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ORIGINAL RESEARCH

Neutrophil Extracellular Traps and Delayed Cerebral Ischemia in Patients With Subarachnoid Hemorrhage

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BACKGROUND: Delayed cerebral ischemia (DCI) after spontaneous subarachnoid hemorrhage (SAH) is not well understood. Recent studies suggest the involvement of neutrophil-mediated immunothrombosis via neutrophil extracellular traps (NETs). We aimed to assess whether trajectories of NET biomarkers in patients with acute SAH are associated with DCI.

METHODS: This was a prospective single-center study of patients within 48 hours of SAH onset. We collected clinical, laboratory, imaging, and 3-month outcome data. NET markers (H3Cit [citrullinated histone H3]-DNA complexes, myeloperoxidase-DNA complexes, cell-free DNA), along with peptidylarginine deiminase 4, and deoxyribonuclease activity were measured in plasma collected on admission (baseline), and post-SAH days 5 and 10. DCI was defined by clinical and imaging criteria. Generalized estimating equations examined differences in NET trajectories comparing patients with and without DCI.

RESULTS: We enrolled 40 patients with SAH (mean age, 57 years; n=21 [53%] women; 32 [80%] aneurysmal). DCI occurred in 12 (30%) patients. All NET levels were similar between groups at baseline. On day 5, those with DCI compared with those without DCI had elevated median H3Cit-DNA complex (63 ng/mL versus 31 ng/mL, P=0.049), myeloperoxidase-DNA complex (5.9 ng/mL versus 0.4 ng/mL, P=0.029), and cell-free DNA (651 ng/mL versus 515 ng/mL, P=0.04) levels, which trended back down on day 10. In the DCI group, generalized estimating equations indicated heightened trajectories of H3Cit-DNA complex (day 5 [compared with baseline]: β=0.7; SE, 0.3; P=0.01; day 10: β=0.5; SE, 0.3; P=0.09), and myeloperoxidase-DNA complex levels (day 5: β=3.1; SE, 0.4; P<0.001; day 10: β=1.6; SE, 1.0; P=0.10).

CONCLUSIONS: In patients with acute SAH, elevated plasma trajectories of NETs are associated with DCI.

Key Words: delayed cerebral ischemia ■ inflammation ■ neutrophil extracellular traps ■ neutrophils ■ subarachnoid hemorrhage

With about one-third of affected individuals dead or severely disabled at 3 months, the contribution of subarachnoid hemorrhage (SAH) to all-stroke mortality and disability is disproportionately high compared with other stroke subtypes.[1] Reasons for poor outcome post-SAH have been delineated and include delayed cerebral ischemia (DCI). DCI is considered as complex pathophysiological process in SAH that involves functional impairment, especially on the neurovascular, cellular, and clinical level, and often includes progression to cerebral infarction within the first days to weeks after index SAH.

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RESEARCH PERSPECTIVE

What Is New?

- Key components of neutrophil extracellular traps (NETs) were found in patients with subarachnoid hemorrhage (SAH), especially in patients with SAH developing delayed cerebral ischemia (DCI), which is a main factor for poor clinical outcome.
- Pathophysiological processes contributing to DCI include endothelial dysfunction and microthrombosis; however, the mechanisms of the action of NETs in the context of DCI are currently unclear. Furthermore, NETs were found elevated before or early in the phase of cerebral vasospasms, suggesting a possible causal role of NETs in the development of DCI.

What Question Should Be Addressed Next?

- Future studies in larger cohorts need to address in more detail (1) trigger factors for elevated NET activity in patients with SAH with DCI and (2) the patho–/physiological processes in DCI that may specifically be influenced by NETs.
- Effects of NET blockade, performed especially early after onset of SAH, should be studied to further characterize the potential role of NETs during the development of DCI. Experimental and clinical results of NET research in SAH should be used to define and study possible targets for future therapeutic interventions to prevent or treat DCI.

Nonstandard Abbreviations and Acronyms

cfDNA	cell-free DNA
DCI	delayed cerebral ischemia
H3Cit-DNA	citrullinated histone H3-DNA
NET	neutrophil extracellular trap
PAD4	peptidylarginine deiminase 4

DCI increases the odds of poor outcome up to 5-fold.[2] However, the pathophysiology of DCI is not well understood, and highly effective therapies are lacking.[3]

On a cellular level, neutrophil-mediated inflammation has been linked to poststroke inflammation and delayed tissue injury after SAH.[4,5] However, neutrophils are not only damaging but have essential roles in resolution of inflammation and tissue repair.[6] In search of more specific injury mechanisms, neutrophil extracellular traps (NETs) have recently been explored in the context secondary injury.[7–9] NETs are a downstream neutrophil-mediated immune mechanism that can cause thrombosis and endothelial injury to blood vessels.[10] Data from SAH mouse models suggest associations between perivascular NETs and microvasospasm, and intravascular NETs and DCI.[9,11] Preliminary data in patients with acute SAH have demonstrated that NETs are abundant in peripheral blood, and that patients with DCI and those without DCI may have distinct NET level trajectories over the first days after the index SAH.[7–9] However, major limitations of the prior studies included the use of serum instead of plasma samples or measurements of biomarkers not specific for NETs.

Here, leveraging prospectively collected detailed clinical and imaging data with blinded multirater DCI assessment from a single-center cohort of patients with acute SAH, we tested the hypothesis that patients with DCI have elevated NET plasma concentrations and trajectories compared with patients without DCI.

METHODS

Data Availability

The statistical analysis and the data that support the findings of this study are available from the corresponding authors upon reasonable request.

Study Design and Participants

We used a prospective observational single-center cohort design. Patients were eligible for enrollment if they were ≥18 years of age and had a spontaneous SAH (aneurysmal or nonaneurysmal perimesencephalic) documented on a computed tomography (CT) scan of the head within 48 hours of symptom onset. Patients were excluded if one of the following conditions was present, because they have been reported to be associated with increased blood NET levels: recent (within 3 months before the index SAH) ischemic stroke or myocardial infarction or venous thromboembolism and a history of rheumatoid arthritis, systemic lupus erythematosus, active cancer, and hereditary coagulopathy. We also excluded those with a prior SAH.

A total of 40 patients were enrolled between November 2021 and January 2023 at a single site, Augsburg University Hospital in Germany. Treatment of patients with SAH followed guidelines set forth by the European Stroke Organization and by the American Heart Association and were supplemented by local standard operating procedures[12,13]; treatment details are reported in Data S1. Results of this study were reported in adherence to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

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Standard Protocol Approvals, Registrations, and Patient Consent

Written informed consent was provided by each patient or their legally authorized representative for study participation. Deferral of consent was allowed for patients who were not able to consent initially. Consent was waived for patients who were not able to consent initially and who died early during hospital stay. Institutional review board approval was obtained from the ethics committee of the Bavarian Medical Association. The study was registered with the German Register of Clinical Studies (www.drks.de, DRKS00025118).

