads to microangiopathy through uncontrolled thrombin and fibrin generation, potentially evolving towards disseminated intravascular coagulation (DIC) [3–6]. During septic shock, DIC exacerbates multiple organ failure and increase the risk of death, underlining the relevance of blood coagulation processes as therapeutic target of interest [7,8]. However, DIC pathophysiological mechanisms are not fully understood [3,9]. Polymorphonuclear neutrophils (PMNs or neutrophils) have recently been identified as potential players in the response to infection by releasing their content, including DNA, histones and granules enzymes [10]. These structures, called neutrophils extracellular traps (NETs), form a large net-like structure in which the pathogens get trapped [10]. NETs are thus involved in host defense against the invading pathogens. NETs also form a large procoagulant surface and activate the contact phase of coagulation [11,12]. Indeed, NET components along with platelets, red blood

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cells and fibrin promote thrombus formation especially in microvessels [13–16] and contribute to subsequent mortality. Furthermore, cytokines released by neutrophils contribute to alter the membrane profile of vascular cells, especially endothelial cells, thus participating to septic shock-induced endothelial dysfunction and coagulation activation [17,18]. Thus, neutrophils may play a major role in immunothrombosis, defined by interactions between innate immunity, inflammation and thrombosis leading to thrombin generation, especially in microvessels [19–21]. Nevertheless, the pathophysiological role of neutrophils as modulators of the interactions between coagulation and immunity during infectious and thrombotic diseases, and their interaction with other blood cells remain a matter of debate [22]. During septic shock-induced DIC, neutrophil activation is deregulated and might result in excessive NET formation [20,23,24]. Herein, we report an original proof of concept, highlighting circulating NETs in human blood during septic shock-induced DIC.

2. Material and methods

2.1. Patients and blood collection

Patients in septic shock with DIC ($n = 3$) and without DIC ($n = 3$) were enrolled in this study, as well as 3 healthy donors. Septic shock was assessed according to the third international consensus definition [1], and DIC was diagnosed if the JAAM (*Japanese Association for Acute Medicine)*-DIC 2016 score was at least equal to 4 during the first 24 h of septic shock [25]. At the admission, 5 mL of blood were drawn on EDTA containing tubes (BD Vacutainer Plus Blood Collection Tubes, Becton Dickinson, Le Pont de Claix, France) that contain K2EDTA ethylenediamine-tetraacetic acid dipotassium dihydrate (EDTA-2K) anticoagulant which is spray-dried to the walls of the tube (final concentration 7.2 mg/5 mL). The Strasbourg University Hospital Ethics Committee approved this study. Informed consent was obtained from the patient or relatives at admission and confirmed by the patient as soon as possible.

2.2. Neutrophil isolation

Neutrophils were immediately purified after blood collection using negative immuno-magnetic sorting method (EasySep®, Stemcell Technologies, Canada) [26]. A total of 5×10^4 cells purified PMNS were subsequently spotted on Thermo Scientific™ SuperFrost™ Microscope Slides (Fisher Scientific, Illkirch, France) using cytocentrifugation at 35 *g* for 5 min, which preserved cell integrity and morphology.

2.3. Positive controls

NET formation was induced *in vitro* by stimulation of purified PNNs from healthy donors ($n = 3$) with 4 μM ionomycin (Calbio-chem, Merk Biodevelopment, France) for 4 h at 37 °C.

2.4. Neutrophil staining and observation

PMNs were fixed on slides using 4% paraformaldehyde for 30 min and stained after three washes with phosphate-buffer saline (PBS, Gibco, Saint Aubin, France). In brief, after permeabilization with 0.2% Tween-20 during 15 min, antigenic sites of PMNs were blocked with 10% fetal bovine serum (FBS) in PBS without EDTA for 30 min. After three more washes, cells were incubated with 125 ng of green FITC anti-myeloperoxidase antibody (mouse anti-human clone 5B8, BD Biosciences™, USA, 1/10 dilution) for 30 min at room temperature in dark. Smears were then washed with PBS and counterstained with the Vectashield mounting medium® containing 1.5 μg/mL of blue-fluorescent DAPI (Vector Laboratories, Burlingame™, USA) that preferentially stains dsDNA. All procedures were performed at room temperature. Immunofluorescence microscopy was performed on Zeiss™ imager.Z2 (Marly Le Roi, France) using Metasystems™ Isis fluorescence software analysis© (Cannes, France). NETs were identified as elongated extracellular DAPI stained DNA fibers associated to myeloperoxidase, as described by Brinkman et al. [10].

