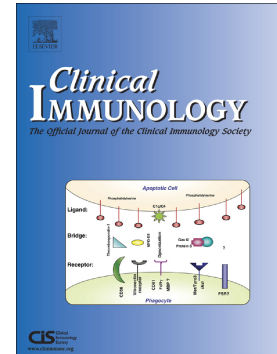


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Nucleosomes and Neutrophil Extracellular Traps in Septic and Burn Patients

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Abstract

NETosis is a host defense mechanism associated with inflammation and tissue damage. Experimental models show that platelets and von Willebrand factor (VWF) are key elements for intravascular NETosis.

We determined NETosis in septic and burn patients at 1 and 4 days post-admission (dpa).

Nucleosomes were elevated in patients. In septics, they correlated with Human Neutrophil Elastase (HNE)-DNA complexes and SOFA score at 1dpa, and were associated with mortality.

Patient's neutrophils had spontaneous NETosis and were unresponsive to stimulation. Although platelet P-selectin and TNF- α were increased in both groups, higher platelet TLR4 expression, VWF levels and IL-6 were found in septics at 1 dpa. Neither platelet activation markers nor cytokines correlated with nucleosomes or HNE-DNA.

Nucleosomes could be indicators of organ damage and predictors of mortality in septic but not in burn patients. Platelet activation, VWF and cytokines do not appear to be key mediators of NETosis in these patient groups.

Keywords: Neutrophils, NETs, Sepsis, Inflammation, Platelets, Burn

Abbreviations

cfDNA: cell free DNA; Cit: citrullinated; dpa: days post admission; HNE: human neutrophil elastase; ICU: intensive care unit; LPS: lipopolysaccharide; NET: neutrophil extracellular trap; PRP: platelet rich plasma; PI: propidium iodide; PGI₂: prostacyclin; SIRS: systemic inflammatory response syndrome; TBSA: total body surface area; TNF- α : tumor necrosis factor alpha; WP: washed platelets; VWF: von Willebrand factor.

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1. Introduction

The formation of neutrophil extracellular traps (NETs) is a novel antimicrobial process named NETosis through which neutrophils can trap and kill microbes [1]. Despite NETosis being initially described as a beneficial host response against pathogens, it is being increasingly recognised that the uncontrolled formation of these traps switches NETosis from a beneficial response into a major cause of tissue damage and organ failure [2-4]. Besides pathogens, cytokines or danger signals can trigger NET formation [5]. Thus, NETs can be formed not only in infectious conditions but also under inflammatory sterile states. In addition, NETs can be found in tissues as well as inside blood vessels [6]. Experimental models show that platelets, von Willebrand factor (VWF) and endothelial cells are key elements for the intravascular formation of NETs [7-9]. The role of these factors in NETosis occurring under clinical conditions is yet to be investigated.

Sepsis and severe burns are two acute inflammatory conditions, one triggered by an infectious insult and the other by tissue injury. Even though they share many aspects, and multiorgan failure is frequent in both pathologies, the evolution of these two entities is not exactly the same. Although early mortality in burn patients is mostly due to hypovolemic shock and inhalation injuries, in septic patients, multiorgan damage is the most frequent cause of death, being respiratory and renal failure the most common among them [10]. Following the initial 5–7 days period, the evolution of the burn patient becomes similar to that of the septic, existing three possible states, early death associated to a highly pro-inflammatory profile, resolution of the infection and progressive improvement of the organ function due to an adequate balance between pro- and anti-inflammatory states, or torpid and lengthy evolution due to a tendency to immunosuppression from the onset of the disease. It is noteworthy that multiorgan failure prevalence is higher in burn patients when they present acute inhalation injury in a similar way to what happens during sepsis-associated respiratory distress [11]. In this context, several studies in animal models have proven that the lung would be a key

organ in the NET formation process [6, 12, 13] and that it is there where a critical interaction between platelets and neutrophils takes place, leading to a systemic release of NETs and inflammatory cytokines.

Despite multiple technological breakthroughs in the support and treatment of the etiology, septic and burn patient's morbi-mortality remains high. Clinical management of these patients is challenging and largely limited to supportive therapies, which is in part related to a limited understanding of the underlying pathophysiology. After the discovery of the NETosis process, some studies in septic and a few in burn patients have analysed the role of cell free DNA (cfDNA) or nucleosomes in these inflammatory diseases. However, these biomarkers can be released upon several conditions including necrosis, apoptosis or NETosis, [14] and therefore, the real involvement of NETs in clinical inflammatory conditions is still not clear. In order to further understand the role of NETs in the inflammatory response, herein we aimed to determine the presence of NETs in septic and burn patients and correlate them with organ damage, patient evolution, platelet activation, VWF and pro-inflammatory cytokines.

2. Materials and Methods

2.1. Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Ethical Committee of the National Academy of Medicine, Bazterrica Clinic and Burns Hospital, Buenos Aires, Argentina. All individuals provided written informed consent for the collection of samples and subsequent analysis.

2.2. Subjects

The study was designed as a prospective cohort study. Inclusion criteria:

The subjects were patients admitted to the intensive care unit (ICU) of Bazterrica Clinic and Burns Hospital from April 2015 to December 2016. The study population consisted of 24 septic and 23 burned patients and 30 healthy adult volunteers who were not receiving any medication at the time of blood sampling. No attempt to match cases and controls was made.

Severe sepsis was diagnosed if all criteria of systemic inflammatory response syndrome (SIRS), evidence of two or more organ dysfunctions, and a proven septic focus were present, according to the ACCP/SCCM consensus conference [15].

