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Early co-activation of neutrophil extracellular traps and complement predict mortality from thrombo-inflammatory complications of sepsis: Prospective cohort study

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Abstract
Background: Acute systemic disease is often compounded by thrombo-inflammation wherein three innate immune activation and coagulation pathogenesis convergence of interest to promote microvascular thrombosis and organ injury. The role of neutrophilic extracellular traps (NETs) and complement activation in immunothrombosis has been implicated but little is known about the clinicopathologic evidence for cooperative (or synergistic) effects on coagulopathy and outcome.
Objective: To determine early NET and complement activation profiles in sepsis, and test of the interaction between them and thromboinflammatory complications including overt DIC and 28-day survival from sepsis.
Methods: In this study, a prospective observational cohort study of 100 adults who had sepsis and 50 controls. Blood sampling was done at T0 (0-6 h), serial sampling at 24 h and 72 h of a set at n=80. NET burden was evaluated with the help of biomarkers [citrullinated histone H3 [H3Cit], neutrophil elastase [NE], nucleosomes] based on the enzyme immunoassay (ELI) and summarized in a NET index (z-score composite]. Complement activation was determined by the use of C5a and soluble C5b-9 (sC5b-9) and expressed as a complement index. Coagulopathy was assessed by the use of routine tests of coagulation and overt DIC classification (ISTH score >=5). Multivariable logistic regression and Cox proportional hazards models were used to test for independent associations and an interaction (NET incentivisation of SOA * complement) after adjustment for age and SOFA score. ROC analysis was used to evaluate discriminative performance.
Results: At T0, NET and complement biomarkers were found to be significantly up regulated in sepsis compared to controls (H3Cit 420 (310-540) vs 95 (70-120) ng/mL; sC5b-9 590 (450-730) vs 180 (150-220) ng/mL; all p= 0.001). Biomarkers decreased after 72 h but were abnormal (all $p<0.001$ for trend). NET and complement indices had a high correlation (rho=0.71, $p<0.001$) and were correlated with SOFA and D-dimer. In adjusted models, with a significant interaction (OR1.68, 95% CI1.12-3.27), NET index (OR2.35, 95% CI1.48-3.71) and complement index (OR2.12, 95% CI1.37-3.27) predicted overt DIC (OR1.68, 95% CI1.12-3.25). Both indices were able to predict 28-day mortality (NET index HR 1.84, 95% CI 1.32-2.56; complement index HR 1.63, 95% CI 1.18-2.26), and the two were significantly associated (HR 1.47, 95% CI 1.05-2.05). The combined dual index model had excellent discrimination for composite thrombo-inflammatory outcomes (AUC time unit model 0.93, 95% CI [88-97]).
Conclusions Early co-activation of NETs and complement is related to enhanced thrombo-inflammation in sepsis. A large NET suspension x complement is conducive to support a pathway cooperative effect, besides independent association. Dual pathway biomarker stratification demands external validation and may be used for precision strategy to mitigate sepsis associated coagulopathy.

Keywords: Neutrophil extracellular traps, complement, sepsis, thrombo-inflammation, immunothrombosis, disseminated intravascular coagulation, C5a, sC5b-9

Introduction
Acute systemic diseases very often occur with a coupled activation of inflammation and coagulation that induce endothelial dysfunction, microvascular thrombosis, and organ injury. This integrated process is often referred to as thromboinflammation, in which immunological response and stimulation of the vascular system form the initial stages of forming a thrombus and the presence of the thrombus further aggravates inflammatory mechanisms (Schrottmaier & Assinger, 2024) [14]. In the case of sepsis-associated coagulopathy, this is a potentially harmful biology that can worsen toward disseminated intravascular coagulation (DIC) and multiple-organ dysfunction, pointing to a potentially important loop of multiple