Quantification was performed by blinded assessment of extracellular DNA release using fluorescent microscopy (20× magnification). Degree of NETs was quantified as the percentage of neutrophils forming NETs divided by the total number of neutrophils X 100. Samples were categorized into 5 groups: absence of NETs (0% neutrophils forming NETs per microscopic field), scarce number of neutrophils forming NETs (1–25%), moderate NETs (26% to 50%), strong NETs (50% to 75%), and very strong NETs (76% to 100%).

PMNs spots were also stained by standard May-Grünwald-Giemsa for patients, normal PMNs and PMNs after induction of NETosis with ionomycin. Three independent operators reviewed blindly the morphology in at least one hundred neutrophils for each condition. A differential count was established between neutrophils harboring a normal morphology and those harboring morphology similar to PMNs after ionomycin induced NETosis. NETosis-associated morphology in these conditions consisted in nuclear decondensation, cytoplasmic vacuolization and the presence of cytoplasmic membrane expansions visible on > 1/3 of the cell circumference or the presence of one or more membrane prolongation larger than 1/3 of the cell size. These modifications reflect neutrophil activation.

2.5. Blood cell count and neutrophil analysis on Sysmex XN

Complete blood cell count was measured on an automated Sysmex™ XN20® analyzer (Sysmex Corporation, Kobe, Japan) according to the manufacturer without intervention. Cell permeabilization was performed by treatment with proprietary lysis reagents allowing the polymethine fluorescent XN-20 basic dye to enter the cells, where it binds to nucleic acids in the cytoplasmic organelles and the nucleus. NEUT-SFL reflects leukocyte activation and is correlated to NETosis [24,27]. NEUT-SFL values were extracted from the research screen of the analyzer software as previously described.

3. Results

Baseline characteristics, outcome and infection features are summarized in Table 1. Despite the limited number of enrolled that patients, no obvious bias concerning gender, age, comorbidities or type of infection was observed between patients with or without DIC. As expected, patients with DIC had a lower platelet and neutrophil count than non-DIC patients.

PMNs from healthy donors stimulated by ionomycin released NETs visualized using fluorescence microscopy as double-stained DAPI-positive chromatin structures decorated with antimicrobial granule MPO in the extracellular space (Fig. 1B, upper panel).

NET structures similar to those observed after ionomycin stimulation were unambiguously visualized in PMNs from septic shock patients with DIC (Fig. 1D, upper panel), but neither in PMNs from septic shock patients without DIC (Fig. 1C, upper panel), nor in unstimulated PMNs from healthy donors (Fig. 1A, upper panel).

All DIC patients and ionomycin-stimulated PMNs were stratified into “strong NETs formation” (> 50% of PMNs displaying DNA release) and “very strong NET formation” (> 75% of PMNs displaying DNA release) groups (Fig. 2A). Conversely, no-DIC patients and unstimulated normal PMNs were stratified into “scarce” or “absence” NET categories.

Ionomycin-stimulated PMNs stained with May-Grünwald-Giemsa harbored chromatin decondensation and vacuolization of the cytoplasm (Fig. 1B, lower panel), features associated to neutrophil activation. NETosis is one of the phenomena that participates to this activation [28]. These features were absent in unstimulated PMNs (Fig. 1A, lower panel). In addition, villous surface projections (also called pseudopods) were detected on the circumference of ionomycin-stimulated PMNs.

Table 1
Description of patient characteristics at baseline.