Diagnosis of SIRS required at least two of the following: temperature $<36^{\circ}\text{C}$ or $>38^{\circ}\text{C}$, heart rate $>90/\text{min}$, respiration rate $>20/\text{min}$ or arterial $\text{PCO}_2 <32\text{mmHg}$ and white blood cell (WBC) count $>12,000/\text{mm}^3$ or $<4000/\text{mm}^3$, or shift to the left of the differential WBC count with band forms 10%. To evaluate organ dysfunction and the severity of illness, the SOFA score was determined.

Burn patients were included when presented with a burn degree of III and IV in the Garces index and samples were collected within 24 hs of the traumatic event. Burn total body surface area (TBSA) percent was determined at admission.

Patients were excluded from this study when anticoagulated for pre-existing pathologies, previous thrombotic events, previous platelet dysfunction or deep vein thrombosis history. We excluded patients less than 18 years of age, pregnant, with

chronic renal failure, and if within 14 days of a surgical procedure. Clinical data related to the course of hospitalization, including need for invasive mechanical ventilation, occurrence of system organ failure and clinical outcomes were recorded.

2.3. Reagents

RPMI 1640 medium and Ficoll–Hypaque (1.077 g/m density) were purchased from GE Healthcare (Buckinghamshire, UK). Dextran from *Leuconostoc* spp, purified LPS derived from *Escherichia coli* O111:B4, poly-L-Lysine, thymus DNA and propidium iodide (PI) were purchased from Sigma Aldrich (St. Louis, MO, USA). SYBR gold and anti-rabbit Alexa 488 were purchased from Invitrogen (Carlsbad, CA, USA). Prostacyclin (PGI₂) was purchased from Cayman, (Ann Arbor, MI, USA). Recombinant TNF- α was from Peprotech (Rocky Hill, NJ, USA). Micrococcal nuclease (MNase), cell death detection ELISAPLUS kit and peroxidase-labeled anti-DNA mAb were purchased from Roche Diagnostics (Mannheim, Germany). Rabbit anti-human neutrophil elastase (HNE) antibody was from Calbiochem–Merck Millipore (Darmstadt, Germany). Human interleukin (IL)-6 and human TNF- α ELISA kits were purchased from eBioscience (San Diego, CA, USA). FITC-conjugated mouse anti-human CD62P and FITC-conjugated irrelevant IgG1 were from Becton Dickinson (San Jose, CA, USA). Anti-human VWF and HRP-conjugated anti-human VWF were obtained from Dako (Glostrup, Denmark). PE-conjugated anti-human TLR-4 and PE-conjugated irrelevant IgG2a were from BioLegend (San Diego, CA, USA).

2.4. Blood sampling and testing

Collection of blood samples from septic patients was performed within 24 hs from admission to the ICU and 4 days later. Venous blood was collected aseptically from each patient and donor into EDTA–coated pyrogen-free tubes. The WBC count and the neutrophil count were determined with an automated analyser (Diatron, Hungary). Blood samples were processed within 2 hs of collection.

2.5. Isolation of neutrophils

Neutrophils were isolated by Ficoll Hypaque gradient centrifugation and dextran sedimentation, as described previously [16]. Cell suspensions contained >98% neutrophils, as determined with an automated analyser. Cells were resuspended in RPMI 1640 supplemented with fetal bovine serum (FBS, 1%).

2.6. Preparation of platelets

Platelet rich plasma (PRP) from normal donors and patients was obtained by the centrifugation of blood samples ($180 \times g$ for 10 min). For washed platelet (WP) suspensions, PRP was centrifuged in the presence of 75 nM PGI₂. After washing in wash buffer, the WP were resuspended in RPMI 1640 supplemented with FBS (1%) at 2.4×10^8 /ml. Platelets were incubated with 1 µg/ml LPS for 30 min at 37°C [16].

2.7. Ex vivo NET formation

Neutrophils (5×10^5 /ml) were incubated alone, with platelets, LPS and LPS-stimulated platelets or 20 ng/ml of TNF- α for 1 and 3 hs, respectively, in a humidified incubator at 37°C with CO₂ (5%). In all experiments, NET formation was visualised by immunofluorescence microscopy and quantified by evaluation of the released DNA in the supernatants by fluorometry [16].

2.8. Immunofluorescence assays

Neutrophils were adhered on poly-L-lysine-treated coverslips and placed in 24-well, flat-bottom plates. After stimulation, the cells were fixed with paraformaldehyde (PFA 1%) and blocked with 5% heat inactivated FBS. Cells were then stained with rabbit anti-HNE (1:1000) or the corresponding IgG controls and the secondary antibody anti-rabbit Alexa 488. DNA was stained with PI (2 mg/ml) and the coverslips were mounted on slides. Mounted specimens were analysed by confocal fluorescence microscopy

using a FV-1000 microscope (Olympus, Tokyo, Japan) equipped with a Plapon 360/NA1.42 objective [16].

2.9. Quantification of extracellular DNA

DNA released from neutrophils during NETosis was digested with MNase (500 mU/ml) for 15 min. EDTA (5 mM) was added to stop the nuclease activity. Supernatants were collected, centrifuged, and DNA was measured in the supernatants using SYBR Gold in a fluorometer (BioTek Instruments, VT, USA). The calibration curve was constructed using thymus DNA of a known concentration [17].

2.10. Measurement of nucleosomes, VWF, IL-6 and TNF- α

Plasma from healthy donors and patients was obtained by blood centrifugation (900 \times g for 10 min). Plasmatic levels of nucleosomes, IL-6 and TNF- α were measured using commercial ELISA kits. Plasmatic levels of VWF were determined by ELISA as described previously using human VWF antibody and HRP-conjugated human VWF antibody as primary and secondary antibodies, respectively. The results were expressed in ng/ml and extrapolated from serial dilutions of normal pooled plasma, assuming a 7 μ g/ml VWF concentration [16].