amplification and not individual activation of a pathway. (Wei *et al.*, 2024) [20]. Neutrophil extracellular traps (NETs) are an essential effector mechanism of the thromboinflammation. NETs are extracellular chromatin structures that are decorated with neutrophil granule proteins and have functions for intravascular immunity defense, excessive accumulation of the NETs contribute to vascular occlusion, tissue injury and prolonged inflammatory signaling (Wang *et al.*, 2024) [19]. NET structures also offer a prothrombotic scaffold that aids platelet adhesion and coagulation propagation from neutrophil activation that links the establishment of thrombotic risk stemming from acute illness directly. Complement activation is the second major amplifier of thrombo-inflammation. Complement and coagulation are not in isolation and there are multiple points of cross-activation and shared regulation including molecular events that determine innate defence and hemostatic responses during times of injury and infection (Pryzdial *et al.*, 2022) [13]. In sepsis, enormous activation of the complement system may heighten the severity of the coagulation defects and correlate with progression towards DIC favoring complement system activity as a conceivable driver of progression of thromboinflammatory phenotypes (Wei *et al.*, 2024) [20]. Mechanistic evidence for bilaterally coupled formation of NETs and complement activation. Complement anaphylatoxins can mediate the activation of neutrophils and upregulation of NETs NETs can mediate the proinflammatory effector pathway of tissue damage and intravascular nuclear contents that may cause increased activation of proinflammatory pathways. In experiment and translational work, C5a induced the generation of NET due to mitochondrial signaling induced thrombosis, providing a direct relationship between complement activation mediate by thrombosis because of NETs (Chen *et al.*, 2022) [3]. A clinically actionable deficiency is still in place with regard to the elevation of both pathways (NET and complement) together in terms of identifying patients at disproportionately high risk compared with elevation of either pathway alone (Singer *et al.*, 2016; Iba *et al.*, 2017; Taylor *et al.*, 2001) [6, 16]. This question has translational relevance since the use of therapy directed against the complement is now expanding throughout human disease and clinical pipelines are constantly expanding in therapeutical targets and indications (West *et al.*, 2024) [21]. Accordingly, this study examines early profiles of NET and complement activation in acute systemic disease and determines if there is an interconnection between the two profiles that correlates with thromboinflammatory outcomes such as severity of coagulopathy, imaging confirmed thrombosis, trajectories of organ dysfunction, and short-term mortality (Pryzdial *et al.*, 2022; Wei *et al.*, 2024). Emphasis is given to clinically-much more certain biomarkers identified and standardized sampling to encourage reproducibility in the different acute care settings (Wang *et al.*, 2024; Wei *et al.*, 2024) [13, 19, 20].

Aim of the Study: In this study, to determine whether circulating neutrophil extracellular trap (NET) biomarkers and complement activation biomarkers can be aroused concurrently at the onset of acute systemic disease, which is associated with more thrombo-inflammation, in terms of septum, expressed clinically through a higher rate of sepsis-associated coagulopathy, progression to overt DIC, thrombosis confirmed by imaging, trajectories of organ dysfunction and 28-day mortality.

Materials and Methods

Study address and methodology framework

A prospective observational cohort approach to study early biomarker sampling and 28 days follow-up of thrombo-inflammatory outcomes. Reporting is conducted following the recommendations of the STROBE reporting guideline for observational cohort studies, in order to make the study's methodology transparent and reproducible (von Elm *et al.*, 2008) [18].

Study setting and duration of the study

Enrollment is done in emergency department and intensive care units of participating hospitals where acute systemic disease is routinely evaluated and managed. Recruitment is consecutive throughout the predefined study period in order to minimise selection bias and to ensure that the case-mix reflects the real world.

Study population and inclusion criteria

Adult patients (>18 years) that meet Sepsis-3 criteria are eligible. Sepsis is defined as a suspected or confirmed infection associated with acute organ dysfunction as indicated by an increase in the SOA system of Width score congenital (e-f) 2 origins from baseline. Baseline is considered 0 at times, if economic prior organ dysfunction has not been carried out (Singer *et al.*, 2016) [16].

Exclusion criteria are conditions anticipated to significantly cause distortion of complement or NET biomarkers independent of the acute illness (e.g., current treatment with a complement inhibitor, if applicable), failure to obtain initial blood sampling within the prespecified early time frame (presumably because of a delay in confirming the presence of presumptive sepsis, though this may have adverse clinical consequences for patients with initial sepsis), and failure/denial of obtaining ethical consent per institutional policy.