Gender	Female	Male	Male	Female	Male	Male
Age (years)	30	32	81	64	67	71
Comorbidities						
DIC	+	+	+	−	−	−
DVT	−	−	+	−	−	−
Arterial thrombosis	−	−	−	+	−	−
Immunosuppression	+	−	−	−	−	−
Chronic heart failure	−	−	−	+	+	−
Cirrhosis	−	−	−	−	−	−
Chronic renal failure	−	−	−	+	−	−
Chronic respiratory failure	−	−	−	+	+	−
Organ failure						
Norepinephrine support	+	+	+	+	+	+
Mechanical ventilation	+	+	+	+	+	+
Extrarenal epuration	−	−	−	+	−	−
SOFA score	13	7	9	9	12	14
Mortality						
Death at day 7	+	−	−	−	−	−
Death at day 28		−	−	−	−	−
CBC						
Leucocytes (G/L) [4.10–10.50]	2.7	21.47	14.77	28.26	30.93	25.2
Neutrophils (G/L) [1.80–7.70]	1.81	20.34	11.5	19.37	26.19	23.18
Platelets (G/L) [150–400]	16	39	169	371	138	306
Hemoglobin (g/dL) [13.0–18.0]	12.5	8.2	9.9	11.9	9.3	10.2
Infection						
Community-acquired	+	−	+	+	−	+
Nococomial	−	+	−	−	+	−
Site of infection	Lung	Abdomen	Urinary system	Urinary system	Abdomen	Lung
Microorganism	*Streptococcus pneumoniae*	Polymicrobial	*Escherichiacoli*	*Klebisiella pneumoniae*	*Enterococcus faecium*	*Streptococcus pneumoniae*
Blood-culture	+	+	+	−	−	+
PMNS imaging						
Elongated extracellular DAPI stained DNA fibers associated to myeloperoxidase	+	+	+	−	−	−
Villous PMNS (%)	70%	82%	79%	2%	5%	3%
PMNS fluorescence[a]	58,1	62,8	55,8	46,5	53,2	57

All patients suffered from septic shock. DIC: Disseminated intravascular coagulation; DVT: Deep Venous Thrombosis; CBC: Complete Blood Cell Count. Normal values of CBC parameters are enclosed in square brackets.

[a] *Side fluorescence (Sysmex NEUT-SFL parameter).

These modifications were characterized by cytoplasmic membrane expansions visible on > 1/3 of the cell circumference or the presence of one or more membrane prolongation larger than 1/3 of the cell size (Fig. 1B, lower panel). The percentage of PMNs harboring a NETosis-associated morphology reviewed by three blinded-operators were strongly elevated in PMNs from DIC patients (Fig. 1D, lower panel and Fig. 2B) but not in no-DIC patients (Fig. 1C, lower panel and Fig. 2B).

Accordingly, the side-fluorescence of PMNs (NEUT-SFL), a Sysmex™ CBC analyzers parameter previously associated to NETosis modifications [24] was increased in normal PMNs stimulated with ionomycin and DIC patients compared to patients without DIC and normal unstimulated normal PMNs (Fig. 3A, B).

4. Discussion

The present report highlights the first direct visualization of circulating NETs in the blood of patients diagnosed with DIC during a septic shock. In this pilot study, PMNs from DIC patients displayed typical NETs structures, that were not observed in PNs from no-DIC patients.

The degree of NETs formation observed in all DIC patients using immunofluorescence microscopy was strong, comparable to the one observed in ionomycin-stimulated PMNs from healthy volunteers.

In previous studies, NETosis was mainly correlated to DIC and thrombosis by serum assays or flow cytometry [24,29,30]. It still remains unclear whether NETs are associated or not to DIC during septic shock [9,21]. We have recently reported that indirect markers of NETosis (MPO-DNA complexes) are elevated in septic shock-induced DIC patients compared to no-DIC patients [24], but this observation was not confirmed by Maruchi et al. [30]. These apparently conflicting results may be explained by the use of different NET measurement methods, the characteristics of the patient studied or the anticoagulation therapy administrated for DIC.