2.11. HNE-DNA complexes ELISA

Quantification of HNE-DNA complexes was performed as previously described [13]. Briefly, 96-well plates were coated with 5 mg/ml anti-HNE antibody overnight at 4°C. After washing three times, plasma samples were added with incubation buffer containing a peroxidase-labeled anti-DNA mAb (dilution 1:25). The plate was then incubated for 2 h, shaking at 300 rpm at room temperature (RT). After three washes, 100 μ l of peroxidase substrate (ABTS) were added. Absorbance at 405 nm wavelength was measured after 20 min incubation at RT in the dark. Values for soluble HNE-DNA complexes were expressed as optical density (OD).

2.12. Flow cytometry

Aliquots of PRP were fixed and stained with a FITC-CD62P (anti-P-selectin) or an equivalent amount of isotype-matched control Ab. For detection of TLR-4, aliquots of PRP were stained with PE-conjugated anti-TLR4 or PE-conjugated irrelevant IgG2a as isotype control.

Staining was performed by 30 minutes incubation in the dark at RT. Then, samples were fixed with PFA (1%) and analysed by flow cytometry on a FACSCalibur using CELLQUEST software (Becton Dickinson, San Jose, CA, USA) and results were expressed as mean of fluorescence intensity (MFI).

2.13. Statistical analysis

Data were analysed for normality using the Shapiro-Wilk test. Univariate analysis for both patient groups was conducted using median and 25–75 interquartile range (IQR). Differences between quantitative data (patients vs. healthy donors) were explored using median nonparametric tests. A Spearman correlation was used to compare nonparametric variables (septic patients SOFA score, burn patients TBSA percent, nucleosome levels and HNE-DNA complexes) and the corresponding R square coefficients were obtained.

Patient's initial response (1 dpa) was compared between patients groups and with healthy donors using Kruskal-Wallis test. In order to assess patients' evolution, the inflammatory response after 4 dpa was compared intra and inter groups.

Logistic regression was conducted to examine the relationships between nucleosome levels and mortality within 30 days from admission to the ICU. Infostat and GraphPad softwares were used for the statistical analyses. Values of $p < 0.05$ (two tailed) were considered statistically significant.

3. Results

3.1. Patients

Demographic and clinical data for the 47 enrolled patients are shown in Table 1. The age difference between septic and burn patients is due to the epidemiology of both pathologies, where at both global and regional level, the average age for sepsis is 69 years old [18] while for burn patients it is 40 years old mostly due to work -related accidents [19].

Even though thrombocytopenia (platelet count $<140 \times 10^9/l$) was observed in 21% of septic patients and 39% of burn patients, overall, no statistical differences with healthy donors were found.

Table 1.

Patient's demographics at admission to the ICU.

Variable	Sepsis	Burns
N	24	23
Age in years (IQR)	70 (64:79)	39 (26:45)
Female sex	11	8
SOFA score (sepsis)	7 (5:10)	-
TBSA % (burn)	-	33 (25:39)
Survived > 31 days (Y:N)	19:5	17:6

Days in ICU (IQR)	20 (9:36)	42 (28:45)
MOF (Y:N)	24:0	
WBC 10⁹/l (IQR)	17 (11:22)	10 (6:12)
Lymphocytes 10⁹/l (IQR)	0.75 (0.6:1.4)	1.1 (0.7:1.4)
Granulocytes 10⁹/l (IQR)	15 (10:20)	9 (4:11)
Platelets 10⁹/l (IQR)	223 (151:305)	186 (125:280)
Plasma VWF (IQR)	33 (25:44)	25 (20:29)
Plasma TNF-α (IQR)	120 (0:292)	90 (25:139)
Plasma IL-6 (IQR)	351 (64:428)	63 (19:156)

Y:N = Yes/No; MOF = multiple organ failure.

3.2. Nucleosomes and HNE-DNA complex are increased in septic and burn patients

To explore the occurrence of NETosis in septic and burn patients, levels of circulating nucleosomes were analysed at 1 and 4 dpa to the ICU. Figure 1 A shows that both groups of patients had significantly increased nucleosomes levels at 1 dpa compared to control subjects and remained elevated by 4 dpa.

Since nucleosomes can be released not only during NETosis, but also as a result of other cellular processes like necrosis or apoptosis [14], we also analysed the presence of HNE-DNA complexes that are specific indicators of NET formation [13]. Figure 1 B

depicts the levels of HNE-DNA complexes determined by ELISA. Similar to nucleosomes, while healthy donors had almost no circulating levels of HNE-DNA complexes, septic and burn patients showed significantly higher levels than controls throughout the study.

Remarkably, while nucleosomes showed good correlation with the HNE-DNA complexes in septic patients either at 1 or 4 dpa, there was no correlation of these two parameters in burn patients at any time studied (Fig. 1 C). Studying patients severity, we found that nucleosomes levels correlated with septic's SOFA score at 1dpa ($p = 0.03$) explaining near 20% of SOFA's variability ($R^2 = 0.195$, Fig. 1 D). No significant correlations were found between nucleosome levels and TBSA% in burn patients (Fig. 1 E).

In septic patients, nucleosome levels at admission were associated with mortality (Wald = 5.31, $p = 0.02$). This was not the case with burn patients (Wald = 0.14, $p = 0.71$).

