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Plasma H3.1 nucleosomes as biomarkers of infection, inflammation and organ failure

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Abstract

Background Neutrophil extracellular traps (NETs) are a vital part of the innate immune response, while excessive NET formation can cause tissue damage. H3.1 nucleosomes, a component of NETs, have emerged as a potential biomarker. This study aimed to evaluate H3.1 nucleosomes in critical illness, assessing their relationship with sepsis, organ failure, inflammatory subphenotypes and outcomes.

Methods The MARS cohort was used, comprising of consecutive Intensive Care Unit patients, with plasma samples collected on days 0, 2 and 4. H3.1 nucleosome concentrations were measured using the Nu.Q® NETs Immunoassay. H3.1 nucleosome concentrations were compared across sepsis presence and organ failure, both at baseline and longitudinally. The relationship between H3.1 nucleosome concentrations and clinical outcomes was investigated.

Results 1713 critically ill patients were included, with a total of 3671 plasma samples. Baseline H3.1 nucleosome concentrations differed between sepsis confirmed by clinical adjudication (740 ng/mL), sepsis unconfirmed by clinical adjudication (416 ng/mL) and non-sepsis (463 ng/mL, $P < 0.001$). H3.1 concentrations were associated with SOFA score ($r = 0.40$) and were higher in patients with disseminated intravascular coagulation, acute kidney injury and hyperinflammatory sepsis. H3.1 concentration was highly predictive for the need of renal replacement therapy (hazard ratio 2.00 per log10 increase), correcting for mortality.

Conclusions Sepsis and organ failure were closely associated with plasma H3.1 nucleosome concentrations. While individual diagnostic performance for sepsis and organ failure remained low, H3.1 levels predicted the need for renal replacement therapy and disseminated intravascular coagulation, revealing unique insights into the innate immune response.

Trial registration: ClinicalTrials.gov identifier NCT01905033; IRB number 10-056C, registered June 16, 2010.

Keywords Neutrophil extracellular traps, Sepsis, Nucleosomes, Organ failure, Critical care

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Introduction

Neutrophil extracellular traps (NETs) have emerged as crucial players in the body's immune response [1]. NETs, composed of DNA, histones, and antimicrobial proteins, are released by neutrophils through a process called NETosis [2]. While they help fight infections by immobilizing and killing microorganisms, excessive NETosis can contribute to tissue damage and organ dysfunction [3, 4]. This dual role makes NETs potential biomarkers for understanding sepsis and organ dysfunction [3, 5–8].

Sepsis, a life-threatening condition driven by a dysregulated immune response to infection, often leads to organ dysfunction [9, 10]. Profiling of the host response can provide valuable insights into disease mechanisms, particularly in critically ill patients. [11]. Considering the central role of NETs in inflammation and immune dysregulation, NETs can be implicated in the pathogenesis of conditions such as acute kidney injury (AKI), disseminated intravascular coagulation (DIC), and acute respiratory distress syndrome (ARDS). These conditions, while distinct, share common pathways of endothelial dysfunction, thrombosis, and inflammation, which are also key features of excessive NET formation [12–15].

Recent in vitro, in vivo, and small-scale clinical studies suggest that NET components may contribute to direct and distant organ injury [16–21]. Due to their large molecular size, NETs are not readily filtered by the glomerulus, implying that elevated plasma levels are unlikely to result from impaired renal clearance [2, 22]. Instead, they may reflect an active inflammatory process contributing to tissue injury, suggesting their potential as a unifying biomarker across different types of organ dysfunction. Furthermore, NETosis is potentially modifiable, with therapeutic interventions such as DNase treatment or inhibition of peptidylarginine deiminase (PAD) enzymes offering avenues for mitigating NET-driven pathology [23].

H3.1 nucleosomes represent a particularly promising biomarker, as they are specifically enriched in chromatin released during NET formation, distinguishing them from nucleosomes derived from apoptosis or necrosis [5, 24]. This specificity provides insights into the processes driving inflammation and organ failure. Despite their potential, H3.1 nucleosomes concentrations in critically ill patients with infection, inflammation and organ failure remain underexplored, particularly in large-scale studies.

Therefore, this study aimed to evaluate plasma levels of H3.1 nucleosomes in critically ill patients. The primary objective of this study was to evaluate the relationship between H3.1 nucleosome concentrations and sepsis, specifically investigating whether levels differ among patients with confirmed sepsis, unconfirmed sepsis, and non-sepsis. The secondary objective was to explore the associations between H3.1 nucleosomes and AKI, DIC, ARDS, inflammatory biomarkers, and inflammatory subphenotypes. Additionally, we aimed to assess whether H3.1 nucleosome levels could predict mortality, organ failure and renal replacement therapy in critically ill patients. We hypothesize that H3.1 nucleosomes are positively associated with sepsis, inflammatory biomarkers, organ failure severity, mortality and RRT initiation.

Methods

Study design and ethics

This is a secondary analysis of plasma samples collected as part of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) study, a prospective cohort study performed in two academic Intensive Care Units (ICUs) in the Netherlands from 2011 through 2013 (ClinicalTrials.gov NCT01905033; IRB 10-056C, registered June 16, 2010). The MARS study included consecutive patients with an expected stay of more than 24 h, regardless of their admission diagnosis, and aimed to compare patients with sepsis to those who were critically ill due to non-infectious causes. Only MARS patients with a comprehensive set of available biomarkers were included in this analysis [25, 26]. We analyzed samples collected at day 0 (typically within a few hours of ICU admission, or the first morning sample when admitted overnight), as well as on days 2 and 4 following admission.

Nucleosome measurements

H3.1 nucleosome concentrations were measured using the Nu.Q® NETs Immunoassay (CE-IVDD, Belgian Volition SRL, Isnes, Belgium), as detailed in Appendix A. This assay uses chemiluminescence technology, performed on the IDS-i10 automated analyzer system. Plasma samples above 1200 ng/mL were automatically diluted 1:5, and samples exceeding 6000 ng/mL were manually diluted before retesting to ensure accurate quantification.