Surrogate markers of NETosis such as cfDNA and DNase have been associated to sepsis. These factors reflect cell injury and are elevated in other conditions like thrombotic events, autoimmune diseases or cancer [31–35].

Flow cytometry assays were also proposed to identify and quantify NETs in blood samples [29,36]. Although this method is more specific than serum markers, the gating of NETs or NETotic PMNs population using side scatter/forward scatter is difficult and may cause measurement variability and interpretation biases [37]. Taken together, flow cytometry may detect only the earliest stage of NETs release (before plasma membrane perforation and cell lysis) and serum markers the components released after NETosis [9,29].

The immunofluorescence methodology used in this study, limited to MPO-DNA complexes visualization, may overpass the limitations of indirect methodologies. It provides a “snapshot” of circulating PMNs at the time of sampling. The staining of citrullinated histones may represent an additional evidence of the occurrence of NETosis in PMNs, but the technical simplicity of the approach used here may facilitate repeated measures during the evolution of the patient in further studies.

The cytological modifications observed in PMNs from DIC patients also need to be further explored, and the different mechanisms of neutrophil activation that cause these changes have to be deciphered with additional markers. However, if the morphological modifications observed in such patients is not entirely explained by NETosis

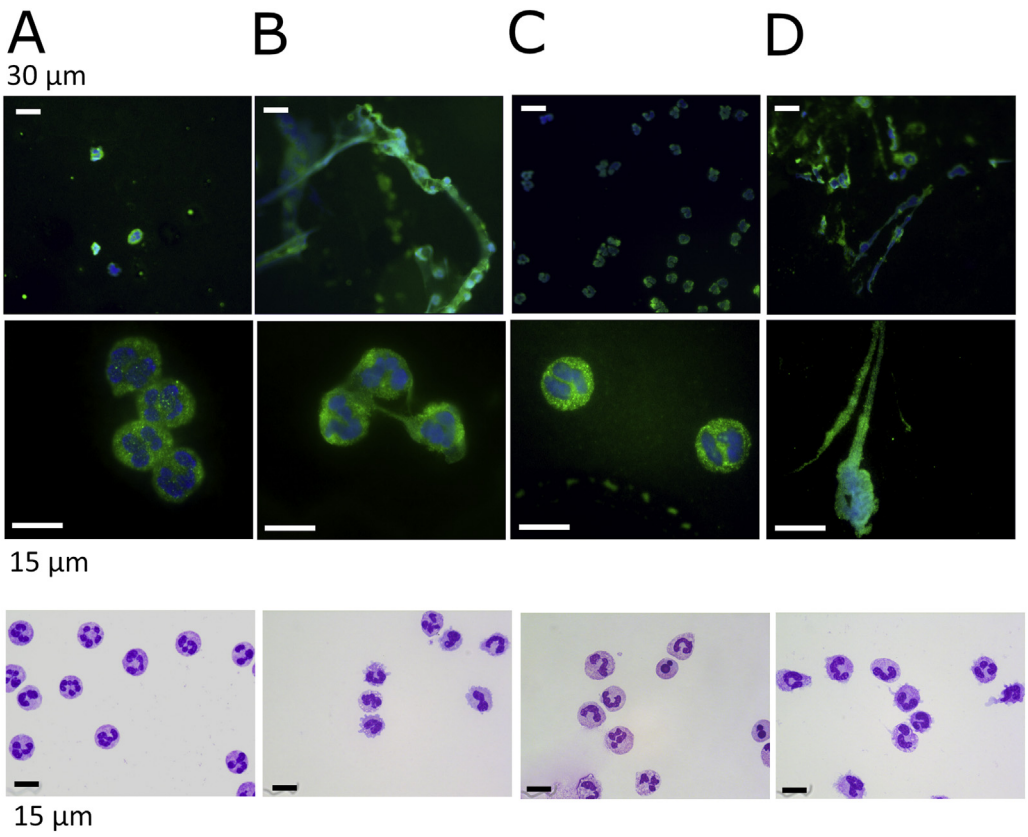

Fig. 1. Representative images of immunofluorescence and May-Grünwald-Giemsa staining showing NETs in purified peripheral blood polymorphonuclear neutrophils (PMNs).
Observations were reproduced at least 3 times in each condition in different donors and patients.
Upper panel: PMNs stained with a FITC-conjugated anti-myeloperoxidase antibody (green) and counterstained using DAPI (blue), ×20 and ×100 original magnification. NETs were identified as elongated extracellular DNA fibers (blue), associated to myeloperoxidase (green).