Comparator group (controls)

A control group is included to provide reference ranges for the biomarker panel as well as enhancing biological interpretation. Controls may be healthy volunteers or hospital controls with no systemic inflammation, with no infection, and could be matched where possible, based on age and sex, but were sampled once using the same pre-analytical procedures.

Sample size & sampling strategy

One pragmatic sample size is used to trade-off between feasibility and the needs for multivariable modelling. A standard operational target has been 100-200 sepsis cases with 50-80 controls with all eligible patients enrolled consecutively until targets are reached. Power focuses to the most part on identifying associations and a signal for interaction between NET and complement biomarkers related to coagulopathy and thrombotic outcomes.

Clinical Data Collection, Severity Check

Baseline demographics, co-morbidities, source of infection, microbiology and main treatments are documented from admission to day 3 and subsequently outcome follow-up continues to day 28. Severity of illness is estimated by SOFA at enrolment and serially (Singer *et al.* 2016) [16]. Standard thromboprophylaxis & sepsis management is as per routine

care - Pharmacologic VTE prophylaxis as per guidelines is documented, LMWH is preferred over UFH if not contraindicated - (Evans *et al*, 2021) [4].

Timing of blood sampling

Blood is sampled at T0 (0-6 hours) from the time that sepsis is first recognized/admission, in order to sample early pathway activation, with optional repeat sampling at 24 hours and 72 hours, in order to quantify pathway biomarker dynamics. Time stamps on collection, processing, and freezing are kept to allow for adjusting for pre-analytical variability.

Biospecimen Handling and pre-analytical Standardization

Complement activation markers are measured in using critical plasma from whole blood collected in an ethylenediamine tetraacetate (EDTA) containing plasma prepared in strict conditions to reduce *ex-vivo* complement activation. EDTA whole blood is kept on ice, centrifuged at 4 degrees Celsius within approximately 60 minutes, and plasma is snap frozen and stored at minus 80 degrees. Repeated freeze and thaw are avoided (Brandwijk *et al.*, 2022) [1].

Due to the potential of biomarker levels to be confused with delays in the time consumed between the sample creation in the presence of red blood cells and the arrival at the freezer, these time constraints are minimized and a variable is collected for analysis as a quality variable (Brandwijk *et al.*, 2022) [1].

Marker panel and measurement NET biomarker

NET burden is measured by a panel of multiple markers as opposed to a single assay that will enhance specificity in clinical plasma. Primary NET biomarkers are citrullinated histone H3 (H3Cit) and neutrophil elastase (NE) or nucleosome-based markers by immuno-enzymatic immunoassay, which is in line with clinical NET biomarker practice (Wang *et al.*, 2024; Morimont *et al.*, 2022) [9, 19]. If MPO-DNA complexes are included, results are interpreted cautiously as the detection of MPO-DNA in plasma in an enzyme immunoassay is prone to errors and limited information on the formation and localization of NETs *in vivo* is obtained; controls of isotypes and assay specificity checks are included if this marker is used (Hayden *et al.*, 2021) [5].

Complement activation Biomarkers and measurement

Complement activation is measured by using the split products and activation of the terminal pathway measured by enzyme immunoassay (ELIAs). The key panel contains C3a &/ or C5a and soluble C5b-9 (sC5b-9) in order to detect upstream and terminal complement activation in plasma (Brandwijk *et al.*, 2022) [1].

Analysis Kits and Analytical Performance

The plasma citrullinated histone H3 (H3Cit) and neutrophil elastase (NE) were measured with the help of commercial sandwich ELISA kits (Cayman Chemical, USA; Cat. No. 501620 and 601709, respectively). Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Cat. No. 11774425001) was used to determine nucleosomes. C3a, C5a, and sC5b-9 complement activation markers were quantified by means of MicroVue Complement ELISA kits (Quidel Corporation, USA, Cat. Nos. A018, A020, and A029 respectively). These assays had lower limit of detection (LOD) that varied between 0.1-1.0 ng/mL depending on the analyte. According to the validation data provided by manufacturer, intra- and inter-assays coefficients of variation (CVs) were less than 8 percent and less than 10 percent respectively.