Measurements

Laboratory Analyses

Peripheral blood samples of patients with acute SAH were collected on admission (within 48 hours from symptom onset), on day 5 (±1 day), and on day 10 (±1 day), or before discharge if the patient was discharged sooner. Samples were centrifuged within 30 minutes of collection twice at room temperature as previously described[8]; supernatants were stored in aliquots at −80 °C. Plasma samples were transferred in batches to KU Leuven. There, NET analyses were performed by laboratory staff blinded to all clinical, imaging, and outcome data. We measured the following NET components: H3Cit (citrullinated histone H3)-DNA complexes, myeloperoxidase-DNA complexes (absolute concentrations and percent of normal plasma pool), and cell-free DNA (cfDNA). We also measured PAD4 (peptidylarginine deiminase 4) and deoxyribonuclease activity (percent of normal plasma pool) as key regulators of NETs. We measured H3Cit-DNA complexes using a sandwich ELISA protocol modified from Thålin et al, with capture antibody anti-histone H3 citrulline R8 (Abcam, ab232939, 1.25 µg/mL) and detection antibody peroxidase-conjugated mouse anti-DNA monoclonal antibody (Cell Death Detection ELISA^{PLUS}, 11 774 425 001, 1:50 dilution; Roche, Basel, Switzerland).[14] We measured myeloperoxidase-DNA complexes using an in-house sandwich ELISA protocol with capture antibody anti-myeloperoxidase polyclonal antibody (Invitrogen; PA5-16672, 1:1000 dilution), protein blocking with 2% low-endotoxin bovine serum albumin (Carl Roth, Karlsruhe, Germany), samples diluted 1:5 in incubation buffer from the Cell Death Detection ELISA kit (11 544 675 001; Roche), and peroxidase-conjugated anti-double-stranded DNA antibody from the Cell Death Detection ELISA diluted 1:40 for detection.[15] In both cases, the reaction was visualized using tetramethylbenzidine substrate (Life Technologies, Carlsbad, CA), stopped with 1N hydrochloric acid, and absorbance measured at 450 nM with 630 nM background subtraction using a Gen5 microplate reader (BioTek, Santa Clara, CA). cf-DNA levels were measured using the Quant-iT Picogreen dsDNA Assay (Invitrogen; P7589). We measured PAD4 levels using the human PAD4 ELISA kit according to the manufacturer's instructions (Cayman Chemical; 501 460). We measured deoxyribonuclease 1 activity using the fluorometric DNase 1 Assay Kit from Abcam (ab234056) as described.[8] Kinetic reads were immediately measured every 30 seconds for 90 minutes at 37 °C using a Cytation 5FV fluorescent plate reader with excitation wavelength at 620 nm and emission wavelength at 680 nm. Deoxyribonuclease activity values were calculated by normalizing the slopes of the measured samples to the average slope of a pool of human citrated (3.2%) plasma (088SER-PLP50-CUSNaC; Tebu-Bio), run in duplicate. A pool of commercially human citrated plasma (SER-PLP50; Zenbio) was run in 4 replicates to obtain reference values for NET biomarkers as well as deoxyribonuclease activity.

Neuroimaging

Based on the consensus set forth by the Neurocritical Care Society multidisciplinary research group,[3,16] DCI was defined as either a neurological worsening (a new focal exam finding or a Glasgow Coma Score reduction ≥2), or a new infarct on brain imaging, not attributable to competing causes. To determine whether a new infarct was present on brain imaging, brain CT scans, and if applicable brain magnetic resonance imaging scans, were evaluated using a 3-raters-consensus approach performed by 2 board-certified neuroradiologists (C.J.M., A.B.) and 1 board-certified neurointensivist (H.S.). Raters were blinded to all clinical, laboratory, and outcome data. DCI was defined as a new ischemic infarction not attributable to aneurysm treatment determined by its timing (occurring between 48 hours after aneurysm treatment and up to 6 weeks following the initial SAH), anatomical location (infarcts in regions not directly manipulated during aneurysm treatment), and its appearance in sequential imaging (new infarcts appearing on delayed imaging rather than immediately postprocedure).

Clinical Outcomes

Clinical outcome (modified Rankin Scale score) was assessed by trained study investigators, either through an in-person assessment or a structured validated phone interview (Rankin Focused Assessment), at hospital discharge and at 3 months.[17]

Statistical Analysis

Clinical, imaging, routine laboratory, and outcome data were prospectively collected in case report forms and

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transferred to an electronic study database. Data were checked for completeness and consistency by 2 investigators (D.M., H.S.) independently before statistical analyses were performed.

For statistics software we used IBM SPSS Statistics (version 29; IBM, Armonk, NY), GraphPad Prism (version 10.1.1; GraphPad Software, Boston, MA), and the generalized estimating equations (GEE) package for R (www.r-project.org; Vienna, Austria). We reported data as counts (percentages) or medians (interquartile ranges [IQRs]), unless specified otherwise. Normality assumption of data was evaluated with a histogram and by performing the Shapiro-Wilk test for each variable. To test for intergroup differences for continuous variables, we used the Mann-Whitney *U* test or an unpaired 2-tailed *t* test depending on the normality of distribution, and the χ^2 test for categorical variables. Paired tests and GEE were performed for analyses of repeated measures of NET biomarkers. To compare trajectories of NET biomarker plasma levels between the groups of patients with and those without DCI, regression models for each NET biomarker were fitted (γ family with log link). To ensure validity and robustness of inferences, we applied the mean–variance relationship visual assessment using residual plot and overdispersion assessment. We had a priori determined to adjust the GEE models for age and disease severity (Hunt-Hess score on admission); however, given the small sample size, addition of any variables overfit the models and caused them to fail. We thus restricted the GEE to the variables NET plasma levels and sampling time points after running a univariate GEE model that did not show an association between patient age and NET levels or between Hunt-Hess grade on admission and NET levels. The threshold for statistical significance was a 2-sided $P<0.05$.

The corresponding authors had full access to all data in the study and take responsibility for their integrity and the data analysis.

