INCREASED SERUM CITRULLINATED HISTONE H3 LEVELS IN COVID-19 PATIENTS WITH ACUTE ISCHEMIC STROKE

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Background and purpose – Prevalence of acute ischemic stroke (AIS) is increased in patients with coronavirus disease 2019 (COVID-19). A proposed hypothesis is increased virus-induced propensity to hypercoagulation resulting in arterial thrombosis. Our aim was to provide evidence regarding the involvement of neutrophil extracellular trap (NET) formation (NETosis) in COVID-19 related AIS.
Methods – Twenty-six consecutively enrolled COVID-19+ pneumonia patients with AIS, 32 COVID-19+ pneumonia patients without AIS and 24 AIS patients without COVID-19 infection were included to the study. Clinical characteristics of recruited patients were collected. Serum levels of citrullinated histone H3 (H3Cit; a factor of NETosis), IL-8 and C5a (mediators associated with NETosis) were measured by ELISA (enzyme-linked immunosorbent assay).
Results – H3Cit levels were significantly higher in COVID-19+ AIS patients, whereas all study groups showed comparable IL-8 and C5a levels. There were no significant differences among etiological subgroups of AIS patients with or without COVID-19. AIS patients with COVID-19 showed relatively increased white blood cell, lymphocyte, neutrophil, D-dimer, C-reactive protein and procalcitonin levels than control groups. H3Cit levels did not correlate with clinical/prognostic features and inflammation parameters. H3Cit and IL-8 levels were correlated in COVID-19 patients without stroke but not in COVID-19 positive or negative AIS patients.
Conclusion – Increased levels of inflammation parameters and H3Cit in COVID-19 related AIS suggest that

AKUT ISCHAEMIÁS STROKE-BAN SZENVEDŐ COVID-19-BETEGEK KÖRÉBEN MEGNŐ A SZÉRUM CITRULLINÁLT HISZTON H3-SZINTJE

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Háttér és cél – A Covid-19-betegek körében megnő az akut ischaemiás stroke (AIS) prevalenciája. Egy hipotetikus mechanizmus szerint a vírus megnöveli a hiperkoagulációs hajlamot, ami arteriális thrombosist eredményez. Vizsgálatunk célja az volt, hogy bizonyítsuk: a neutrophil extracelluláris csapdaképződés (NETosis) közreműködik a Covid-19-cel összefüggő AIS kialakulásában.
Módszerek – A vizsgálatba n = 26, AIS-ban szenvedő Covid-19-pneumoniás beteget, n = 32 AIS nélküli Covid-19-pneumoniás beteget és n = 24 AIS-ban igen, de Covid-19-ben nem szenvedő beteget vontunk be. Összegyűjtöttük a betegek klinikai adatait. ELISA-val mértük a citrullinált hiszton H3 (H3Cit; a NETosis egy faktora), az IL-8 és a C5a (NETosis-asszociált faktorok) szérumszintjét.
Eredmények – A Covid-19 + AIS betegekben szignifikánsan magasabb voltak a H3Cit-szint, míg az IL-8- és C5a-szintek hasonlóak voltak valamennyi csoportban. A covidos és nem covidos AIS-betegek etiológiaalapú alcsoportjaiban nem találtunk szignifikáns különbségeket. A kontrollcsoportokkal összehasonlítva, a Covid-19 + AIS betegekben megemelkedett a fehérvérsejt-, a lymphocyta-, és a neutrophilszám, továbbá megnőttek a D-dimer-, C-reaktív protein- és prokalcitoninszintek. A H3Cit-szintek nem függtek össze sem a klinikai/prognosztikus jellemzőkkel, sem a gyulladásos paraméterekkel. A H3Cit- és az IL-8-szintek összefüggésben álltak egymással az AIS nélküli Covid-19-betegek esetében, azonban nem korreláltak a Covid-pozitív vagy -negatív AIS-betegek esetén.
Következtetés – A Covid-19-cel szövődött AIS esetében a gyulladásos paraméterek és a H3Cit megnövekedett szint-

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NETosis may cause susceptibility to arterial thrombosis. However, H3Cit levels do not correlate with clinical severity measures and inflammation parameters diminishing the prognostic biomarker value of NETosis factors. Moreover, the link between IL-8 and NETosis appears to be abolished in AIS.