Sepsis and infection likelihood

Sepsis was classified according to the Sepsis-3 criteria, with further categorization based on the Sequential Organ Failure Assessment (SOFA) score and the likelihood of infection according to CDC criteria [27]. Our critically ill patients were classified into three categories: (1) non-sepsis, comprising patients who did not meet Sepsis-3 criteria and in whom an infection was not considered to play a role; (2) confirmed sepsis, defined as meeting Sepsis-3 criteria with a probable or culture-confirmed infection, as determined by two independent assessors; and (3) unconfirmed sepsis, referring to patients who met Sepsis-3 criteria but in whom infection was not considered probable by both assessors. The adjudication was based on a structured, post hoc review

of all available clinical, radiological, and microbiological data. This process is described in detail in a separate publication [27]. Among patients with confirmed sepsis, a further subdivision of septic shock was made based on lactate levels (> 2 mmol/L) and vasopressor usage.

Organ failure

Organ failure was assessed across all patients (sepsis confirmed, sepsis unconfirmed, and non-sepsis) and was defined using the following criteria: Acute Kidney Injury (AKI) was diagnosed according to the RIFLE criteria [28], defined as an abrupt decline in kidney function with either a ≥ 1.5-fold increase in serum creatinine (Risk), a ≥ twofold increase (Injury), or a ≥ threefold increase (Failure), or an absolute creatinine level of ≥ 4.0 mg/dL with an acute rise of at least 0.5 mg/dL (Failure). Urine output criteria included < 0.5 ml/kg/h for ≥ 6 h (Risk), ≥ 12 h (Injury), or ≥ 24 h (Failure) despite optimized volume status. Disseminated Intravascular Coagulation (DIC) was assessed using the ISTH scoring system [29] based on platelet count, fibrin markers (e.g., D-dimer), prothrombin time (PT), and fibrinogen levels, with a score of 5 or more indicating overt DIC and less than 5 suggesting non-overt DIC [30]. Acute Respiratory Distress Syndrome was defined according to American-European-consensus criteria and later checked for consistency with the Berlin definition [31]: acute onset, bilateral infiltrates consistent with pulmonary edema, not explained by cardiac failure or fluid load alone, and a PaO_2/FiO_2 ratio below 300 mmHg. An additional subdivision was created to classify severity levels as mild (PaO_2/FiO_2 < 300), moderate (< 200), and severe (< 100). See appendix B for a detailed description of these organ failure scores.

Inflammatory host response

Using the admission day sample, the following biomarkers were measured using a cytometric bead assay; Interleukin (IL)-6, IL-8, Protein C and Tumor necrosis factor (TNF-α). Established inflammatory subphenotypes Hypoinflammatory and Hyperinflammatory—characterized by distinct patterns of immune response, clinical outcomes and treatment susceptibility—were assigned using a previously published biomarker model consisting of IL-6, bicarbonate and Protein-C (Appendix C) [32, 33].

Event outcomes

We used the need for RRT as a time-dependent outcome as we considered this a relatively objective endpoint for severe kidney failure. In the participating hospitals, RRT therapy was delivered using continuous veno-venous hemodialysis (CVVHD) and it was typically initiated based on standard indications: severe hyperkalemia, persistent metabolic acidosis or fluid overload. We considered mortality up to 90 days as a competing risk, and as an outcome on itself. Based on a prior study involving a smaller cohort of critically ill patients compared to healthy controls, as well as supporting in vitro research, we applied a predefined H3.1 nucleosome concentration cut-off of 1000 ng/mL [15, 22, 24].

Statistical analysis

H3.1 nucleosome concentrations were visualised using boxplots. To evaluate the diagnostic performance of H3.1 nucleosome concentrations, the area under the receiver operating characteristic curve (AUROC) was calculated along with their 95% confidence intervals by bootstrapping. Associations between nucleosome concentrations, other biomarkers, and laboratory values were assessed using Spearman's rank correlation coefficient. These associations were visualized with scatter plots including trend lines, annotated with correlation coefficients and corresponding *P* values. Alluvial plots were used to illustrate the development of organ failure over time. To compare H3.1 nucleosome concentrations over time across different types of organ failure, linear mixed-effects models were applied. These included fixed effects for time, organ failure, their interaction, and random intercepts for individual patients. To explore the predictive value of H3.1 nucleosome concentration on RRT initiation, a joint model was used; combining a linear mixed-effects model for H3.1 with a competing risk model for RRT and mortality, and including age and SOFA as potential confounders (*JMbayes2* package [34]). The joint model output was visualized in a dynamic plot, integrating the predicted longitudinal trajectory of H3.1 with the cause-specific cumulative risks for RRT initiation and mortality, along with their respective confidence intervals. To assess the association between baseline H3.1 nucleosomes and mortality, logistic regression was used, while additionally correcting for baseline risk stratifiers age, sex and SOFA.

Continuous variables were summarised as either mean with standard deviation (SD) or median with interquartile range (IQR), depending on their distribution. Categorical variables were expressed as counts with percentages. Differences between groups were tested using the appropriate methods: t-test for normally distributed data, Wilcoxon signed-rank test for non-normally distributed data, and chi-squared test for categorical variables. All tests were two-sided, with a significance level set at $P < 0.05$. All statistical analyses were performed using R software (version 4.2.1), through the RStudio interface.

Results

1713 critically ill patients were included with a total of 3671 plasma samples (day 0 = 1638, day 2 = 1194, day 4 = 839, Table S1). Median H3.1 nucleosome concentrations were 568 ng/mL [IQR 175–1419] on day 0, 784 ng/mL [IQR 322–1774] on day 2 and 1004 ng/mL [IQR 441–2248] on day 4 ($P < 0.001$, Table S1). Baseline characteristics of patients are described in Table 1.