RESULTS

Cohort Characteristics

Among 72 patients with CT-documented SAH admitted to the critical care unit during the study period, 40 patients were enrolled. Reasons for study inclusion and exclusion are shown in Figure 1. Most patients not

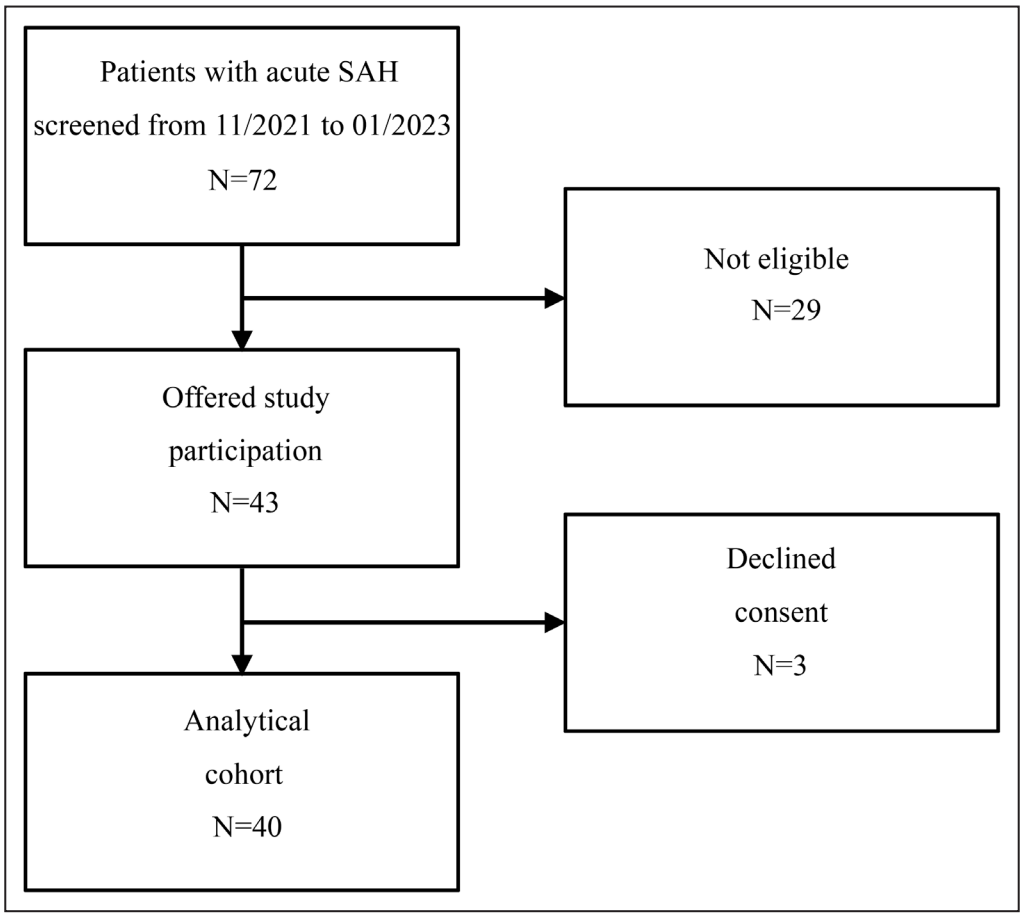

Figure 1. Flowchart of selected patients.
SAH indicates subarachnoid hemorrhage.

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Table 1. Characteristics of Patients With Spontaneous Subarachnoid Hemorrhage, Stratified by Presence or Absence of Delayed Cerebral Ischemia

Characteristic	DCI (n=12)	No DCI (n=28)	*P* value
Age, y, mean±SD	51.5±13.1	60.3±9.2	0.02, 2-tailed *t* test
Women, n (%)	7 (58)	14 (50)	0.63, χ^2 test
Hunt-Hess Scale score on admission, median (IQR)	4 (2–4)	3 (2–5)	0.88, MWU
Glasgow Coma Scale score on admission, median (IQR)	10 (7–15)	15 (3–15)	0.54, MWU
Modified Fisher Scale score (on first imaging), median (IQR)	4 (3–4)	3 (3–4)	0.14, MWU
Motor deficit on admission, n (%)	3 (25)	6 (21)	0.31, χ^2 test
Intracranial aneurysm detected, n (%)	11 (92)	21 (75)	0.23, χ^2 test
On home antiplatelet before admission, n (%)	1 (8)	3 (11)	0.82, χ^2 test
On home anticoagulant before admission, n (%)	0 (0)	3 (11)	0.24, χ^2 test
Time interval from symptom onset to first NET sampling, h, median (IQR)	34.0 (21.3–36.8)	37.5 (26.3–45.0)	0.16, MWU

DCI indicates delayed cerebral ischemia; IQR, interquartile range; MWU, Mann-Whitney *U* test; and NET, neutrophil extracellular trap.

included during the recruitment period were admitted or screened outside the time window for inclusion. Clinical and imaging baseline characteristics of the analytical cohort (mean±SD; age, 57.7±11.2 years; 21 [53%] women) are reported in Table 1. Twelve patients (30%) developed DCI. Those with DCI compared with those without DCI were significantly younger (52 versus 60 years, *P*=0.02) and had a higher rate of aneurysmal SAH cause (92% versus 75%, *P* not significant). The time from symptom onset to first NET sample collection was similar comparing participants with DCI and those without DCI (median 34 hours; IQR, 21–37 versus 38; IQR, 26–45; *P*=0.16). Aneurysm repair was predominantly endovascular in both groups, and aneurysm treatment modalities were otherwise similar considering the larger subgroup of those without identified aneurysm (and therefore no need for aneurysm repair) in the group without DCI (Table 2). During the hospital course, patients with DCI had higher rates of external ventricular drain (92% versus 64%, *P*=0.08) and tracheostomy requirements (33% versus 4%, *P*=0.009) and had a higher rate of indwelling ventriculoperitoneal shunt at discharge (33% versus 14%, *P*=0.2). The modified Rankin Scale scores at 3 months were worse overall in the DCI group (median, 6; IQR, 1–6 versus median, 2; IQR, 1–6; *P*=0.25), as was mortality (50% versus 29%, *P*=0.2).

Plasma NETs in Patients Versus Controls

Plasma samples in patients with SAH were collected at a median (IQR) of 36 hours (25–42) (baseline), 123 hours (112–155) (day 5), and 235 hours (217–253) (day 10). In comparison to reference values of human controls, the SAH cohort had 3- to 5-fold higher H3Cit-DNA-complex levels, and ≈1.5-fold higher cfDNA levels, across all 3 plasma sampling time points (Table 3). Numerically, but not statistically significant, myeloperoxidase-DNA levels were ≈3-fold elevated, and deoxyribonuclease plasma activity was mildly elevated in patients with SAH compared with controls. PAD4 levels were similar to control levels.