Keywords: *COVID-19, ischemic stroke, citrullinated histone H3, NETosis, IL-8*

je azt sugallja, hogy a NETosis arteriális thrombosis iránti fogékonyságot eredményezhet. Mindazonáltal az az eredmény, miszerint a H3Cit-szintek nem korrelálnak a klinikai súlyossággal és a gyulladásos paraméterekkel, lehetetlenné teszi a NETosis-faktorok prognosztikus biomarkerként való használatát. Ráadásul úgy tűnik, hogy az IL-8 és a NETosis közötti kapcsolat megszűnik AIS esetén.

Kulcsszavak: *Covid-19, ischaemiás stroke, citrullinált hiszton H3, NETosis, IL-8*

The ongoing Coronavirus disease 2019 (COVID-19) pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which primarily afflicts the respiratory system. Although lung is the major target organ, almost all organ systems including the nervous system may be disturbed directly or indirectly by the SARS-CoV-2 virus[1]. Neurological symptoms appear to be triggered by a myriad of virus-induced mechanisms that are still in the process of being fully characterized. Angiotensin-converting enzyme-2 (ACE-2) receptor, one of the main targets of the virus, is present in most tissue types and is involved in the renin-angiotensin-aldosterone system, complement system, coagulation cascade and kallikrein-kinin pathway[1]. Improved understanding of the mechanisms by which SARS-CoV-2 alters the functions of these systems is required for development of novel anti-viral treatments.

Severe thrombotic events including deep vein thrombosis, pulmonary embolism, myocardial infarction and stroke may be encountered in COVID-19 patients[2]. COVID-19 has been associated with an increased incidence of ischemic stroke[3], prompting better understanding of the association between COVID-19 and stroke, which is the main contributor of neurological morbidity and mortality in this disease. Acute ischemic stroke (AIS) constitutes the majority of stroke cases in COVID-19 and increased propensity to coagulation appears to be involved in COVID-19-associated stroke[2].

Various factors have been suggested in the activation of platelets in COVID-19, such as hypoxia, vessel damage, inflammatory factors, neutrophil extracellular trap (NET) formation (NETosis) and autoimmune reactions. Histones, the most abundant proteins in the nucleus, are released into the extracellular space, where they induce platelet aggregation, neutrophil migration and endothelial cell death[4]. Platelet aggregation generally results from cross-linking of platelet integrin aIIbB3 by plasma fibrinogen. Histones bound to platelets induce calcium influx, recruit plasma adhesion proteins such as fibrinogen to induce platelet aggregation and delay fibrin digestion[4, 5]. A crucial step during NET formation is citrullination of histones by peptidylarginine deiminase 4 (PAD4). Subsequent decrease in positive charge of the histone results in a weaker binding to the DNA and chromatin decondensation. Therefore, H3Cit plays a central role in neutrophil release of decondensed and web-like nuclear chromatin. H3Cit is released into the bloodstream upon NETosis and thus serum H3Cit levels reflect the degree of NETosis[6, 7].

In severe COVID-19, neutrophils may gain a prothrombotic phenotype characterized by degranulation, increased oxidative burst and enhanced NET formation and by this directly initiate the coagulation and complement cascades in blood vessels[8]. Number of neutrophils is increased in the circulation and lungs of COVID-19 patients and neutrophil quantity correlates with the severity of the disease. Moreover, neutrophils and NETs are observed in alveoli and lung parenchyma[8]. While several mediators may trigger NETosis, IL-8 (endothelial chemokine inducing neutrophil chemotaxis) and C5a (complement cascade breakdown product with inflammatory and thrombotic action) have been found to be specifically involved in COVID-19-related NETosis[9, 10].