Sepsis

Overall, sepsis occurrence in our cohort was 1013 (59%), of which 662 (65%) were confirmed by clinical adjudication and 351 (35%) were unconfirmed by clinical adjudication. At baseline, median H3.1 nucleosome concentrations significantly differed across sepsis presence: 741 ng/mL [IQR 233–1677] in the confirmed sepsis group, 460 ng/mL [IQR 134–1140] in the unconfirmed sepsis group, and 386 ng/mL [IQR 138–1135] in the non-sepsis group (Fig. 1A, $P < 0.001$, Table S2a). The H3.1 concentration increased over the days, irrespective of sepsis diagnosis (Table S2a). Baseline H3.1 nucleosomes demonstrated low diagnostic accuracy for distinguishing between confirmed and unconfirmed sepsis (AUROC 0.59, 95% CI 0.56–0.60, Fig. S1) and between confirmed and non-sepsis (AUROC 0.59, 95% CI 0.57–0.60, Fig. S1).

In patients with confirmed sepsis, baseline H3.1 nucleosome levels were significantly higher in those with septic shock compared to those without (881 ng/mL [IQR 303–1974] vs. 444 ng/mL [IQR 143–1084], respectively; $P < 0.001$, Table S2b). However, the diagnostic accuracy for distinguishing septic shock from non-septic shock was low (AUROC = 0.62, 95% CI 0.57–0.67, Fig. S1).

Organ failure

In patients with acute kidney injury (AKI), median baseline nucleosome concentrations demonstrated a severity-dependent relationship (no AKI = 372 ng/mL [IQR 133–974], at risk = 807 ng/mL [IQR 358–2169], injury = 970 ng/mL [IQR 278–1919], failure = 1225 ng/mL [IQR 555–3909], $P < 0.001$, Fig. 1B, Table S3), while trajectories of nucleosomes were not distinct in AKI patients ($\beta = 0.97$, $P = 0.14$, Table S4, Fig. S3), and the diagnostic accuracy was moderate (AUROC 0.68, 95% CI 0.67–0.70, Fig. S2). Similarly, in patients with disseminated intravascular coagulation (DIC), baseline H3.1 nucleosome concentrations were higher (no DIC = 667 ng/mL [IQR 220–1587], non-overt DIC = 1216 ng/mL [IQR 643–3737], overt DIC = 1111 ng/mL [IQR 323–2557], $P < 0.001$, Fig. 1C, Table S3), and changes over time were not moderated by DIC severity ($\beta = -81.5$, $P = 0.64$, Table S4, Fig. S3), resulting in a diagnostic accuracy of AUROC 0.64, 95% CI 0.58–0.70, Fig. S2. In patients with ARDS, baseline H3.1 nucleosome concentrations were also elevated (no ARDS = 484 ng/mL [IQR 160–1200], mild ARDS = 552 [195–2707], moderate ARDS = 751 [209–2113], severe ARDS = 1039 [432–2481], $P < 0.001$, Fig. 1D, Table S3). Changes over time were not significantly moderated by ARDS ($\beta = -66.73$, $P = 0.42$, Table S4, Fig. S3), and the diagnostic accuracy remained low (AUROC 0.59, 95% CI 0.57–0.60, Fig. S2).

H3.1 nucleosome concentrations were consistently higher in patients with a higher number of failing organ systems ($P < 0.001$, Fig. 1E). Additionally, there was a positive association between H3.1 nucleosome concentrations and the SOFA score ($r = 0.40$, Fig. 1F, $P < 0.001$).

Association with inflammatory host response

Median baseline H3.1 nucleosome concentrations were higher in Hyperinflammatory (1005 ng/mL [IQR 366–2488]) compared to Hypoinflammatory confirmed sepsis patients (493 ng/mL [IQR 193–1142], Fig. 2A, Table S5), with moderate diagnostic accuracy at baseline (AUROC 0.66, 95% CI 0.60–0.69, Fig. S4). The associations between H3.1 nucleosomes and various biomarkers are shown in Fig. 3: IL-6 ($r = 0.27$, $P < 0.001$), IL-8 ($r = 0.39$, $P < 0.001$), Protein C ($r = -0.08$, $P = 0.002$) and TNF-α ($r = 0.07$, $P = 0.064$). Additionally, H3.1 nucleosome concentrations showed weak positive associations with CRP (r = 0.19, $P < 0.001$) and white blood cell count (r = 0.08, $P = 0.002$).

Prediction of clinical outcomes

Organ failures were frequently already present at ICU admission and their development could therefore not be predicted (Fig. S5). The need for renal replacement therapy (RRT) frequently occurred on days after ICU admission and was therefore selected as a clinically relevant intermediate outcome (Fig. S5). Baseline H3.1 was associated with RRT initiation with an odds ratio (OR) of 3.03 per log10 increase (95% CI 2.44–3.78, $P < 0.001$). Patients with baseline H3.1 concentrations exceeding a cutoff of 1000 ng/mL had an OR of 3.14 compared to those below 1000 ng/mL (95% CI 2.40–4.12, $P < 0.001$; Fig. 3). Longitudinal H3.1 nucleosome concentration, in a competing-risk joint model analysis, was prognostic of the need for RRT while adjusting for age, sex and SOFA (HR: 2.00, 95% CI 1.44–2.62 per log10 increase, $P < 0.001$, Fig. S6–S7, Table S6), but not of mortality for patients who did not need RRT (HR: 1.03, 95% CI 0.97–1.11 per log10 increase, $P = 0.39$, Fig. S7, Table S6).