3.3. Patient's neutrophils spontaneously form NETs and are unresponsive to stimuli

Besides pathogens, NETs can be triggered by several stimuli including cytokines [20] and activated platelets [13, 16, 21]. To determine the ability of patient's neutrophils to generate NETs, we examined NETosis induced by LPS in the absence or presence of platelets and by the pro-inflammatory $TNF-\alpha$. As expected, immunofluorescence studies showed that non-stimulated neutrophils from healthy donors in the absence or presence of platelets did not undergo NETosis (Fig. 2 A). Although addition of LPS or $TNF-\alpha$ (Fig. 2 A) significantly induced NETs, pre-stimulation of platelets with LPS increased NETosis. In contrast, neutrophils from septic or burn patients formed NETs spontaneously and they did not respond to further stimulation with LPS-activated

platelets or TNF- α (Fig. 2 A). Similar results were obtained when DNA release was quantified by fluorometry (Fig. 2 B).

3.4 Platelet Activation, VWF levels and NETs formation

In experimental mice models of sepsis, platelets and VWF appeared to be required for intravascular NET formation [7-9]. To analyse whether a similar situation occurs under inflammatory clinical conditions we evaluated platelet count, expression of P-selectin and circulating VWF levels in septic and burn patients.

Even though it has been reported that sepsis and burns often leads to thrombocytopenia [22], we encountered a great variability in the platelet count in both groups of patients resulting in no statistical differences between them and healthy donors (Fig. 3 A). However, flow cytometry studies revealed that platelets from septic and burn patients showed an augmented expression of P-selectin (Fig. 3 B).

Considering that platelet TLR4 is a critical mediator of NET formation [6], we also analysed this receptor's expression. Septic patients showed an increased expression of TLR4 compared to healthy donors at 1 dpa and remained elevated at 4 dpa. In contrast, in burn patients, the TLR4 increased was only observed after 4 dpa (Fig. 3 C). As expected, ELISA quantitation showed significantly higher values of VWF in septic and burn patients compared to control samples. However, in burn patients, the levels of VWF were higher at 4 dpa than at 1 dpa. (Fig. 3 D)

Despite evidence of platelet activation and increased levels of VWF in septic and burn patients, linear regression analysis showed no correlation between these biomarkers and nucleosomes or HNE-DNA complexes.

3.5 Inflammatory cytokines are not associated with NETs formation neither in septic nor in burn patients

Considering the involvement of the pro-inflammatory cytokines IL-6 and TNF- α in the acute phase response of sepsis and severe burns [23, 24] and their role as triggers of NETosis [5, 20], we studied their levels in the plasma of septic and burn patients.

Although IL-6 and TNF- α were not detectable in the plasma of healthy donors, septic and burn patients had very high levels of both cytokines at 1 dpa. IL-6 was higher in septics compared to burn patients at admission and in the former, these values were significantly reduced at 4 dpa but still remained higher than the control group (Fig. 4).

On the other hand, TNF- α levels showed no variation after 4 dpa in either group.

None of these cytokines showed any association with levels of nucleosomes or HNE-DNA complexes nor did they seem to play a role in organ damage scores or mortality.

4. Discussion

In this study, we have shown that nucleosomes and HNE-DNA complexes are elevated in both septic and burn patients, but only in septic patients, nucleosomes correlated with organ damage and were predictors of mortality. Despite platelet activation and increased levels of inflammatory cytokines, neither of them appeared to be triggers of DNA release on the population studied.

Increased levels of cfDNA leading to high discriminative power to predict ICU mortality in patients with severe sepsis have been previously reported [25-27]. However, since cfDNA can be released not only during NETosis, but through various cellular processes including necrosis or apoptosis [14], the real role of NETs could not be extrapolated from these studies. The positive correlation between nucleosomes and HNE-DNA complexes observed in septic patients in our study indicates that nucleosomes most probably reflect NETs formation. On the other hand, the direct relationship between levels of nucleosomes, but not of HNE-DNA complexes with SOFA score, together with the fact that nucleosomes are good predictors of mortality, suggests that extracellular DNA is, among other factors, involved in the organ damage of septic patients and a more sensitive biomarker than HNE-DNA complexes. Moreover, we found that neutrophils from septic patients had a higher level of spontaneous NETosis and they were not capable of responding to further stimuli compared to control neutrophils. Several factors have been reported to be involved in the induction of NETs. Therefore, it is possible that exposure of leukocytes to high levels of endogenous and exogenous mediators released during sepsis could have rendered these cells unresponsive to stimuli. In fact, plasma levels of inflammatory cytokines known to prime and activate neutrophils such as IL-6 and TNF- α were elevated in the population of septic patients studied. Our data are in agreement with the study of Masamitsu Hashiba et al [27] who found not only an impaired NET formation in response to PMA stimulation but also an ineffective *E. coli* killing by NETs formed by neutrophils purified from septic patients. The inability of neutrophils from septic patients to form NETs upon stimulation might

represent another mechanism by which neutrophil dysfunction promotes sepsis and even leads to organ failure [28].

In experimental mice models, it has been shown that platelets, endothelium and VWF are intimately involved in the formation of intravascular NETs [7-9]. Our observation that platelets from septic patients had increased expression of P-selectin and TLR4 indicated an ongoing process of platelet activation. In addition, we also found increased levels of VWF in these patients. However, neither of these biomarkers correlated with HNE-DNA complexes nor nucleosomes suggesting that at least in this studied population and contrary to animal models, platelets and VWF do not appear to take part in the development of NETs. TLRs are well known receptors involved in recognising pathogens and initiating innate immune responses [29]. Even though platelets express all members of the TLR family [30, 31], their role in the physiopathology of the inflammatory response is still not clear. It was suggested that platelet TLR4 functions as a barometer for systemic infection, binding avidly to sequestered neutrophils during septicemia and endotoxemia in the capillaries of lungs and liver leading to the rapid formation of NETs, which maintain their integrity under flow conditions [6]. Thus, even though we could not find a positive correlation with NETs, we do not discard the possibility that the increased expression of platelet TLR4 observed in our study was associated with an augmented recruitment and adhesion of neutrophils.