In this study, our main goal was to provide evidence regarding the involvement of NETosis in COVID-19 related AIS through measurement of citrullinated histone H3 (H3Cit) levels and investigation of putative correlations between H3Cit levels and clinical parameters of stroke[7]. Another target was to seek for evidence regarding the involvement of IL-8 and C5a, involved in NETosis and other inflammation mechanisms, in COVID-19-related NETosis and AIS, and look for potential correlations between these inflammation factors versus clinical features and serum H3Cit levels of COVID-19 patients.

Materials and methods

PARTICIPANT

We consecutively enrolled 26 COVID-19+ pneumonia patients admitted to our inpatient clinic within a few hours after the onset of AIS (C+S). Baseline parameters measured on admission were the National Institutes of Health Stroke Scale (NIHSS) scores and inflammation-related blood count/biochemistry parameters (white blood cells [WBC], lymphocytes, neutrophils, platelets, neutrophil/lymphocyte ratio [NLR], C-reactive protein [CRP], D-dimer, procalcitonin). Maximum modified Rankin Scale (mRS) during hospital stay, prevalence of admission to intensive care unit (ICU) and prevalence of death in the ICU were also recorded (**Table 1**). As control groups, COVID-19+ pneumonia patients without AIS (C, n=32) and AIS patients without COVID-19 infection (S, n=24) were enrolled.

AIS was diagnosed on the basis of clinical features and cranial MRI (T1-, T2-, FLAIR- and diffusion-weighted) findings. Stroke subtypes were classified according to Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification[11]. During their hospital stays, all AIS patients underwent Doppler ultrasonography of the carotid arteries, electrocardiogram (ECG), transthoracic echocardiogram, 24-hour Holter monitoring, cranial and cervical computed tomography angiography (CTA) investigations on a routine basis. Patients with no pathological findings in these investigations were considered as AIS of undetermined etiology. In case of neurological deterioration, intracranial hemorrhage was ruled out by neuroimaging. None of the included patients had a history of central nervous system disorder or any other coexisting infections. Vascular risk factors were hypertension, type 2 diabetes mellitus, coronary artery disease, atrial fibrillation, hyperlipidemia and heart valve replacement. COVID-19 patients were only under antiviral treatment (favipiravir) during inclusion and patients who had received immunosuppressive medications were excluded.

COVID-19 was diagnosed with viral RNA detection using reverse transcriptase-polymerase chain reaction (RT-PCR) on nasopharyngeal swabs in all patients and found negative in control AIS patients. COVID-19 associated pneumonia was diagnosed with clinical and thorax CT findings. Days between the onset of pneumonia and AIS ranged between 0 and 8 (mean ± standard deviation; 2.5±3.0 days) in the C+S cohort. In C group, duration of pneumonia was similar during serum sampling (1-9 days; 3.7±3.1 days). All patients received a standard treatment protocol, as per national guidelines for COVID-19 and international guidelines for AIS management[12]. Ethical approval was obtained from the Institutional Review Board (Health Sciences University Clinical Research Ethics Board, no: 2779-2021) and written consent forms were obtained from the participants.

ELISA (ENZYME-LINKED IMMUNOSORBENT ASSAY)

Sera were obtained from AIS patients immediately after admission within the first few hours of stroke onset. None of the patients were under anti-coagulant, immunosuppressive or immunomodulating treatments during blood sampling. Inflammation- (listed in **Table 1**) and NETosis-associated factors were measured using the same serum samples. Serum levels of H3Cit (sensitivity=0.3 ng/ml; assay range: 0.15-10 ng/ml; Cayman Chemical, Ann Arbor, MI, USA), IL-8 (sensitivity=5.9 pg/ml; assay range: 15.6-1000 pg/ml; Cloud Clone, Katy, TX, USA) and C5a (sensitivity=27 pg/ml; assay range: 78-5000 pg/ml; Cloud Clone) were determined by ELISA kits as per manufacturer's recommendations. The results were converted to ng/ml or pg/ml under the guidance of the curves generated from the values of standards.