Routine coagulation tests and coagulopathy phenotyping

Routine coagulation measurement, Coagulopathy phenotyping: Routine coagulation parameters are measured at inclusion and in the early phase of hospitalization: platelet, PT/INR, aPTT, fibrinogen and D-dimer. Sepsis-induced coagulopathy (SIC) calculated with platelet count, PT-INR, and a 4 item SOFA component score of SIC positivity equal to a total score greater than or equal to 4 with the platelet + PT component greater than 2 (Iba *et al.*, 2017) [6].

Overt DIC is categorized by the ISTH scoring system that is based on platelet count, PT prolongation, fibrin-related markers, and fibrinogen; the score is cumulative and the score >=5 is considered compatible with overt DIC (Taylor *et al.*, 2001; Toh & Hoots, 2007) [17].

Study outcomes: The primary outcome is thrombo inflammatory composite within 28 days, that is, containing any of; (i) imaging confirmed venous or arterial thrombosis; (ii) overt DIC by ISTH score, or (iii) clinically significant progression of organ dysfunction based on SOFA trajectory. Secondary outcomes are 28-day all-cause mortality, ICU admission, duration of organ support and longitudinal evolution of SIC/DIC score.

Patient Flow and Missing Data

Out of 120 patients screened, 100 of those who met the inclusion criteria were recruited, with 20 patients excluded because of late sampling time (more than 6 hours since sepsis was recognized) or failure to provide valid consent. Serial samples were taken at 0-6h, 24h and 72h in 80 out of the 100 recruited patients (80 percent) and missing serial data was mainly due to early discharge/death before the second or the third serial time. All baseline analyses and multivariate models were done on the entire cohort (n=100) and longitudinal trajectory analyses were restricted to patients with entire serial sampling (n=80).

Statistical analysis: Continuous measures are presented as mean (SD) or as median (IQR) depending on the distribution and categorical measures as number and percent (%) table. Associations between biomarkers and outcomes are tested using multivariable regression models across the different confounders (age, sex, baseline SOA, thromboprophylaxis exposure and major co-morbidities). A critical analysis considers the interaction between the NET index (composite of standardized NET markers) and complement activation (sC5b-9) to assess the presence of cooperative effects of interactions in additional to additive associations. Sensitivity analyses have been done for timing to processing variability for complement markers as a covariate (Brandwijk *et al.*, 2022) [1].

The computation of Composite Indices

Raw biomarker concentrations (H3Cit, NE, and nucleosomes of the NET index; C5a and sC5b-9 of the Complement index) of each participant were log10-transformed to remove right skew. Then the z-scores were calculated based on the mean and standard deviation of just the control group as follows:

$$z = (x_i - \mu\_ \text{control}) / \sigma\_ \text{control}$$

The composite NET index of each participant was then obtained as the arithmetic mean of the three standardized values of the three NET markers:

- NET index = z H3Cit / z NE/ z nucleosome/3.
- Likewise, the Complement index was estimated as:
- Complement index = (z_C5a + z_sC5b-9) / 2

Both indexes were again centered and standardized across the entire dataset and then incorporated in the regression models to reduce the effects of multicollinearity and to interpret the interaction term (NET × Complement) to represent the effect of an equivalent 1-SD increase in both pathways.

This process is important to assure that z-scores are used to assess biomarker deviation compared to healthy controls and still can be compared across scales when used together in multivariate models.

Ethical Approval and Consent

The protocol is submitted to the ethics committee of the institution, Sampling has little or no additional risk beyond routine phlebotomy and confidentiality is safeguarded by coded identifiers as well as limited access. Consent procedures are in accordance with the local policy for critically ill patients including the use of proxy consent where appropriate.

Results

Demographics and comorbidities were not similar between groups (p>0.05). Most sepsis patients were manifested by moderate to severe organ dysfunction (median SOFA 8), with 72% requiring admission to ICU and half being on vasopressors.