Table 2. Hospital Course, Complications, and Outcomes Stratified by Presence or Absence of Delayed Cerebral Ischemia

Characteristic	DCI (n=12)	No DCI (n=28)	*P* value
Aneurysm treatment: clipping, n (%)	1 (8.3)	6 (21.5)	0.20, χ^2 test
Aneurysm treatment: endovascular, n (%)	10 (83.3)	15 (53.6)	
Aneurysm treatment: none/NA, n (%)	1 (8.4)	7 (24.9)	
Mechanical ventilation during index hospitalization, n (%)	7 (58)	14 (50)	0.11, χ^2 test
External ventricular drain, n (%)	11 (91.7)	18 (64.3)	0.076, χ^2 test
Tracheostomy, n (%)	4 (33.3)	1 (3.6)	0.009, χ^2 test
Ventriculoperitoneal shunt at discharge, n (%)	4 (33.3)	4 (14.3)	0.17, χ^2 test
Discharge mortality, n (%)	5 (41.7)	8 (28.6)	0.42, χ^2 test
Discharge mRS score, median (IQR)	5 (1–6)	2 (0–6)	0.27, MWU
3-month mortality, n (%)	6 (50)	8 (28.6)	0.19, χ^2 test
3-month mRS score, median (IQR)	6 (1–6)	2 (1–6)	0.25 MWU

DCI indicates delayed cerebral ischemia; IQR, interquartile range; mRS, modified Rankin Scale; NA, not applicable; and MWU, Mann-Whitney *U* test.

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Table 3. NETs Plasma Levels in Patients With SAH Compared With Human Controls

NETs plasma biomarker	SAH (n=40)	Reference value (n=4)	*P* value*
H3Cit-DNA, ng/mL, median (IQR)			
Visit 1	23.5 (12.6–43.9)	6.4 (5.5–7.3)	0.002
Visit 2	32.2 (16.9–70.1)		0.003
Visit 3	30.6 (12.1–54.0)		0.02
Myeloperoxidase-DNA, ng/mL, median (IQR)			
Visit 1	0.66 (0.095–4.48)	0.19 (0.04–4.6)	0.28
Visit 2	0.73 (0.13–4.38)		0.15
Visit 3	0.66 (0.000–3.56)		0.37
PAD4, ng/mL, median (IQR)			
Visit 1	0.0185 (0.0013–0.093)	0.025 (0.020–0.032)	0.78
Visit 2	0.034 (0.013–0.092)		0.82
Visit 3	0.025 (0.001–0.108)		0.98
cfDNA, ng/mL, median (IQR)			
Visit 1	509 (431–665)	360 (282–391)	0.001
Visit 2	556 (444–737)		0.001
Visit 3	533 (457–689)		0.002
Deoxyribonuclease activity, % of normal plasma pool, median (IQR)			
Visit 1	120 (100–149)	100	NA
Visit 2	126 (101–166)		NA
Visit 3	118 (92–182)		NA

cfDNA indicates cell-free DNA; H3Cit-DNA, citrullinated histone H3-DNA complex; IQR, interquartile range; NA, not applicable; NET, neutrophil extracellular trap; PAD4, peptidylarginine deiminase 4; and SAH, subarachnoid hemorrhage.
*Statistical test: Mann-Whitney *U* test.

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Plasma NETs in DCI Versus No DCI

At baseline, median NET levels in plasma were similar in patients with DCI compared with those without DCI (Figure 2, Table S1).

On post-SAH day 5, those with DCI compared with those without DCI had elevated median H3Cit-DNA complex (*P*=0.049), myeloperoxidase-DNA complex (*P*=0.029), and cfDNA levels (*P*=0.038). Both groups had similar day-5 levels of PAD4 (*P*=0.61) and deoxyribonuclease activity (*P*=0.3).

On post-SAH day 10, intergroup differences were less pronounced, although the median H3Cit-DNA and myeloperoxidase-DNA complex levels remained ≈2-fold elevated in the DCI group. The median cfDNA level in the DCI group remained slightly but significantly elevated (*P*=0.03). As on day 5, we found similar day-10 levels of PAD4 and deoxyribonuclease activity in both groups (Figure 2, Table S1).

NET Trajectories in DCI Versus No DCI

In patients with DCI, GEE models demonstrated elevated trajectories on day 5 for H3Cit-DNA (compared with baseline: β=0.7; SE, 0.3; *P*=0.01) and myeloperoxidase-DNA complex levels (compared with baseline: β=3.1; SE, 0.4; *P*<0.001) (Table 4 and Figure 3). We found trend level elevations for myeloperoxidase-DNA and H3Cit-DNA complexes on day 10. Additionally, in the group without DCI, there was a significant downtrend of myeloperoxidase-DNA levels (compared with baseline, day 5: β=–1.3; SE, 0.3; *P*<0.001; day 10: β=–1.0; SE, 0.2; *P*<0.001). There were no significant up or down trends within the remainder of NET-related marker trajectories (Table 4). In a post hoc subgroup analysis, from which we excluded those with nonaneurysmal SAH, GEE results were similar for myeloperoxidase-DNA complex level trajectories (DCI group, day 5 compared with baseline: β=3.1; SE, 0.52; *P*<0.001), but the statistical significance of the elevated H3Cit-DNA trajectory was not reproduced in this subgroup (DCI group, day 5 compared with baseline: β=0.36; SE, 0.26; *P*=0.16) (Table S2).

DISCUSSION

In patients with acute SAH, we found that peripheral plasma levels of H3Cit-DNA complexes and myeloperoxidase-DNA complexes, 2 specific NET biomarkers, were elevated about 3-fold compared with reference values of human controls. Those patients with SAH who developed DCI had H3Cit-DNA and myeloperoxidase-DNA complex level peaks on postbleed day 5 and distinct elevated trajectories over time compared with patients without DCI. A subgroup analysis limited to those with aneurysmal SAH