STATISTICS

Statistical analysis was conducted with the GraphPad Prism software (Version 5.01). Non-parametric tests were used for comparison of patient and healthy control groups due to uneven distribution of data. Thus variables were compared with Kruskal–Wallis, chi square and Mann–Whitney U tests, as required. Dunn's post-hoc test was used for post-hoc analysis in multiple-group comparisons. Correlation analysis was done with Spearman's correlation test. $p < 0.05$ was considered statistically significant.

Results

LEVELS OF NETOSIS-ASSOCIATED MEDIATORS AND CORRELATION ANALYSIS

C+S patients showed significantly higher serum levels of H3Cit than S and C patients (p=0.015). By contrast, serum levels of IL-8 (p=0.145) and C5a (p=0.376) were comparable among study groups (**Figure 1**). In both C+S and S groups, H3Cit, IL-8 and C5a levels were identical among

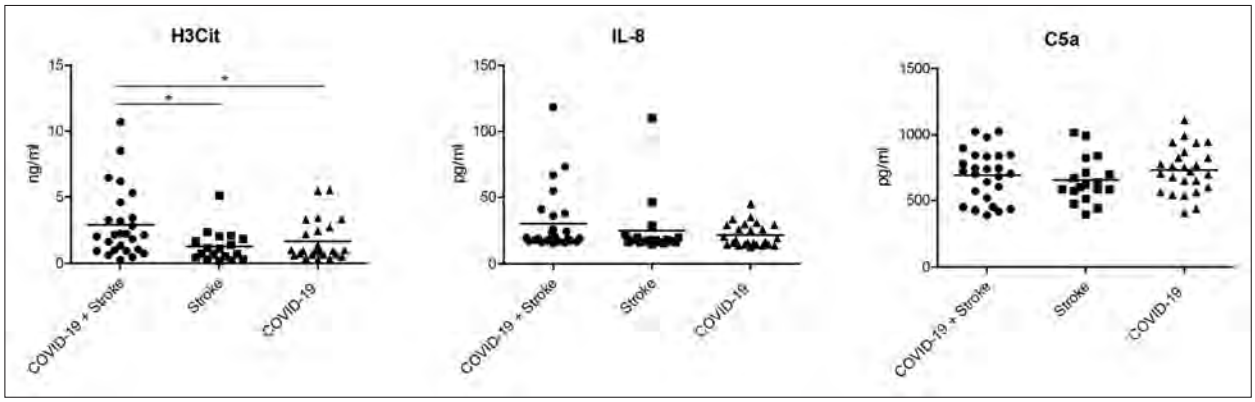

Figure 1. *Serum citrullinated histone H3 (H3Cit), IL-8 and C5a levels of patients with COVID-19 pneumonia and acute ischemic stroke (C+S), acute ischemic stroke without COVID-19 pneumonia (S) and COVID-19 pneumonia without acute ischemic stroke (C). Horizontal lines indicate mean values*

* indicates p<0.05 by Dunn's post-hoc analysis

TOAST classification subtypes (p>0.05 for all comparisons; **Figure 2**). No significant correlation was found among H3Cit, IL-8 and C5a levels versus clinical and laboratory variables (listed in **Table 1**) of C+S, C and S groups. However, H3Cit and IL-8 levels were significantly correlated in the C group. By contrast, these two variables were not significantly correlated in C+S and S groups **(Figure 3)**.