H3.1 nucleosome concentrations were significantly associated with 30-day mortality while correcting for age and sex (OR 1.63 per log10 increase, 95% CI 1.38–1.93), $P < 0.001$), but this association disappeared after also correcting for SOFA (OR 1.19 per log10 increase,

Table 1 Baseline characteristics stratified by sepsis presence

	Sepsis Confirmed	Sepsis Unconfirmed	Non-sepsis	*P* value
n	662	351	700	
Demographics				
Age (years)	63 [53, 71]	63 [51, 71]	64 [53, 73]	0.23
Male (%)	391 (59)	211 (60)	413 (71)	< 0.001
BMI (kg/m^2)	24.7 [22.2, 28.2]	24.9 [22.2, 29.2]	26.1 [23.4, 29.4]	< 0.001
Severity				
SOFA	8 [6, 10]	7 [5, 9]	7 [5, 9]	< 0.001
APACHE IV	81 [65, 104]	75 [59, 101]	69 [53, 91]	< 0.001
Admission diagnosis type				< 0.001
Cardiovascular (%)	81 (12)	122 (35)	344 (49)	
Endocrine (%)	2 (0.3)	4 (1)	11 (2)	
Gastrointestinal (%)	120 (18)	29 (8)	80 (11)	
Hematological & metabolic (%)	8 (1)	11 (3)	8 (8)	
Neurological (%)	42 (6)	25 (7)	62 (9)	
Oncological (%)	2 (0)	0 (0)	11 (2)	
Overdoses & Poisoning (%)	5 (1)	4 (1)	14 (2)	
Postoperative & Trauma (%)	31 (5)	28 (28)	110 (16)	
Renal & Genitourinary (%)	10 (2)	5 (1)	13 (2)	
Respiratory (%)	227 (34)	95 (27)	37 (5)	
Sepsis (%)	131 (20)	27 (8)	0 (0)	
Other (%)	3 (1)	1 (1)	10 (1)	
Site of infection				< 0.001
Cardiovascular (%)	66 (10)	66 (19)	0 (0)	
Neurological (%)	16 (3)	14 (4)	0 (0)	
Intra-abdominal (%)	150 (23)	30 (9)	0 (0)	
Lower respiratory tract (%)	287 (44)	189 (54)	0 (0)	
Skin, Soft Tissue & Bone (%)	45 (7)	7 (2)	0 (0)	
Upper respiratory tract (%)	8 (1)	2 (1)	0 (0)	
Urinary tract (%)	65 (10)	25 (7)	0(0)	
Other (%)	15 (2)	18 (5)	0 (0)	
Comorbidities				
Diabetes mellitus (%)	140 (21)	61 (17)	95 (16)	0.08
Congestive heart failure (%)	29 (4)	22 (6)	63 (11)	< 0.001
Chronic dialysis (%)	21 (3)	10 (3)	5 (1)	0.02
Chronic renal insufficiency (%)	103 (16)	39 (11)	37 (6)	< 0.001
Respiration				
Mechanical ventilation (%)	472 (71)	266 (76)	525 (90)	< 0.001
Respiratory rate, max (/min)	34 [28, 40]	32 [26, 38]	28 [23, 35]	< 0.001
PaO_2/FiO_2 (mmHg)	148 [98, 208]	145 [93, 200]	205 [134, 273]	< 0.001
$PaCO_2$ (mmHg)	41 [34, 50]	43 [38, 51]	42 [37, 47]	0.04
pH	7.33 [7.25, 7.41]	7.33 [7.26, 7.40]	7.34 [7.28, 7.39]	0.57
HCO_3 (mmol/L)	18.8 [15.5, 22.9]	19.9 [16.6, 23.2]	20.3 [17.2, 22.4]	0.01
Hemodynamics				
Vasopressor use (%)	483 (73)	244 (70)	462 (79)	0.002
Heart rate, max (/min)	131 [115, 148]	123 [108, 145]	114 [100, 131]	< 0.001
Mean arterial pressure (mmHg)	78 [69, 87]	81 [72, 91]	80 [71, 91]	< 0.001
Laboratory values				
Creatinine (mg/dL)	1.4 [0.9, 2.2]	1.2 [0.9, 1.8]	1.1 [0.9, 1.6]	< 0.001
Sodium (mmol/L)	139 [136, 143]	140 [137, 143]	140 [138, 142]	0.07

Table 1 (continued)

	Sepsis Confirmed	Sepsis Unconfirmed	Non-sepsis	*P* value
Potassium (mmol/L)	4.5 [4.2, 5]	4.6 [4.2, 5]	4.7 [4.3, 5.2]	< 0.001
Lactate (mmol/L)	2.8 [1.7, 5.2]	2.7 [1.6, 4.6]	2.3 [2, 3.8]	0.001
CRP (mg/L)	166 [70, 269]	53 [8, 155]	9 [2, 43]	< 0.001
Biomarkers				
IL6 (pg/mL)	276 [54, 2073]	77 [21, 372]	74 [26, 220]	< 0.001
IL8 (pg/mL)	157 [50, 757]	68 [26, 200]	56 [23, 132]	< 0.001
TNFA (pg/mL)	2.66 [1.22, 6.43]	2.83 [1.34, 9.32]	2.23 [1.23, 6.85]	0.47
Protein-C (% activity)	107.7 [82.8, 147.6]	130.5 [97.3, 173.9]	139.7 [104.5, 172.8]	< 0.001
Hyperinflammatory (%)	315 (47.7)	102 (29.1)	112 (19.2)	< 0.001
Organ failure				
Septic shock (%)	308 (46%)	0 (0)	0 (0)	
DIC (%)	76 (13)	26 (12)	30 (9)	0.28
AKI (%)	665 (47)	297 (37)	232 (27)	< 0.001
ARDS (%)	499 (34)	242 (30)	112 (13)	< 0.001

Values are presented as median [IQR], unless noted with a (%), in which case they are displayed as counts with percentages. The data in this table is categorized by sepsis classifications (SOFA scores combined with clinical adjudication, see methods for details). All measurements were taken at baseline or derived from the first plasma sample collected