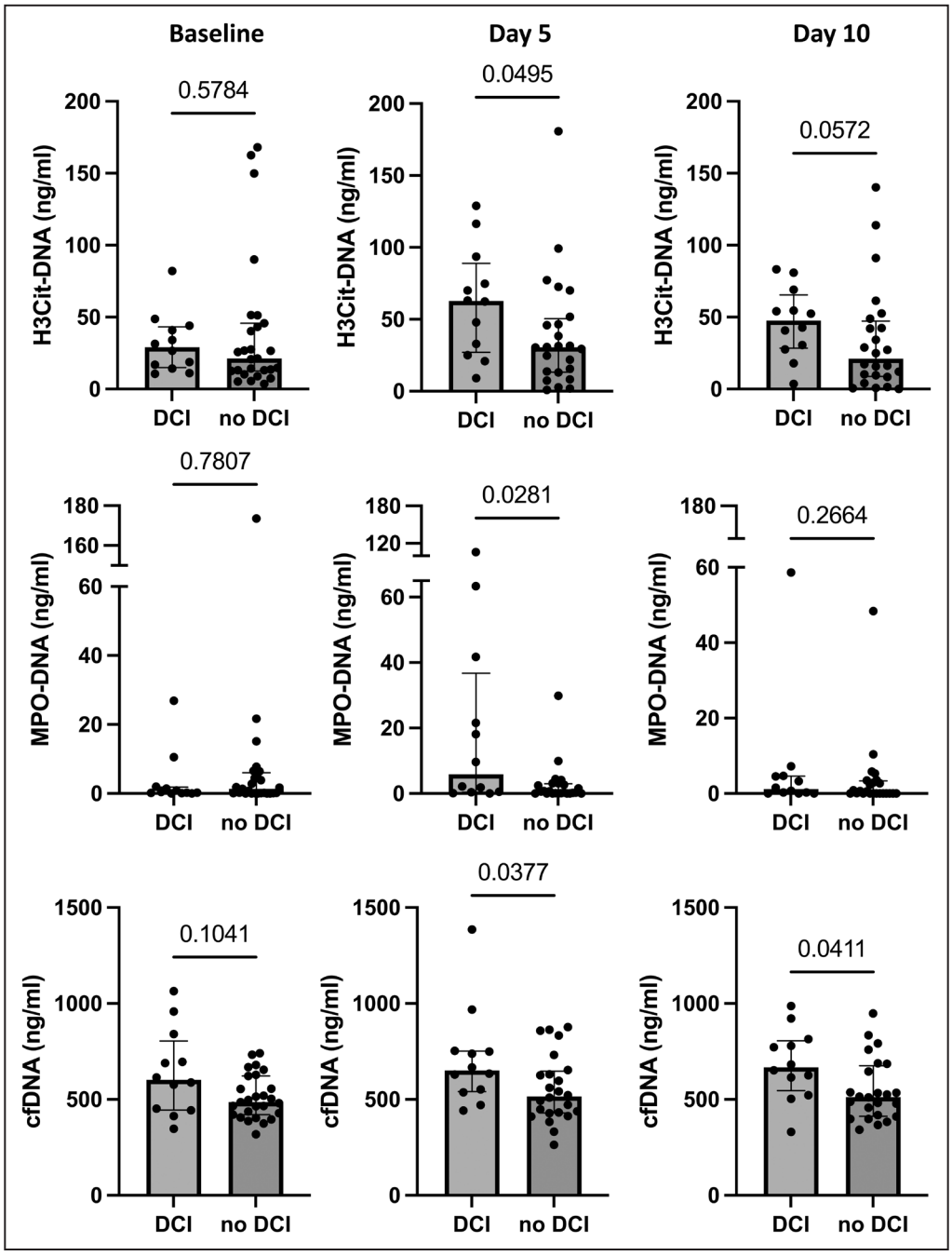

Figure 2. NET levels at 3 time points (baseline, day 5, day 10) after the index subarachnoid hemorrhage, stratified by presence vs absence of delayed cerebral ischemia.
cfDNA indicates cell-free DNA; DCI, delayed cerebral ischemia; H3Cit-DNA, citrullinated histone H3-DNA complex; MPO-DNA, myeloperoxidase-DNA complex; and NET, neutrophil extracellular trap.

showed NET trajectories that were in line with the main analysis.

Framing these results in a biologically plausible way, we found that plasma markers of cellular breakdown (cfDNA) and DNA clearance (deoxyribonuclease) were elevated in patients with SAH compared with human controls. Moreover, cfDNA was more elevated in patients with SAH with DCI than in those without DCI and continued to be elevated in patients with DCI beyond post-SAH day 5, although this did not translate to elevated trajectories in the repeated measures GEE analysis. This can be interpreted as a qualitative difference between patients with SAH and controls (presence of cellular breakdown in patients with SAH), and that there was quantitatively more injured tissue in those who developed DCI in addition to the directly

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Table 4. Effects of Plasma Sampling Time Point on NET or NET-Related Marker Levels Modeled by Generalized Estimating Equations, Stratified by Presence or Absence of Delayed Cerebral Ischemia

	H3Cit-DNA complexes		Myeloperoxidase-DNA complexes		PAD4		cfDNA		Deoxyribonuclease	
Variable	β (SE)	*P* value	β (SE)	*P* value	β (SE)	*P* value	β (SE)	*P* value	β (SE)	*P* value
DCI										
Time, day 5	0.67 (0.27)	0.014	3.10 (0.43)	<0.001	−0.17 (0.28)	0.55	0.05 (0.12)	0.65	−0.03 (0.25)	0.89
Time, day 10	0.51 (0.30)	0.09	1.60 (0.99)	0.10	0.26 (0.20)	0.180	0.03 (0.12)	0.82	−0.25 (0.25)	0.32
No DCI										
Time, day 5	0.009 (0.23)	0.97	−1.28 (0.34)	<0.001	−0.21 (0.16)	0.17	0.05 (0.05)	0.36	0.18 (0.20)	0.37
Time, day 10	−0.13 (0.21)	0.55	−0.97 (0.24)	<0.001	−0.18 (0.13)	0.15	0.04 (0.06)	0.52	0.26 (0.20)	0.20

cfDNA indicates cell-free DNA; DCI, delayed cerebral ischemia; H3Cit-DNA, citrullinated histone H3-DNA; NET, neutrophil extracellular trap; and PAD4, peptidylarginine deiminase 4.

hemorrhage-related primary tissue injury (more cellular breakdown in those with DCI). These findings of increased cellular breakdown in patients with DCI lend biochemical support to our clinical and blinded radiographic DCI attribution. The sustained cfDNA elevation up to post-SAH day 10 demonstrates that the time-limited NET (H3Cit-DNA and myeloperoxidase-DNA complexes) level elevation around post-SAH day 5 is not merely another marker of DCI-related cell death but more likely a correlate of increased NET activity