Table 1: Baseline characteristics

Variable	Sepsis (n = 100)	Controls (n = 50)	*P* value
Age (years), mean ± SD	57 ± 15	54 ± 13	0.28
Male sex, n (%)	58 (58%)	27 (54%)	0.64
BMI (kg/m²), mean ± SD	27.1 ± 4.8	26.4 ± 4.3	0.39
Diabetes mellitus, n (%)	33 (33%)	10 (20%)	0.11
Chronic kidney disease, n (%)	18 (18%)	4 (8%)	0.12
Cardiovascular disease, n (%)	29 (29%)	9 (18%)	0.15
Pulmonary source of infection, n (%)	41 (41%)	—	—
Urinary source of infection, n (%)	28 (28%)	—	—
Abdominal source of infection, n (%)	17 (17%)	—	—
Culture positive (blood), n (%)	46 (46%)	—	—
ICU admission, n (%)	72 (72%)	—	—
Mechanical ventilation, n (%)	39 (39%)	—	—
Vasopressors, n (%)	52 (52%)	—	—
SOFA score at T_0, median (IQR)	8 (6-10)	—	—
VTE prophylaxis within 24 h, n (%)	78 (78%)	—	—

NET as well as complement biomarkers were significantly elevated in sepsis. Median H3Cit and sC5b-9 levels were about four-fold higher than controls (p<0.001), which confirmed good activation of both pathways in developing systemic inflammation.

Table 2: Early NET and complement activation at T0 (0 - 6h)

Biomarker (T_0)	Sepsis median (IQR)	Control median (IQR)	Effect size (SMD)	*P* value
H3Cit (ng/mL)	420 (310-540)	95 (70-120)	1.62	< 0.001
Neutrophil elastase (ng/mL)	370 (250-460)	110 (80-150)	1.28	< 0.001
Nucleosomes (AU)	2.4 (1.8-3.2)	0.8 (0.6-1.1)	1.49	< 0.001
C5a (ng/mL)	48 (37-62)	14 (10-19)	1.71	< 0.001
sC5b-9 (ng/mL)	590 (450-730)	180 (150-220)	1.85	< 0.001
NET index (z-score)	1.42 ± 0.51	-0.73 ± 0.42	—	< 0.001
Complement index (z-score)	1.58 ± 0.55	-0.69 ± 0.47	—	< 0.001

NET and complement levels decreased after 72 h significantly (p<0.001), but it was noted that they are still at a higher level than medians of controls, indicating prolonged albeit diminishing activation during the acute phase.

Table 3: Temporal dynamics of biomarkers (T_0 - 24 hours - 72 hours) of sepsis (n = 80 with serial samples)

Biomarker	T_0 median (IQR)	24 h median (IQR)	72 h median (IQR)	*P* (repeated-measures Friedman)
H3Cit (ng/mL)	430 (320-540)	380 (270-470)	250 (170-330)	< 0.001
NE (ng/mL)	365 (260-460)	320 (210-410)	230 (150-300)	< 0.001
C5a (ng/mL)	49 (37-62)	44 (30-55)	31 (22-40)	< 0.001
sC5b-9 (ng/mL)	600 (460-720)	550 (410-670)	400 (310-510)	< 0.001

Coagulation tests showed the systemic activation of pro and anti-coagulant pathways. Platelet counts were significantly low and poorer fibrinogen and was found in favor of thromboinflammatory phenotype consistent with early SIC.

Table 4: Routine coagulation and thromboinflammatory on T_0

Marker	Sepsis mean ± SD	Control mean ± SD	*P* value
Platelets (×10⁹/L)	145 ± 60	262 ± 55	< 0.001
PT (INR)	1.42 ± 0.29	1.03 ± 0.08	< 0.001
aPTT (s)	44 ± 9	33 ± 6	< 0.001
Fibrinogen (g/L)	5.1 ± 1.3	3.3 ± 0.9	< 0.001

Strong positive correlations were found between NET and complement indices (rho 0.71) and each and clinical severity (SOFA, D-dimer). The inverse relationship with platelets (rho = -0.52) makes the NET-complement activation linked to consumptive coagulopathy.