BMI = Body Mass Index, SOFA = Sequential Organ Failure Assessment, APACHE IV = Acute Physiology And Chronic Health Evaluation IV, CCI = Charlson Comorbidity Index, WBC = White Blood Cells, DIC = Disseminated Intravascular Coagulation, AKI = Acute Kidney Injury, ARDS = Acute Respiratory Distress Syndrome

95% CI 0.99–1.43, $P = 0.070$, Table 2). This association between H3.1 nucleosome concentrations and 30-day mortality was not different between inflammatory subphenotypes (interaction $P = 0.22$). Similar findings were noted for 90-day mortality (Table 2). Patients with a baseline H3.1 nucleosome concentration > 1000 ng/mL had higher odds of 30-day mortality (OR 1.68, 95% CI 1.35–2.10, $P < 0.001$) than those below. Over time, H3.1 nucleosome concentrations were associated with mortality in a joint-model analysis while correcting for age and SOFA (HR: 2.67, 95% CI 2.46–2.91 per log10 increase, $P < 0.001$, Table S7).

Discussion

In this large observational study, we demonstrated that plasma nucleosomes, represented by H3.1 nucleosome concentration, are associated with the presence of sepsis, the severity of organ dysfunction, and a Hyperinflammatory host response. While H3.1 nucleosome concentration appears central to these key aspects of sepsis pathophysiology, its diagnostic accuracy for identifying sepsis or organ failure was limited. Notably, plasma H3.1 nucleosome concentration showed predictive value for the need for renal replacement therapy.

In line with previous studies [5, 20, 24, 35], we identified a higher plasma H3.1 nucleosome concentration in patients with sepsis, compared to two control groups. Based on the case-definition available in the MARS cohort, we were in the unique position to compare patients with sepsis and a confirmed infection to patients without such confirmation and a non-infected control group. H3.1 nucleosome concentrations were highest in patients with confirmed infection and lowest in the patients without suspected infection. Nonetheless, there was considerable overlap between the three groups. This suggests that, although the presence of a pathogen may increase NETosis, it certainly is not the only contributing factor.

There was also an association between the different types, the severity, and the number of organ dysfunctions and an increased concentration of H3.1 nucleosomes. DIC was infrequent in this population, but when it was present, it was associated with significantly raised NETosis. This was expected as NETosis is distinctly related to microthrombosis, one of the hallmark features of DIC [36, 37]. Acute kidney injury was much more prevalent and was also associated with increased H3.1 nucleosome concentrations. One may speculate that AKI would limit clearance of H3.1 nucleosomes, but the molecule is too large to be filtrated and the lack of a difference in biomarker slope over time makes this an unlikely explanation. Furthermore, the H3.1 nucleosome concentration predicted the need for renal replacement therapy, and the biomarker trajectory was not influenced by the initiation of RRT. There is considerable evidence that thromboinflammation plays an important role in the development of AKI, at least in part of the patients, and our findings support that [38].

We identified that patients with a Hyperinflammatory phenotype had much more NETosis than patients who