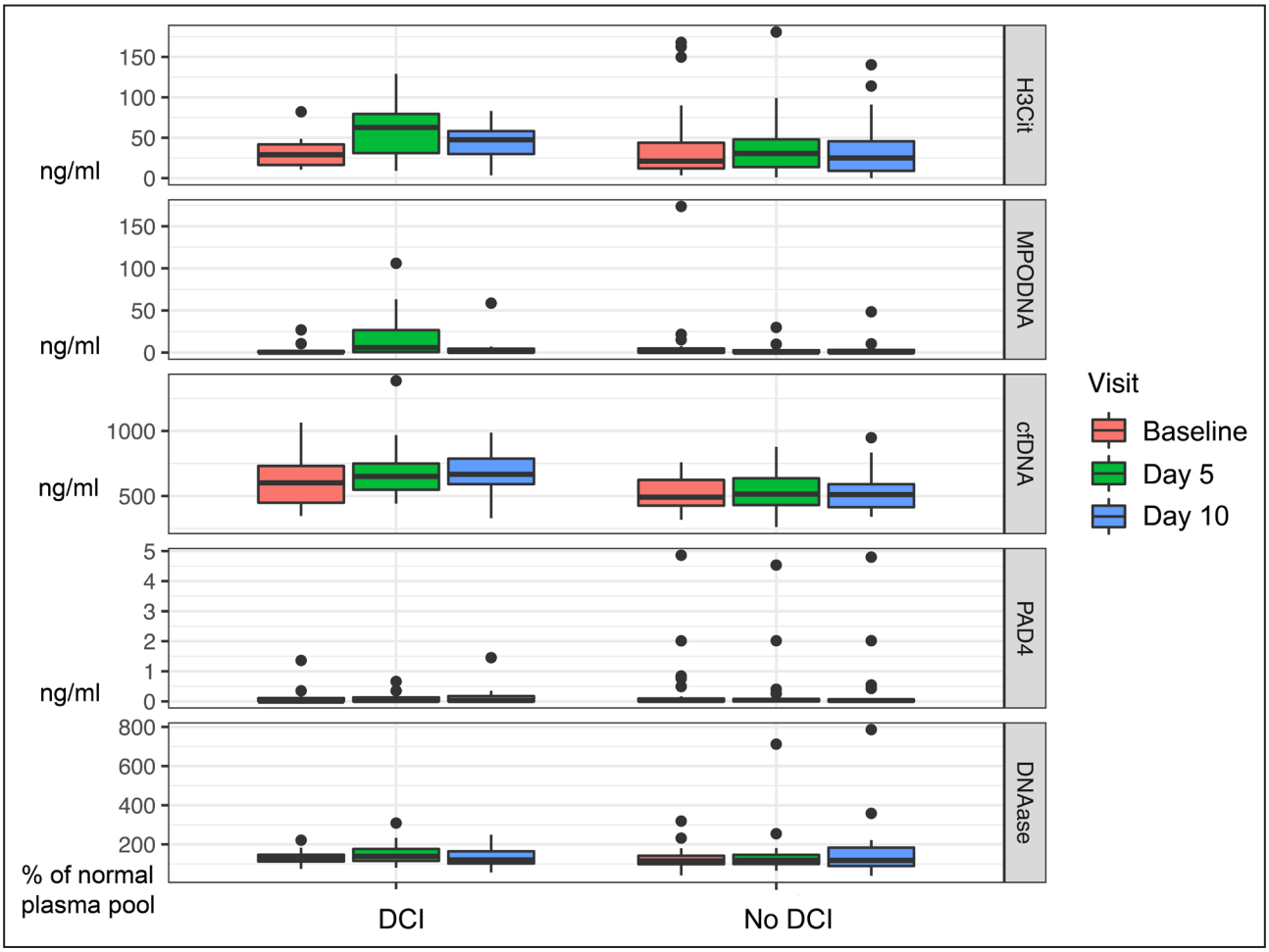

Figure 3. NET plasma-level trajectories.
Absolute NET plasma levels at baseline, post-SAH days 5 and 10, stratified by presence or absence of DCI. cfDNA indicates cell-free DNA; DCI, delayed cerebral ischemia; H3cit-DNA, citrullinated histone H3-DNA complex; MPO-DNA, myeloperoxidase-DNA complex; and NET, neutrophil extracellular traps.

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before DCI development. This may point to a causal role of NETs in the development of DCI.

Considering the results of the post hoc subgroup analyses of patients with aneurysmal SAH only, showing GEE trajectories for myeloperoxidase-DNA in line with main results, we assume that the increase of NETs at day 5 after SAH may be interpreted as specific for DCI. Including patients without aneurysmal DCI in our exploratory study broadened the spectrum of analyzable patients with SAH. However, larger cohorts of aneurysmal patients with SAH should be considered for future studies to detect the full spectrum of NET responses observed in our main cohort.

Our results should be considered in the context of prior studies. They align with recently published results by Zeineddine et al obtained in plasma samples of patients with acute SAH, where the authors found trends toward higher plasma levels in their selection of biomarkers in those patients with DCI compared with those without DCI.[9] Importantly, Zeineddine et al's study measured neutrophil elastase, a marker of neutrophil (not necessarily NETs-mediated) inflammation and a previously used commercially available histone H3 (not H3Cit-DNA complex) ELISA kit. In contrast, our study used a recently recommended approach measuring H3Cit-DNA complexes via a custom made in-house kit, in addition to our measurements of 2 additional NET-specific markers.[14] These methodological differences limit the comparability between ours and Zeineddine and coworkers' studies, which found no significant differences in H3Cit-DNA levels between their DCI and no-DCI groups before post-SAH day 10.

Our study stands in contrast to our own prior findings obtained in serum samples from patients with SAH, which, antidromic to the plasma trajectories, suggested an overall reduction (not an increase) in NET levels from admission to post-SAH day 4, and a more pronounced NET level reduction in those with DCI.[7] This could be due to NETs in plasma being removed during serum preparation at the time of centrifugation along with clotted proteins and platelets.

Our study in synopsis with the prior work illustrates the need for methodological standardization to obtain reliable NET measurements. Serum and plasma NET levels cannot be equated, possibly because ongoing procoagulatory processes after serum sample collection skew NET measurements. Laboratory markers should be NET-specific and align with broad scientific consensus.[14]

Our study adds several novel aspects to the existing literature. It is the first to demonstrate the presence of NET-specific markers in plasma of patients with acute SAH and to detect higher NET levels in those with DCI at a time that follows peak neutrophil inflammation and precedes peak DCI detection in patients with SAH.[9,18] On the one hand, these findings add plasma NETs to a list of biomarkers worthy of further investigation as potential indicators of impending DCI.[19] On the other hand, more importantly, our findings are well aligned with emerging data assigning pathophysiologic relevance to neutrophil-mediated inflammation and NETs in secondary injury mechanisms post-SAH.[9,20,21] For example, a recent investigation in an SAH mouse model visualized NETs in the perivascular space of small brain vessels and associated them with microvasospasm, implying a role in DCI pathophysiology.[11] In the same rodent study, inhibition of NETs via intracisternal administration of deoxyribonuclease reduced perivascular NET presence and microvasospasm.