Table 5: Marker correlation with indices of severity of disease

Variable 1	Variable 2	Spearman ρ
H3Cit ↔ SOFA	0.61	< 0.001
C5a ↔ SOFA	0.58	< 0.001
sC5b-9 ↔ D-dimer	0.63	< 0.001
NET index ↔ Complement index	0.71	< 0.001
NET index ↔ Platelets	-0.52	< 0.001

When adjusting for each other, both NET and complement indices were independently associated with overt DIC. Sympathetic and parasympathetic degminent first, synergistic potentiation confirms presence of Synergistic (concurrently high activations of both pathways increased the odds of DIC more than additive expectation) Fact

Table 6: Logistic regression for overt DIC (ISTH (>= 5)

Predictor	Adjusted OR (95% CI)	*P* value
NET index (z-score)	2.35 (1.48-3.71)	< 0.001
Complement index (z-score)	2.12 (1.37-3.27)	< 0.001
Interaction (NET×Complement)	1.68 (1.12-2.55)	0.014
Age	1.02 (0.99-1.04)	0.18
SOFA score	1.21 (1.09-1.36)	< 0.001

Nets and complement were higher indices predicting more 28-day mortality independently of age and SOFA. The interaction term was still significant, meaning that the simultaneous activation of both the cascades is linked to worst survival.

Table 7: Predictors of 28-day mortality time (multivariable Cox model)

Variable	Hazard ratio (95% CI)	*P* value
NET index (z)	1.84 (1.32-2.56)	< 0.001
Complement index (z)	1.63 (1.18-2.26)	0.003
NET × Complement interaction	1.47 (1.05-2.05)	0.023
SOFA score	1.11 (1.05-1.18)	< 0.001
Age	1.02 (1.00-1.04)	0.07

ROC analysis showed very good discrimination ability for composite thromboinflammatory result using both indices (AUC 0.93). Single markers had good, but lower performance (AUC 0.79 - 0.88). The dual-index model enhanced the sensitivity without affecting the specificity.

Table 8: Predictive ability to identify composite thrombo-inflammatory outcome

Biomarker / Index	AUC (95% CI)	Cut-off	Sensitivity%	Specificity%
H3Cit	0.82 (0.74-0.90)	> 300 ng/mL	78	80
C5a	0.79 (0.70-0.87)	> 40 ng/mL	74	78
sC5b-9	0.85 (0.78-0.92)	> 500 ng/mL	80	82
NET index	0.88 (0.82-0.94)	> 1.0 z	84	81
Complement index	0.86 (0.79-0.92)	> 1.1 z	83	80
Combined (NET + Complement index)	0.93 (0.88-0.97)	> 1.0	88	85

Outcomes and Event Rates

Eighty-eight percent (88 out of 100 sepsis patients, 28 of which met overt DIC criteria (ISTH score 5 or higher)) received overt DIC in hospital and 22 (22 out of 100 sepsis patients) patients died within 28 days. The rest 78 of the patients survived after day 28 or were censored at discharge. These numbers of events were sufficient to give the multivariate logistic and Cox regression model that was used to assess the DIC outcome and mortality and reduced the chance of model over fitting.

Discussion: This study examined the concerted activation of neutrophil extracellular traps (NETs) and the complement system in acutely ill systemic disease patients and their combined effect on thrombo-inflammation, coagulopathy and clinical outcome. The results showed that both of these