COMPARISON OF CLINICAL FEATURES AND INFLAMMATION PARAMETERS

All three study groups had comparable age, gender and vascular risk factor distribution. Likewise, C+S and S patients showed comparable distribution of stroke subtypes and large artery atherosclerosis, cardioembolism and small-vessel occlusion were the most frequently detected stroke etiologies in both

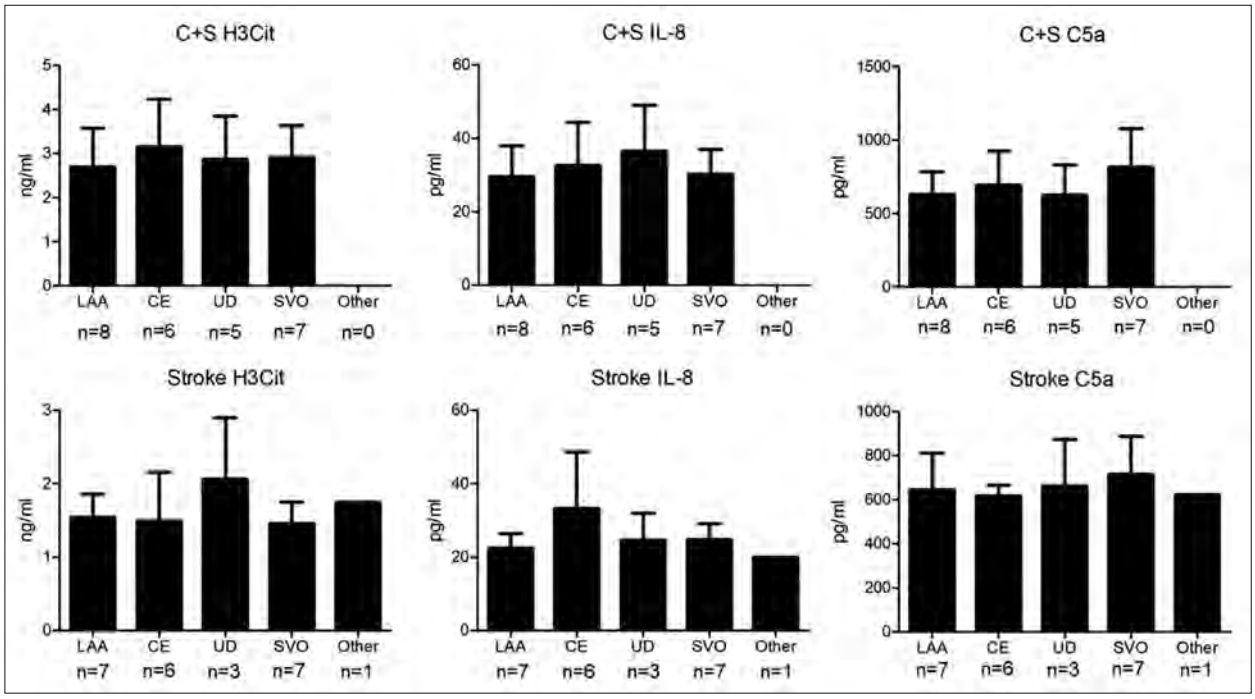

Figure 2. *Distribution of serum citrullinated histone H3 (H3Cit), IL-8 and C5a levels of patients with COVID-19 pneumonia and acute ischemic stroke (C+S) and acute ischemic stroke without COVID-19 pneumonia (stroke) among TOAST criteria subgroups (LAA, large-artery atherosclerosis; CE, cardioembolism; SVO, small-vessel occlusion; UD, undetermined etiology; other, other determined etiology). Vertical bars indicate standard errors. Number of patients for each TOAST subgroup was indicated at the bottom of the panels*

Table 1. *Comparing characteristics of patients with COVID-19 pneumonia and acute ischemic stroke (COVID-19+stroke; C+S), COVID-19 pneumonia without acute ischemic stroke (COVID-19; C) and acute ischemic stroke without COVID-19 pneumonia (Stroke; S)*