Similar to septic patients, NETosis is not yet clearly identified in burn patients. Three studies showed that cfDNA levels are significantly increased already on day 1 after admission [32] and two others that cfDNA is a valuable marker for prediction of mortality [33, 34]. However, the source of this DNA and whether it reflected the formation of NETs was not addressed in either of the studies. In contrast, in a recent study, it was observed that cfDNA was elevated only after 7 days in burn patients during septic episodes. Interestingly, they also found the presence of plasmatic citrullinated (Cit)-H3, a direct indicator of NET formation, in nine patients. However, as

there were no quantitative data of Cit-H3 levels, correlation between these two biomarkers was not established [35]. In our study, we found increased levels of nucleosomes and HNE-DNA complexes at admission to the ICU and 4 days after. In contrast to septic patients, there was not a positive correlation between these two biomarkers indicating that the origin of nucleosomes include other sources of cellular death beyond NETosis and highlighting that measurement of cfDNA cannot be considered an index of NET formation in these patients. Moreover, neither nucleosomes nor HNE-DNA complexes correlated with the burn surface area suggesting that they are not directly involved in nor are a consequence of tissue damage. Like septic patients, burn patients showed an increase *ex vivo* basal formation of NETs and the neutrophils were unresponsive to further stimulation. These data are in agreement with those obtained by Hampson et al who also found reduced NETosis by PMA stimulation in burn-injured patients at days 3 and 7 post injury. In addition, burn patients had increased P-selectin expression as well as high levels of VWF and pro-inflammatory cytokines at day 1 post admission compared to healthy controls. However, the degree of the inflammatory response in burns was milder than in septic patients as they showed no increase in platelet TLR4 expression and the levels of VWF and IL-6, despite being higher than healthy controls, were significantly lower in burn than in septic patients. Notably, at 4 day after admission, platelet TLR4 expression increased together with plasmatic level of VWF. Even though it could be reasoned that the increase of these biomarkers was associated to a septic episode, none of the patients studied showed clinical signs of sepsis or had positive bacteria cultures. In conclusion, our results indicate that regardless whether the inflammatory insult is infectious or not, nucleosomes and HNA-DNA complexes are augmented *in vivo* and *ex vivo* in septic and burn patients. Although nucleosomes appear to be involved in the tissue damage of sepsis and are potential predictors of mortality, they do not have the same relevance in burn patients.

Despite progress being made in improving treatment for sepsis and severe burns, they both are pathologies with high mortality rates. The relevance that nucleosomes seem to have in the evolution of sepsis, points towards nucleosomes quantification being a feasible new biomarker which could be used alongside well-established ICU severity scores in order to improve outcome prediction in patients and provide new tools for physicians in their effort to advance on bedside care.

Larger studies with a longer follow-up are needed in order to better assess the sensitivity and specificity of NETs as potential predictors of death in septic patients.

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Figure captions**Fig. 1.** Circulating nucleosomes and NETs.

A) Nucleosome levels and B) HNE-DNA complex levels (OD) were measured in plasma by ELISA in septic (n = 24) and burned (n = 23) patients compared with controls (n = 30). **p < 0.01, ****, p < 0.001, Kruskal–Wallis. Correlation between nucleosome levels and HNE–DNA complexes for C) septic and B) burned patients at 1 and 4 dpa, Spearman test. Correlation between nucleosome levels and D) SOFA Score for septic or E) Total Body Surface Area percentage (TBSA %) for burned patients at 1 and 4 dpa, Spearman test.

Fig. 2. *Ex vivo* NETs formation.

A) Neutrophils ($5 \times 10^5/\text{ml}$) were placed on poly-L-lysine-treated coverslips in 24-well, flat-bottom plates and incubated alone, with LPS, platelets (Plts), and LPS-stimulated platelets or 20ng/ml of TNF- α for 1 and 3 hs, respectively, in a humidified incubator at 37°C with CO₂ (5%). Cells were then fixed and stained for PI (red) and HNE (green). B) DNA release was quantified in the supernatants by fluorometry. Results were compared vs non-stimulated healthy donors and between groups, *p < 0.05, Kruskal–Wallis.

Fig. 3. Platelets and endothelium activation.

A) Platelet count was obtained using an automated analyser and expressed as cells per liter. B) Platelet P-selectin and C) TLR4 expression were measured by flow cytometry and expressed as MFI, *p < 0.05, **p < 0.01, Kruskal–Wallis. D) VWF plasmonic levels were measured by ELISA and are expressed as $\mu\text{g}/\text{ml}$, *p < 0.05, ***p < 0.001, Kruskal–Wallis.

Fig. 4. Plasma levels of IL-6 and TNF- α in septic and burn patients.

Plasma A) IL-6 and B) TNF- α were quantified by ELISA and results are expressed as pg/ml. *p < 0.05, ***p < 0.001, Kruskal–Wallis.