Lower panel: neutrophils from the same samples stained with May-Grünwald-Giemsa (×60 original magnification), showing extracellular cytoplasmic extensions associated with NET images
A: unstimulated PMNs from healthy donors
B: NETs induced in healthy donors PMNs exposed to ionomycin for 4 h
C: PMNs isolated from patients with septic shock without disseminated intravascular coagulation (DIC)
D: PMNs isolated from patients with septic shock with DIC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

occurrence, chromatin decondensation associated to the modifications of plasma membrane may indicate that NET formation occurs by a "vital NETosis" mechanism, as depicted by de Buhr et al. [28].

Nevertheless, our data support the *in vivo* existence and relevance of NETs in humans. For the first time, evidence of the presence of circulating NETs in the blood of patients with septic shock-induced DIC was provided, in accordance with the observation of McDonald et al. who showed that NETs promote intravascular coagulation in mice [38].

Intravascular NETs may induce platelet trapping and microvascular occlusion leading to multi-organ failure. Accordingly, destruction of NETs *via* DNAse treatment was associated to the impairment of intravascular coagulation, suggesting that the integrity of NETs is critical for coagulation activation [38]. New treatments targeting NET formation such as DNAse or Peptidyl arginine deiminase-4 (PAD4) enzyme that mediates histone citrullination may represent an interesting therapeutic approach during septic shock [10,39]. Indeed, DNAse or PAD-4 inhibitors improved organ perfusion and survival in animal models of sepsis [38–42]. NET components, especially histones, also stand out as potential therapeutic targets [43]. For instance, anti-histone antibody infusion reduced neutrophil-induced cell damages and lung congestion

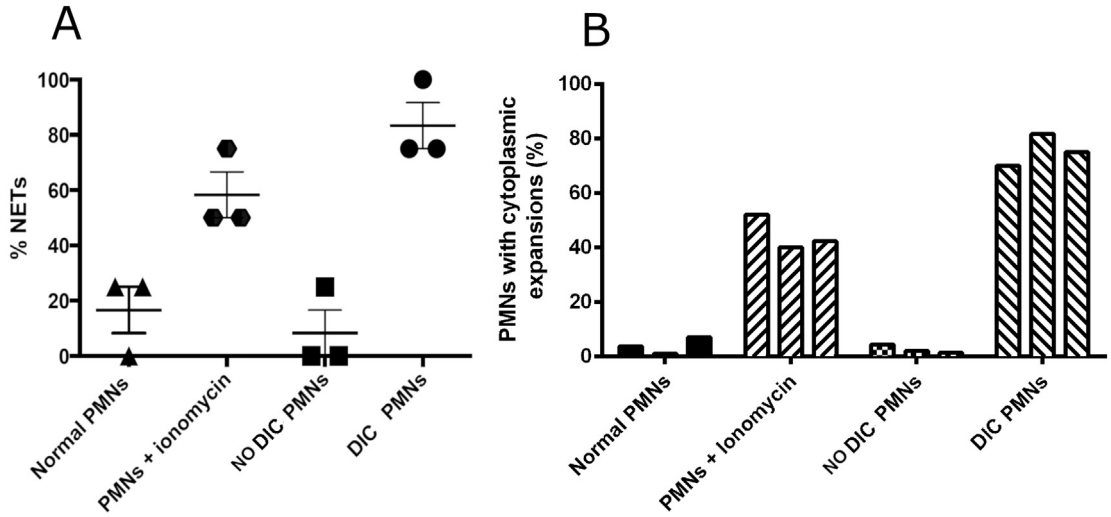

Fig. 2. Quantification of NETS and cytological modifications.
A. NETs quantification using immunofluorescence microscopy (% neutrophils forming NETs per microscopic field, ×20 magnification)
B. Representation of cells harboring cytoplasmic expansions, expressed in percentage of PMNs, determined blindly by 3 different cytologists in all samples.