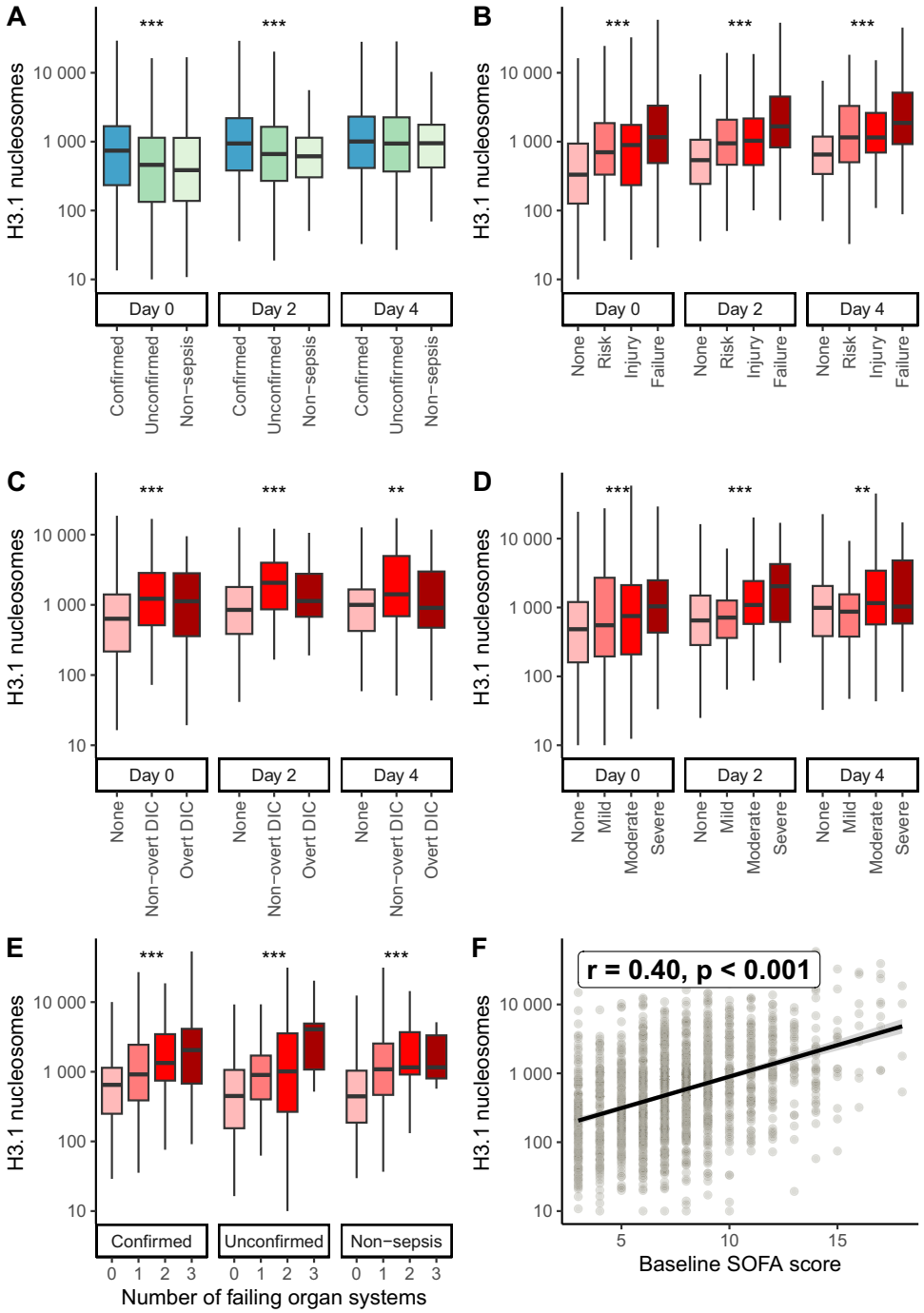

Fig. 1 H3.1 nucleosome concentrations by sepsis presence and clinical scores. Panels **A** to **D** present boxplots across different sample days, stratified by various categories: Panel A = sepsis presence, Panel B = AKI score, Panel **C** = DIC score, and Panel **D** = ARDS severity. Supplementary Tables S2–S4 and Figs. S1–S3 contain more information on this. Panel **E** shows the number of failing organ systems per sepsis category, while Panel **F** is a scatterplot illustrating the relationship with baseline SOFA scores. Asterisks indicate significance levels from the Kruskal–Wallis test: *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$. Abbreviations: AKI = Acute kidney injury, DIC = Disseminated intravascular coagulation, ARDS = Acute respiratory distress syndrome, SOFA = Sequential Organ Failure Assessment

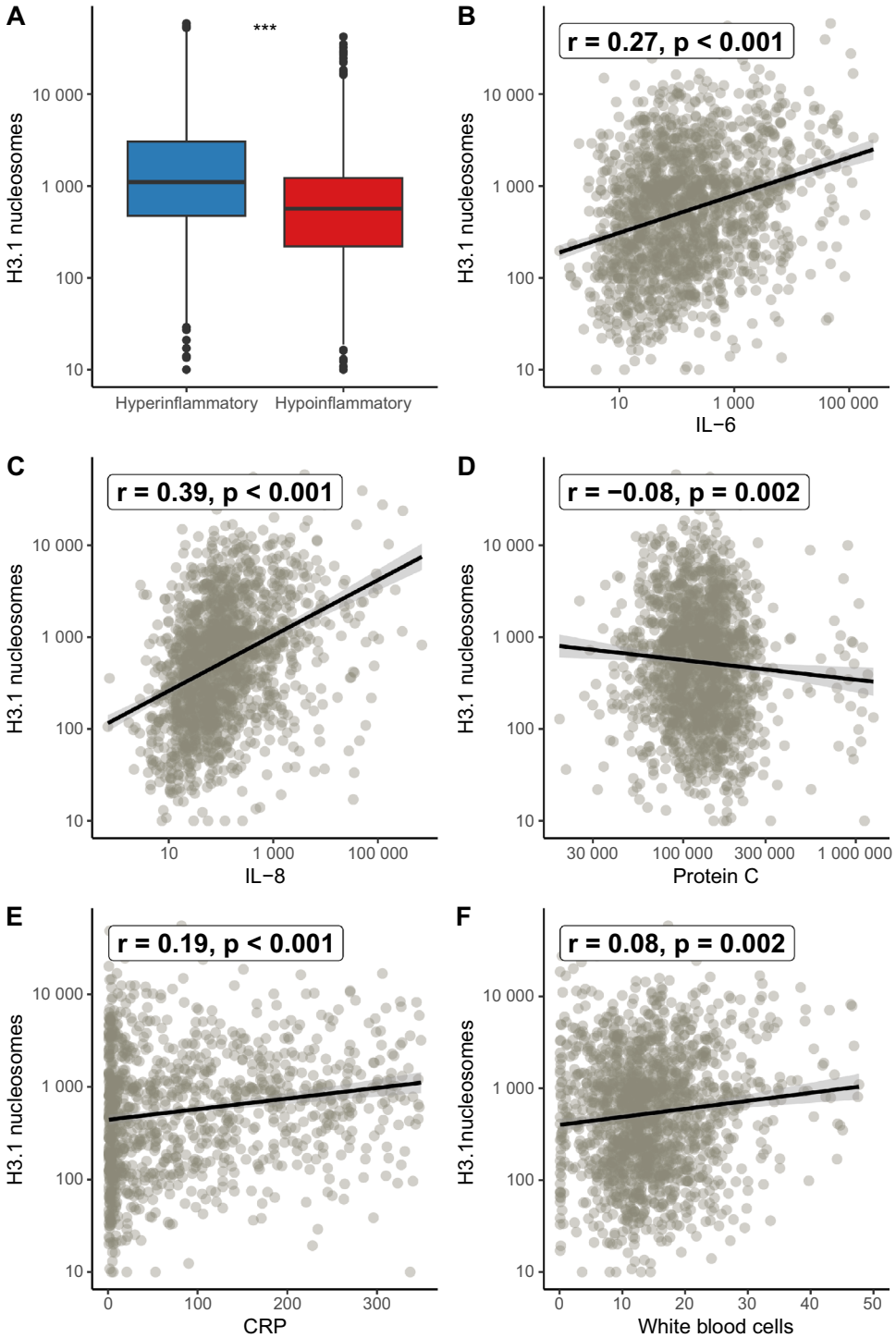

Fig. 2 H3.1 nucleosome concentrations and the host response. Panel **A** shows boxplots stratified by Hyperinflammatory and Hypoinflammatory sepsis. Panels **B** to **F** display the associations between baseline H3.1 nucleosomes and the following biomarkers: Panel **B** = IL-6, Panel **C** = IL-8, Panel **D** = Protein C, Panel **E** = CRP, and Panel **F** = White blood cell count. Asterisks indicate significance levels from the Kruskal–Wallis test: *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$. Abbreviations: Interleukin 6 = IL-6, Interleukin 8 = IL-8, C-reactive protein = CRP