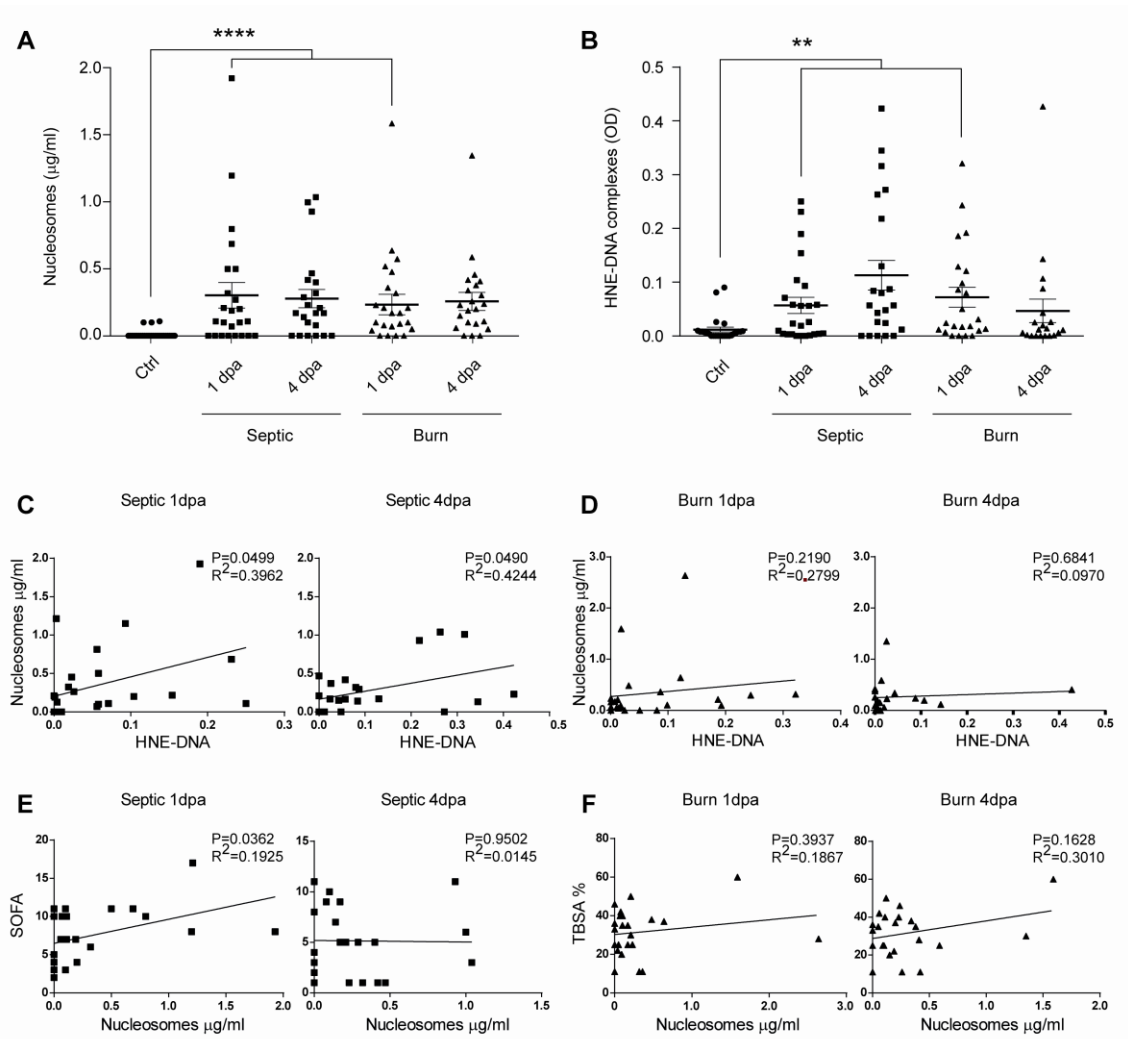


Figure 1

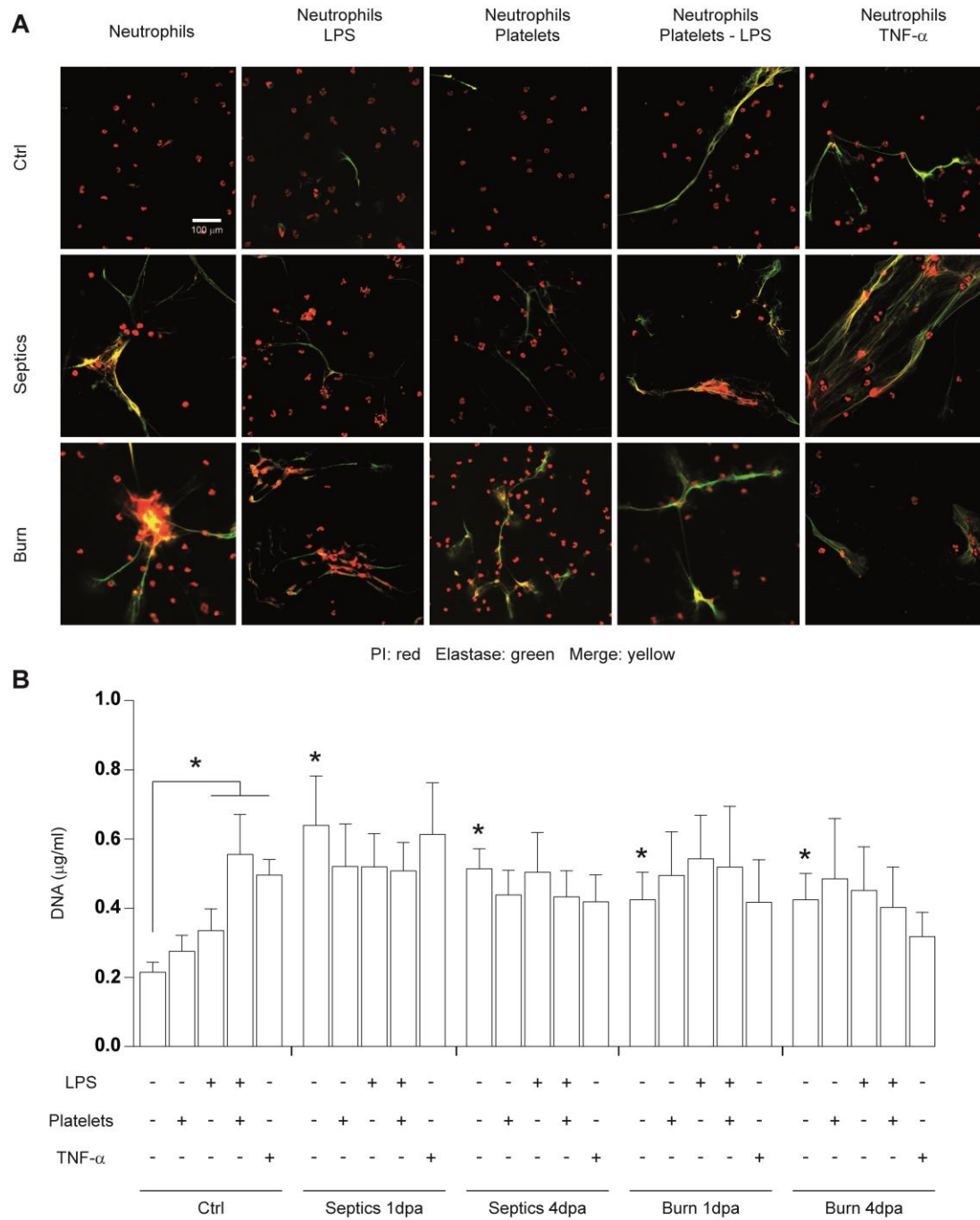


Figure 2

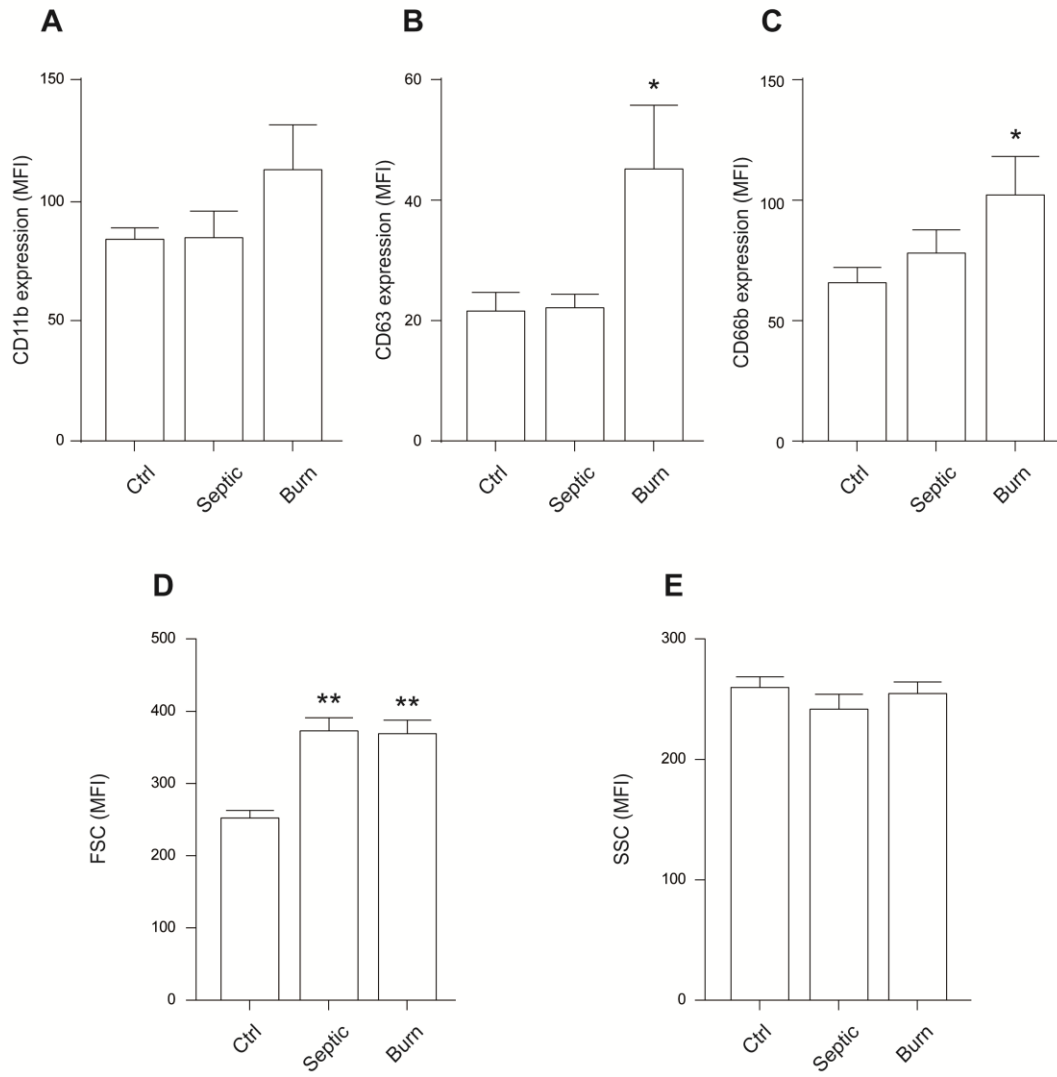


Figure 3

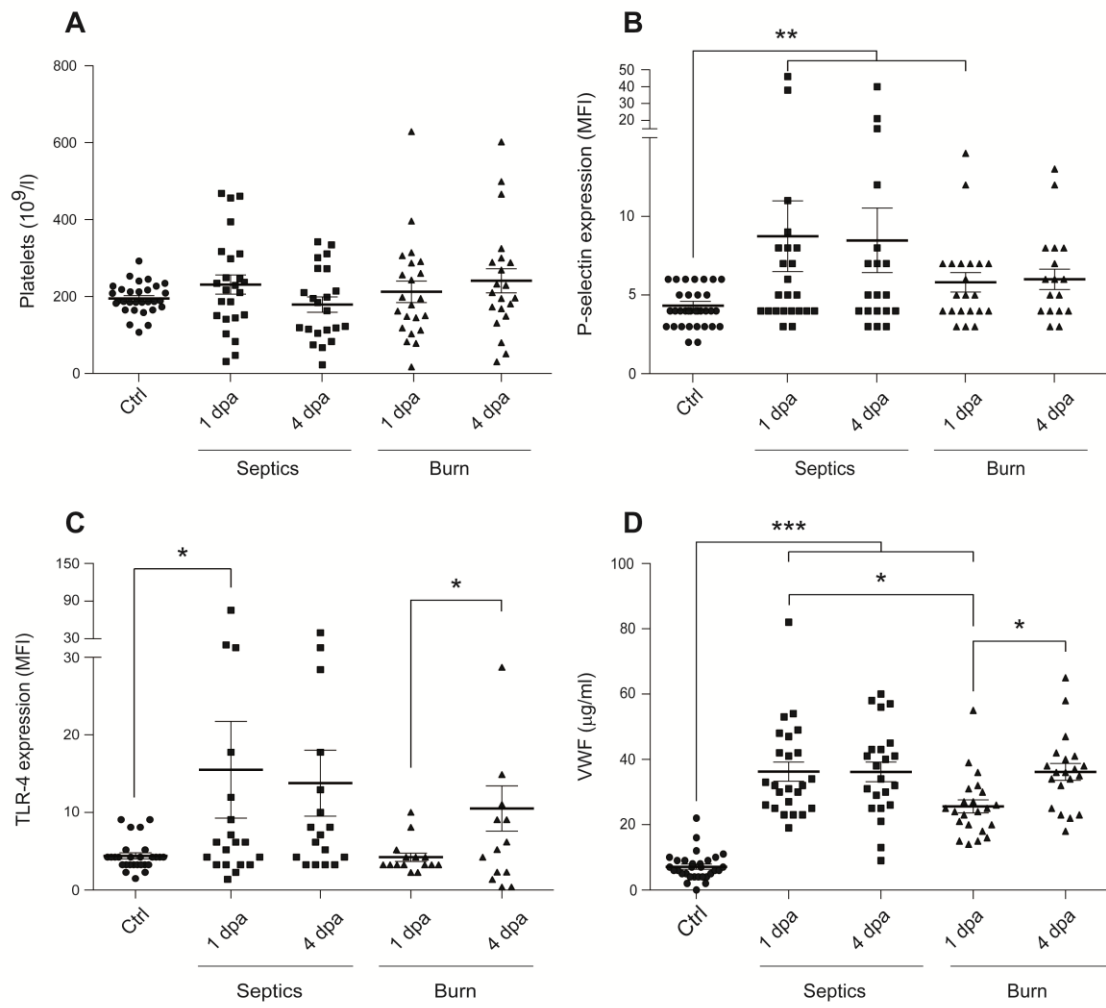


Figure 4

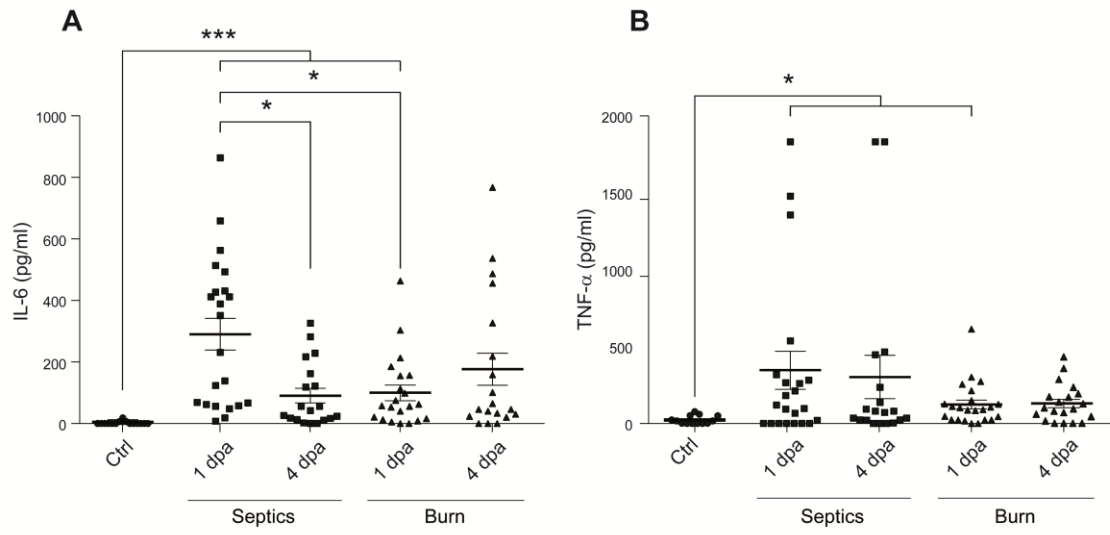


Figure 5

Highlights

- Nucleosomes and HNE-DNA complexes are elevated in septic and burn patients.
- Septics' nucleosomes correlate with SOFA at 1dpa and were associated with mortality.
- Patient's neutrophils exhibit spontaneous NETosis and are unresponsive to stimulation.
- Nucleosomes could be organ damage indicators and mortality predictors in septics.
- Platelets, VWF and cytokines do not seem to mediate NETosis in these patients.

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