	COVID-19 + stroke (C+S) n=26	COVID-19 (C) n=32	Stroke (S) n=24	p value C+S vs C	p† for C+S vs S	p† for C vs S	p† for
Demographic data							
Age	67.5 ± 13.0	62.6 ± 17.9	63.6 ± 15.6	0.427	ns	ns	ns
Gender (men/women)	19/7	19/13	17/7	0.488	na	na	na
Medical history and acute ischemic stroke characteristics							
TOAST (n)				0.829	na	na	na
Large artery atherosclerosis	8	na	7				
Cardioembolism	6	na	6				
Small-vessel occlusion	7	na	7				
Undetermined etiology	5	na	3				
Other determined etiology	0	na	1				
Large vessel occlusion	8	na	9	0.616	na	na	na
Vascular risk factors (n)							
Hypertension	16	13	12	0.855	na	na	na
Type 2 diabetes mellitus	12	8	7				
Coronary artery disease	10	9	6				
Atrial fibrillation	6	5	7				
Hyperlipidemia	14	12	17				
Heart valve replacement	0	2	1				
ICU admission (n)	7	8	na	0.868	na	na	na
Death in ICU (n)	2	1	na	0.435	na	na	na
NIHSS	6.4 ± 4.2	na	5.8 ± 2.1	0.280	na	na	na
Maximum mRS	2.3 ± 1.7	na	2.0 ± 1.1	0.285	na	na	na
Laboratory findings							
WBC (x10^3/μl)	10.8 ± 4.9	7.5 ± 2.4	8.5 ± 2.7	*0.014*	*	*	ns
Lymphocytes (x10^3/μl)	1.7 ± 1.1	1.1 ± 0.6	1.8 ± 1.1	*0.003*	*	ns	**
Neutrophils (x10^3/μl)	8.2 ± 4.7	5.9 ± 2.5	5.8 ± 2.5	0.094	ns	ns	ns
NLR	6.8 ± 5.5	7.4 ± 5.7	4.8 ± 3.9	0.071	ns	ns	ns
Platelets (x10^3/μl)	288.2 ± 122.9	243.3 ± 91.9	257.6 ± 84.8	0.172	ns	ns	ns
D-dimer (mg/L)	1.7 ± 1.7	0.9 ± 0.6	0.9 ± 0.8	0.100	ns	ns	ns
CRP (mg/L)	97.3 ± 98.2	73.6 ± 49.9	16.2 ± 37.5	*<0.001*	ns	***	***
Procalcitonin (ng/mL)	0.3 ± 0.4	0.2 ± 0.1	0.1 ± 0.1	*0.010*	ns	**	*
Citrullinated histone H3 (ng/ml)	2.9 ± 2.6	1.7 ± 1.5	1.3 ± 1.2	*0.015*	*	*	ns
IL-8 (pg/ml)	30.4 ± 23.9	21.9 ± 8.7	25.1 ± 22.5	0.145	ns	ns	ns
C5a (pg/ml)	692 ± 192	654 ± 171	723 ± 175	0.376	ns		

TOAST, trial of ORG 10172 in acute stroke treatment classification; na, not applicable; ns, not significant; vs, versus; ICU, intensive care unit; NIHSS, National Institutes of Health Stroke Scale; mRS, modified Rankin scale; WBC, white blood cells; NLR, neutrophil-lymphocyte ratio; CRP, C-reactive protein

*p<0.05; **p<0.01; ***p<0.001 by Dunn's post-hoc analysis

†Significant p values are denoted by italic characters.

groups. A single case in the S group had internal carotid artery dissection (other determined etiology). Severity of COVID-19-pneumonia was not different among C+S and C patients in terms of prevalence of ICU admission and death in the ICU. Also, C+S and S patients showed identical NIHSS and maximum mRS scores. C+S patients showed higher WBC counts than control groups. Notably, while C patients displayed lower lymphocyte counts, C+S patients had lymphocyte counts comparable to S patients. C+S patients showed trends toward displaying relatively increased neutrophil counts and D-dimer levels without reaching statistical significance. NLR values, CRP and procalcitonin levels were higher in