systems are highly activated in the early stages of sepsis and remain elevated over the first 72 hours of sepsis and interact to increase the severity of thrombo-inflammatory injury, consistent with emerging immunothrombotic paradigms. At the time presented, NET biomarkers such as H3Cit, NE, and nucleosomes and complement activation biomarkers such as C5a, sC5b-9 were markedly elevated in sepsis vs control, thereby confirming simultaneous activation in this innate immune and coagulation mechanism pathways. Temporal assessment demonstrated evidence of partial depression within 72 hours, but lasting abnormality, which is in line with previous reports stating that both systems are active for longer than the first inflammatory relay chain (Yang *et al.*, 2023) [22]. Strong positive correlations between NET and indices of complement (r = 0.71), and their inverse relation with platelet count suggest a role for cross amplification in consumptive coagulopathy. Regression analyses showed that high NET and complement indices were independent predictors of overt disseminated intravascular coagulation (DIC) and 28-day mortality and that the interaction term (NET * Complement) was significant even after adjustment for age and SOFA score. This indicates a biologically relevant synergy (as opposed to co-occurrence). The worst performance was in the individual markers and the biomarker model combination had excellent diagnostic performance (AUC = 0.93), underlining the clinical and diagnostic utility in firstly risk stratification. The results become consistent with accumulating evidence that NETs and complement behave as tentative cooperative effectors in the immunothrombosis state. Studies were conducted in the context of severe infection and Covid-19 finding parallels between increased levels of H3Cit and sC5b-9 and thrombotic events and multiorgan failure (Kaltenmeier *et al.*, 2022; Middleton *et al.*, 2023) [7, 11]. Experimental work has shown complement component C5a to be responsible for triggering the formation of NETs through the inhibition of mitochondrial STAT3, and this is one direct mechanistic link between both systems (Chen *et al.*, 2022) [3]. On the other hand, both the Nora histones and the proteases produced by the NET, can activate the other and terminal pathways of the complement, thus supporting a feed-forward cycle (Noris *et al.*, 2023) [12]. The presence of high levels of sC5b-9 and NET markers over 3 days is comparable to several studies in septic shock, finding that long-lasting complement consumption and NETosis were linked to organ dysfunction trajectories (Lupu *et al*, 2024) [10]. The observed link between the activation of the dual pathway and a decrease in platelet number is similar to those which reported the NET-complement crosstalk was promoting platelet aggregation and microvascular thrombosis (Lefrançais *et al.*, 2022) [8]. Collectively these data reinforce the notion that the synergism in the concept of combined targeting of NETosis and complement may have an attenuation effect on coagulation-driven inflammation more potently than single treatment options. NETs present a matrix with many DNA and histones that contribute to the speed of coagulation via the activation of factor XII and platelet adhesion. Complement fragments, especially C5a and C3a, further increase this process of neutrophil activation and harming of the endothelium. The present results prove that the risk for DIC is not additive but is actually multiplied by simultaneous high activation of both systems, suggesting non-additive synergism. This could be explained by:

1. C5a induced priming of neutrophils for producing NETs;
2. histones bound to NET activating the complement cascade; and
3. Formation of platelet-neutrophil aggregates with release of more C5a and thrombin (Carvelli *et al.*, 2022) [2].

These mechanisms combine to perpetuate thrombo-inflammatory circuits, endothelial dysfunction and microthrombosis with end organ dysfunction. The combined NET-complement index may play a useful role as a biomarker to identify early those patients at risk of sepsis-associated coagulopathy and thrombotic complications. Since the introduction of complement-directed inhibitors (eg, eculizumab, narsoplimab) into the clinical setting,1, 2 integrating NET biomarker into patient selection for trials of complement modulation or adjunctive DNase therapy could better separate patients for trial determination. Moreover, use of these markers in sepsis stratification models may lead to an improvement in predicting mortality outside of the current scoring systems. Main strengths include: prospective sampling, biopsy within 6 hours of specifics, serial biomarker assessment and adjustment for main confounders. Limitations include single center design, small sample size, and importance of the method of assay, in this case, the use of enzyme immunoassays (ELAZA) which may differ according to the manufacturer. Functional assays confirmation of complement pathway activity was not done. Therefore, causal inferences still remain limited and multicenter validation with mechanistic endpoints is in order.

Conclusion

Early in sepsis, Achara (NET biomarker) (H3Cit, NE, nucleosome), complement activation biomarkers (C5a, sC5b-9) were extremely elevated, in comparison to controls, with evidence of sustained yet falling activation during the first 72 hours. Strong correlation between NET and complement indices, in particular, with an inverse correlation with platelet counts and positive correlation with an index of SOFA and D-dimer, support for a thrombo-inflammatory axis being coordinated. In adjusted models, both NET and complement indices were independent prognosticators of overt DIC and 28-day death with the presence of a significant NET x complement interaction indicating cooperative risk amplification exceeding that of additive effects of the 2 models. A combination model of the two indices showed good discriminative performance for thrombo-inflammatory results compared with the results obtained using individual biomarkers. These results provide insight into a mechanistic basis whereby NET--complement co-activation contributes to the promotion of sepsis-associated coagulopathy and poor clinical outcomes and have justified definitive validation and evaluation of biomarkers-guided strategies to target both of these pathways in multicenter trials.

Confidentiality and Data Protection (optional add-on)

All study data was kept in password-protected electronic data files which could be accessed only by the research team. Any printed records were kept locked in cabinets in secure research facilities. Results were reported in aggregate format so as to prevent the identification of individual participants.

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- **Conflicts of Interest:** The authors have declared that they have no conflict of interest.

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