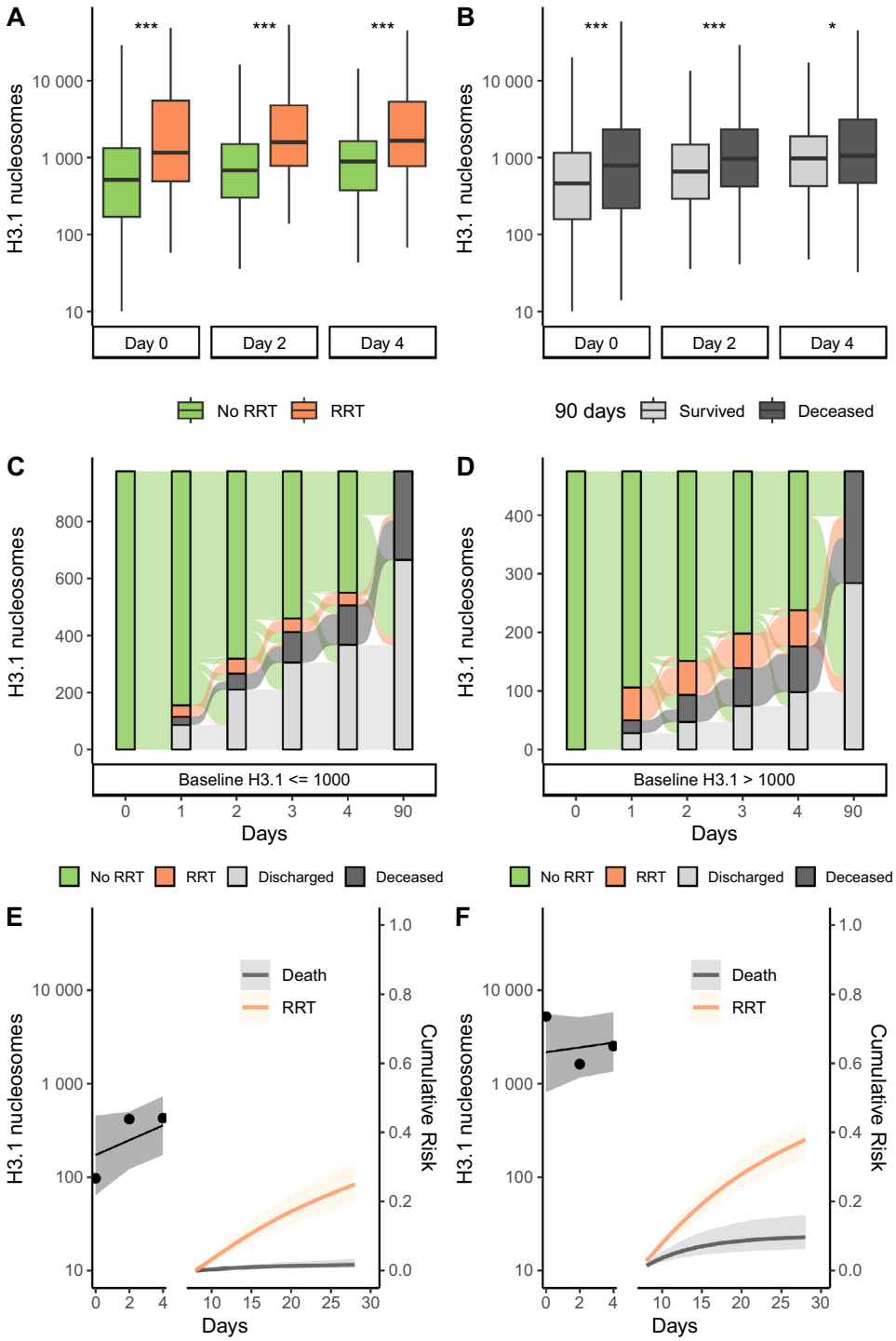

Fig. 3 H3.1 nucleosome concentrations and clinical outcomes. In panels **A** and **B**, H3.1 nucleosome concentrations (ng/mL) are displayed stratified by renal replacement therapy (panel A) and 90-day mortality (panel B). Asterisks indicate significance levels from the Kruskal–Wallis test: *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$. Panels **C** and **D** show alluvial plots of patient outcomes over time, stratified by baseline H3.1 nucleosome concentration ≤ 1000 ng/mL (panel C) and > 1000 ng/mL (panel D). Panels **E** and **F** display dynamic predictions of the competing risk joint model for two patients. The left y-axes in panels E and F illustrate the observed (dots) and predicted (lines) trajectories for H3.1 nucleosome concentrations over time, with shaded areas representing the 95% confidence intervals for the predictions. The right y-axes in panels E and F depict the predicted cause-specific cumulative risks for RRT initiation and mortality, with respective confidence intervals (shaded regions). Panel E corresponds to a 45 years old patient with SOFA score 3, and Panel F corresponds to a 53 years old patient with SOFA score 8

were classified as Hypoinflammatory. Although these phenotypes were first identified in ARDS [33], they have now also been established in patients with sepsis and more generally in critically ill patients [39, 40]. Several studies have confirmed that patients with an inflammatory phenotype show a more activated neutrophil response and this may explain the presence of more NETosis [40]. There was a positive association between H3.1 nucleosome concentrations and pro-inflammatory cytokines IL-6 and IL-8, whereas the association with activated protein C was comparatively weaker. Based on these findings, we could speculate that therapies aiming to reduce harmful NETosis could be targeted at the Hyperinflammatory phenotype. However, there was considerable spread in both phenotypes and H3.1 was associated with organ dysfunction irrespective of inflammatory phenotype. A high H3.1 nucleosome concentration by itself may therefore be a more suitable trait for such therapies.