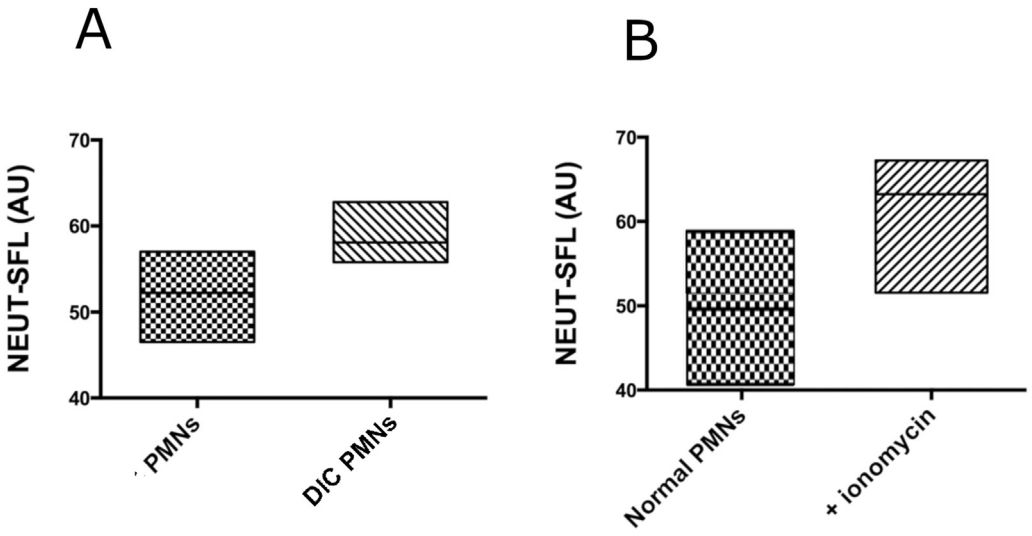

Fig. 3. Neutrophil Side Fluorescence Light (NEUT-SFL) measured on Sysmex XN20.
A. NEUT-SFL in no-DIC patients and DIC patients
B. NEUT-SFL in healthy donors without stimulation and after ionomycin stimulation.
The floating bars represent minimum to maximum values and the black bar their mean.

in sepsis mice model [43].

Most of the treatments administered to DIC patients exhibits anticoagulant activity that increase the risk of bleeding. Standardization of the evaluation of NETs may help identify patients at different levels of risk in future randomized controlled trials. Multispectral imaging flow cytometry proposed by Zhao et al. is a promising sophisticated method to identify NETs [44], but is not routinely available in clinical practice. If confirmed, observation of the morphology of PMNs after cytocentrifugation and evaluation of their chromatin decondensation level using neutrophil side fluorescence (*e.g.* NEUT-SFL, [27]) may represent interesting tools.

5. Conclusions

In this pilot study, we have shown for the first-time direct evidence of circulating NETs in peripheral blood of patients with septic shock-induced DIC using immunofluorescence. These NETs are undistinguishable from NET features observed following *in-vitro* ionomycin stimulation of normal PMNs. NET images were furthermore associated to modifications of PMNs morphology observed with May-Grünwald-Giemsa staining. These features may of great interest for the stratification of patients undergoing future treatment protocols targeting NETs.

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Declaration of competing interest

Pr. Meziani's institution received funding from Stago.

The remaining authors have disclosed that they do not have any potential conflicts of interest.

Trial registration: Clinicaltrial.gov identifier: NCT #02391792.

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