The observational nature of our study allows limited insights into how NETs may be mechanistically linked to DCI. However, the existing literature in other disease entities firmly links NETs to thrombosis and endothelial injury.[10,22] Translating these maladaptive mechanisms of NETs into a hypothetical role in the cause of DCI, NETs may directly lead to small vessel thrombosis causing brain infarction or via endothelial compromise trigger microvasospasm and secondary hypoperfusion.[11] Synergy between vasospasm, microthrombosis, and other contributors is conceivable.

Our study may foreshadow clinical implications if future research can confirm a mechanistic contribution of NETs to DCI. Data from animal models suggest that NETs can be targeted at the stage of NET formation (NETosis) using Janus kinase (JAK) inhibitors, IL(interleukin)-17, or disulfiram, or at the stage after NETs have been released into the extracellular space through dismantling of NET-associated DNA using deoxyribonuclease.[22–24] Our study in synopsis with the study by Zeineddine et al demonstrates a peak peripheral plasma concentration of NETs around post-SAH days 4 to 6. This occurs at a time that is after the peak neutrophil brain tissue infiltration in the first 1 to 3 days post-SAH, which may suggest an early time window to prevent DCI in the first days after hospital admission for acute SAH.[9]

LIMITATIONS

Our study has several limitations. First, this was an observational study that cannot provide definitive evidence of a causal relationship between NETs and DCI. For instance, in addition to measuring plasma NET markers, capturing concomitant information on intraparenchymal or cerebrospinal fluid NETs could provide additional mechanistic insights. Second, the single-center design and cohort size limited generalizability and statistical power of our findings. This may have impacted the statistical significance of our NET level comparisons between patients and healthy controls as well as our subgroup analysis. The low number of subjects and measures preclude reliable estimation of higher-order

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interactions or a fully adjusted model that includes multiple covariates simultaneously. Future studies with expanded sample sizes will be needed to explore more complex multivariable relationships. Third, the largely CT (not magnetic resonance imaging)-based DCI definition may not have identified those who develop smaller delayed infarcts only visible on magnetic resonance imaging; such less-pronounced pathology, although not part of the consensus definition of DCI, may also fall on a DCI spectrum and may be worth correlating with plasma NET levels in the future. Furthermore, intensified imaging monitoring (eg, by serial CT perfusion) may identify more patients with DCI or risk for DCI and should be applied in future studies. Fourth, when designing this explorative study, we had prioritized feasibility and thus planned for only 3 plasma sampling time points per participant; more samples and thus a higher temporal resolution of NET trajectories would be desirable and should be considered in follow-up studies. Fifth, given our limited sample size, we were unable to adjust our GEE analysis for other factors known to influence NET plasma levels and did not capture all of them (eg, effects of aneurysm repair, acute hospital acquired infections, or new acute deep vein thromboses during hospitalization). Sixth, at the time of conceptualization of our study, any correlation between NETs and DCI was unknown; therefore, the study was not powered to validate NETs as potential clinical biomarkers to help predict DCI. This would have required preliminary data to conduct a statistical power calculation and a more expansive study design including an independent cohort for external biomarker validation. Last, patients were recruited in a single German tertiary care center where the majority of patients are White; results may therefore not be generalizable to other countries, races, and health care systems.

CONCLUSIONS

In patients with acute SAH, we detected plasma biomarkers specific for NETs, an immunothrombotic product of neutrophils, and found elevated H3Cit and myeloperoxidase-DNA complex biomarker trajectories that preceded the development of DCI. It may be worthwhile to study the role of NETs in DCI further, because NETs may become useful as clinical markers heralding DCI or as targets of therapies aiming to prevent DCI.

ARTICLE INFORMATION

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Disclosures

Dr Martinod has received consulting fees from PEEL Therapeutics. She is an inventor on granted US patent US10617742B2 for the methods for treating and preventing neutrophil-derived NET toxicity and thrombosis and granted US patent US11426405B2 on Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling inhibition preventing NET formation. The remaining authors have no disclosures to report.

Supplemental Material

Data S1
Tables S1–S2

REFERENCES

1. De Marchis GM, Lantigua H, Schmidt JM, Lord AS, Velander AJ, Fernandez A, Falo MC, Agarwal S, Connolly ES Jr, Claassen J, et al. Impact of premorbid hypertension on haemorrhage severity and aneurysm rebleeding risk after subarachnoid haemorrhage. *J Neurol Neurosurg Psychiatry*. 2014;85:56–59. doi: 10.1136/jnnp-2013-305051
2. Veldeman M, Rossmann T, Haeren R, Vossen LV, Weiss M, Conzen C, Siironen JO, Korja M, Schmidt TP, Hollig A, et al. Delayed cerebral infarction after aneurysmal subarachnoid hemorrhage: location, distribution patterns, infarct load, and effect on outcome. *Neurology*. 2024;103:e209607. doi: 10.1212/WNL.0000000000209607
3. Vergouwen MD, Vermeulen M, van Gijn J, Rinkel GJ, Wijdicks EF, Muizelaar JP, Mendelow AD, Juvela S, Yonas H, Terbrugge KG, et al. Definition of delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage as an outcome event in clinical trials and observational studies: proposal of a multidisciplinary research group. *Stroke*. 2010;41:2391–2395. doi: 10.1161/STROKEAHA.110.589275
4. Coulibaly AP, Pezuk P, Varghese P, Gartman W, Triebwasser D, Kulas JA, Liu L, Syed M, Tvrdik P, Ferris H, et al. Neutrophil enzyme myeloperoxidase modulates neuronal response in a model of subarachnoid hemorrhage by venous injury. *Stroke*. 2021;52:3374–3384. doi: 10.1161/STROKEAHA.120.033513
5. Witsch J, Roh D, Oh S, Iadecola C, Diaz-Arrastia R, Kasner SE, Mayer SA, Murthy SB. Association between soluble intercellular adhesion Molecule-1 and intracerebral hemorrhage outcomes in the FAST trial. *Stroke*. 2023;54:1726–1734. doi: 10.1161/strokeaha.123.042466
6. Zhao X, Ting SM, Sun G, Roy-O'Reilly M, Mobley AS, Bautista Garrido J, Zheng X, Obertas L, Jung JE, Kruzel M, et al. Beneficial role of neutrophils through function of Lactoferrin after intracerebral hemorrhage. *Stroke*. 2018;49:1241–1247. doi: 10.1161/STROKEAHA.117.020544
7. Witsch J, Spalart V, Martinod K, Schneider H, Oertel J, Geisel J, Hendrix P, Hemmer S. Neutrophil extracellular traps and delayed cerebral ischemia in aneurysmal subarachnoid hemorrhage. *Crit Care Explor*. 2022;4:e0692. doi: 10.1097/CCE.0000000000000692
8. Hendrix P, Witsch J, Spalart V, Schneider H, Oertel J, Geisel J, Martinod K, Hemmer S. Neutrophil extracellular trap biomarkers in aneurysmal