This study has several strengths and limitations. We report on the largest study of NETosis in ICU patients to date. The MARS cohort is unique in the extensive and frequent clinical adjudication of the included patients, which allowed us to separate between patients with suspected and non-suspected sepsis, and between proven and rejected infection. However, adjudication relied on the availability of clinical and microbiological data as well as judgement of the clinical team, all by no means perfect and misclassifications will have occurred. Considering the size of the cohort, these will likely have minimal consequences. Organ failure scores also have limitations, and all the results ought to be interpreted in that light. RRT was initiated based on strict guidelines and clinical practice during the study period may not reflect practice elsewhere. Finally, the H3.1 nucleosome assay measures one component of NETosis and other biomarkers reflective of the same biological signal could have yielded other results.

We did not identify H3.1 nucleosome as a standalone diagnostic biomarker for sepsis, organ dysfunction or hyperinflammation. Considering the consistency of this signal, this is unlikely due to problems in case-definitions. Measurement error was minimum and is also unlikely to contribute. In recent years, the concept of "treatable traits" and disease-overarching biological dysfunction has gained popularity [11, 41, 42]. NETosis can be triggered by a wide variety of processes and has many consequences. Therefore, there likely is a subpopulation that has NETosis driven organ failure, potentially preceding clinical manifestation by SOFA score, irrespective of the presence of sepsis and other dysregulations in the immune response. The implications of our results are that NETosis, and H3.1 nucleosomes in particular, plays at the intersection of infection, organ failure and inflammation and is not simply a surrogate of any of these in particular. Based on experimental data, we know that NETosis can be initiated by infection and causes tissue injury, inflammation and immunothrombosis. Our study supports an important role of NETosis in these processes in part of the patients, in particular related to the

Table 2 Baseline H3.1 nucleosomes and mortality

	All		**Hypoinflammatory sepsis**		**Hyperinflammatory sepsis**	
	n = 1713		**n = 463**		**n = 198**	
	OR (95% CI) per log10 increase	***P* value**	**OR (95% CI) per log10 increase**	***P* value**	**OR (95% CI) per log10 increase**	***P* value**
30-day mortality						
Unadjusted	1.65 (1.4–1.95)	< 0.001	1.12 (0.7–1.8)	0.649	1.62 (1.13–2.36)	0.010
				Interaction *P* value = 0.22		
Adjusted for age and sex	1.63 (1.38–1.93)	< 0.001	1.13 (0.7–1.83)	0.617	1.63 (1.12–2.41)	0.013
				Interaction *P* value = 0.20		
Adjusted for age, sex and SOFA	1.19 (0.99–1.43)	0.070	1.02 (0.61–1.68)	0.948	1.21 (0.8–1.84)	0.36
				Interaction *P* value = 0.21		
90-day mortality						
Unadjusted	1.6 (1.37–1.87)	< 0.001	0.93 (0.61–1.42)	0.739	1.41 (1–2.01)	0.054
				Interaction *P* value = 0.14		
Adjusted for age and sex	1.58 (1.35–1.85)	< 0.001	0.92 (0.60–1.41)	0.690	1.37 (0.96–1.98)	0.086
				Interaction *P* value = 0.14		
Adjusted for age, sex and SOFA	1.14 (0.96–1.36)	0.14	0.85 (0.54–1.34)	0.493	1.06 (0.72–1.57)	0.77
				Interaction *P* value = 0.14		

Values are presented as odds ratio (OR) per log10 increase of baseline H3.1 nucleosome concentration, along with the 95% confidence interval (CI). Values are derived by a logistic regression

presence of DIC and the development of AKI requiring RRT. Further study on how NETosis can be modulated in patients with evidence of a high NETosis phenotype, for example measured by H3.1 nucleosome, is timely.

Conclusion

Sepsis, organ failure, and inflammation are closely associated with NETosis, a critical process in the innate immune response. Elevated NET levels, measured by plasma H3.1 nucleosome concentrations, are observed in sepsis patients compared to non-sepsis patients. Additionally, patients with AKI, DIC, and ARDS exhibit significantly higher H3.1 levels compared to those without these conditions. However, the individual diagnostic utility of H3.1 for identifying sepsis and organ failure remains limited. While H3.1 nucleosome concentrations show promise in predicting the need for RRT, further research is required to enhance their diagnostic capability and to identify clinically relevant subgroups and populations.

Abbreviations

AKI	Acute kidney injury
ARDS	Acute respiratory distress syndrome
AUROC	Area under the receiver operating characteristic curve
CI	Confidence interval
CVVHD	Continuous veno-venous hemodialysis
DIC	Disseminated intravascular coagulation
HR	Hazard ratio
ICU	Intensive care unit
IL-6	Interleukin-6
IL-8	Interleukin-8
IQR	Interquartile range
MARS	Molecular diagnosis and risk stratification of sepsis study
NET	Neutrophil extracellular trap
OR	Odds ratio
RRT	Renal replacement therapy
SD	Standard deviation
SOFA	Sequential organ failure assessment
TNFR1	Tumor necrosis factor receptor 1

Supplementary Information

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Supplementary material 1

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Author contributions

DF, AR, TW, LB contributed to the study design, DF, LB, MJ accessed and verified the data. DF, LK, MJ, LB performed statistical analyses. DF, LB, LK drafted the manuscript. DF, MJ, LK, TH, AR TP, OC, LB critically revised the manuscript. All authors gave final approval for publication.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Ethics approval for this study was obtained from the institutional review boards of both hospitals (IRB number 10-056C, registered June 16, 2010). Patients were included via an opt-out consent method, which was approved by the same institutional review boards.

Consent for publication

Not applicable.

Competing interests

Two clinical scientists (AR, TH) who are employed by VolitionRx part-time were involved in the design of the study, the interpretation of the data and contributed to the manuscript. VolitionRx had no access to the clinical data and was not involved in data analysis. The other authors declare no competing interests.

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