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subarachnoid hemorrhage: early decline of DNase 1 activity associated with delayed cerebral ischemia. *Front Neurol*. 2024;15:1354224. doi: 10.3389/fneur.2024.1354224
9. Zeineddine HA, Hong SH, Peesh P, Dienel A, Torres K, Thankamani Pandit P, Matsumura K, Huang S, Li W, Chauhan A, et al. Neutrophils and neutrophil extracellular traps cause vascular occlusion and delayed cerebral ischemia after subarachnoid hemorrhage in mice. *Arterioscler Thromb Vasc Biol*. 2024;44:635–652. doi: 10.1161/ATVBAHA.123.320224
10. Martinod K, Wagner DD. Thrombosis: tangled up in NETs. *Blood*. 2014;123:2768–2776. doi: 10.1182/blood-2013-10-463646
11. Nakagawa RIT, Shibuya N, Kishima H, Ymashita T. Perivascular neutrophil extracellular traps exacerbate microvasospasm after experimental subarachnoid hemorrhage. *Stroke*. 2024;12:2872–2881. doi: 10.1161/STROKEAHA.124.047574
12. Steiner T, Juvela S, Unterberg A, Jung C, Forsting M, Rinkel G; European Stroke O. European stroke organization guidelines for the management of intracranial aneurysms and subarachnoid haemorrhage. *Cerebrovasc Dis*. 2013;35:93–112. doi: 10.1159/000346087
13. Connolly ES Jr, Rabinstein AA, Carhuapoma JR, Derdeyn CP, Dion J, Higashida RT, Hoh BL, Kirkness CJ, Naidech AM, Ogilvy CS, et al. Guidelines for the management of aneurysmal subarachnoid hemorrhage: a guideline for healthcare professionals from the American Heart Association/american Stroke Association. *Stroke*. 2012;43:1711–1737. doi: 10.1161/STR.0b013e3182587839
14. Thalin C, Aguilera K, Hall NW, Marunde MR, Burg JM, Rosell A, Daleskog M, Mansson M, Hisada Y, Meiners MJ, et al. Quantification of citrullinated histones: development of an improved assay to reliably quantify nucleosomal H3Cit in human plasma. *Journal of Thrombosis and Haemostasis*. 2020;18:2732–2743. doi: 10.1111/jth.15003
15. Vanderbeke L, Van Mol P, Van Herck Y, De Smet F, Humblet-Baron S, Martinod K, Antoranz A, Arijs I, Boeckx B, Bosisio FM, et al. Monocyte-driven atypical cytokine storm and aberrant neutrophil activation as key mediators of COVID-19 disease severity. *Nat Commun*. 2021;12:4117. doi: 10.1038/s41467-021-24360-w
16. de Oliveira Manoel AL, van der Jagt M, Amin-Hanjani S, Bambakidis NC, Brophy GM, Bulsara K, Claassen J, Connolly ES, Hoffer SA, Hoh BL, et al. Common data elements for Unruptured intracranial aneurysms and aneurysmal subarachnoid hemorrhage: recommendations from the working group on hospital course and acute therapies-proposal of a multidisciplinary research group. *Neurocrit Care*. 2019;30:36–45. doi: 10.1007/s12028-019-00726-3
17. Saver JL, Filip B, Hamilton S, Yanes A, Craig S, Cho M, Conwit R, Starkman S; Investigators F-M, Coordinators. Improving the reliability of stroke disability grading in clinical trials and clinical practice: the Rankin focused assessment (RFA). *Stroke*. 2010;41:992–995. doi: 10.1161/STROKEAHA.109.571364
18. Schmidt TP, Weiss M, Hoellig A, Nikoubashman O, Schulze-Steinen H, Albanna W, Clusmann H, Schubert GA, Veldeman M. Revisiting the timeline of delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage: toward a temporal risk profile. *Neurocrit Care*. 2022;37:735–743. doi: 10.1007/s12028-022-01545-9
19. Megjhani M, Terilli K, Weiss M, Savarraj J, Chen LH, Alkhachroum A, Roh DJ, Agarwal S, Connolly ES Jr, Velazquez A, et al. Dynamic detection of delayed cerebral ischemia: a study in 3 centers. *Stroke*. 2021;52:1370–1379. doi: 10.1161/STROKEAHA.120.032546
20. Al-Mufti F, Amuluru K, Damodara N, Dodson V, Roh D, Agarwal S, Meyers PM, Connolly ES Jr, Schmidt MJ, Claassen J, et al. Admission neutrophil-lymphocyte ratio predicts delayed cerebral ischemia following aneurysmal subarachnoid hemorrhage. *J Neurointerv Surg*. 2019;11:1135–1140. doi: 10.1136/neurintsurg-2019-014759
21. Witsch J, Witsch T, Martinod K. Emerging role of neutrophil extracellular traps in subarachnoid hemorrhage. *Stroke*. 2024;55:2882–2884. doi: 10.1161/STROKEAHA.124.049321
22. Sorvillo N, Cherpokova D, Martinod K, Wagner DD. Extracellular DNA NET-works with dire consequences for health. *Circ Res*. 2019;125:470–488. doi: 10.1161/circresaha.119.314581
23. Denorme F, Portier I, Rustad JL, Cody MJ, de Araujo CV, Hoki C, Alexander MD, Grandhi R, Dyer MR, Neal MD, et al. Neutrophil extracellular traps regulate ischemic stroke brain injury. *J Clin Invest*. 2022;132:e154225. doi: 10.1172/JCI154225
24. Wolach O, Sellar RS, Martinod K, Cherpokova D, McConkey M, Chappell RJ, Silver AJ, Adams D, Castellano CA, Schneider RK, et al. Increased neutrophil extracellular trap formation promotes thrombosis in myeloproliferative neoplasms. *Sci Transl Med*. 2018;10:10. doi: 10.1126/scitranslmed.aan8292

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