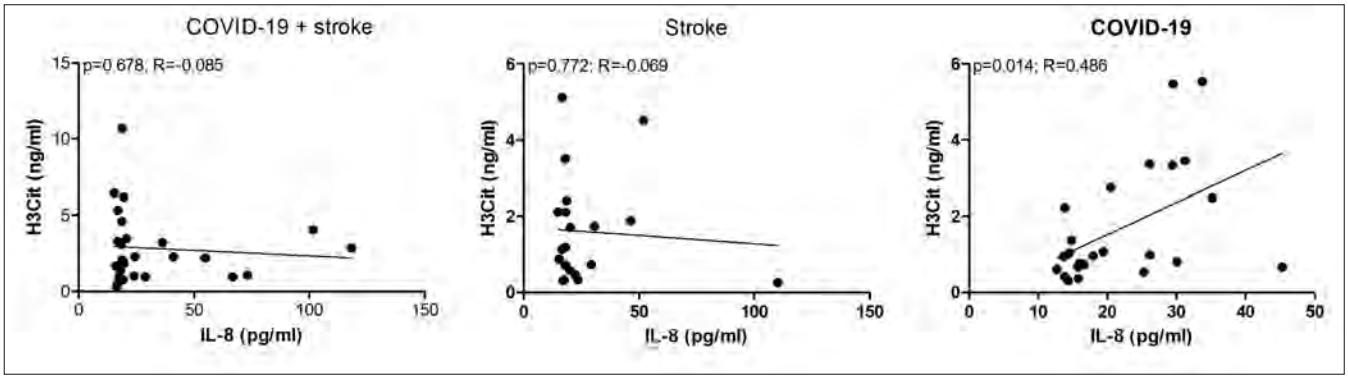

Figure 3. *Correlation between serum IL-8 and citrullinated histone H3 (H3Cit) levels in patients with COVID-19 pneumonia and acute ischemic stroke (COVID-19+stroke; C+S), acute ischemic stroke without COVID-19 pneumonia (stroke; S) and COVID-19 pneumonia without acute ischemic stroke (COVID-19; C). p and R (correlation coefficient) values denoted on the upper left corners of the quadrants were obtained by Spearman correlation test*

C+S patients than those of control groups. These differences attained statistical significance only for CRP and procalcitonin levels **(Table 1)**.

Discussion

In this study, we measured serum levels of H3Cit, a crucial element of NETosis, IL-8 and C5a in patients with COVID-19 and/or AIS, matched in terms of age, gender and disease severity and found increased levels of H3Cit in COVID-19 patients with AIS. Since H3Cit is crucially involved in NET formation[6, 7], our finding implies increased NET formation in AIS patients with COVID-19 and provides a modest support regarding the involvement of NETosis in arterial thrombosis of the brain in COVID-19 patients. Nevertheless, H3Cit levels were not correlated with measures of disease severity or levels of inflammation factors such as IL-8, C5a and procalcitonin. This finding disagrees with the potential prognostic biomarker value of NETosis factors. A likely hypothesis was that NETosis mediated immunothrombosis could be the underlying etiological factor in AIS of undetermined etiology. However, we found similar levels of H3Cit in all subgroups of TOAST classification arguing against this assertion.

NETosis is a complicated process triggered by a variety of factors. For instance, for induction of NET formation, neutrophils can release their chromatins not only by PAD4-mediated histone citrullination, but also through gasdermin-g facilitated cell membrane rupture[13]. Moreover, there are various factors of NETosis such as myeloperoxidase and neutrophil elastase, latter of which is particularly involved in anti-viral NETosis[8]. Thus, measuring levels of an extended spectrum of NETosis-related factors might result in the discovery of immunothrombosis mediators that are more closely associated with AIS occurrence and prognosis.

As a remarkable finding of our study, COVID-19+ AIS patients exhibited increased levels of inflammation-related parameters (e.g., WBC, lymphocyte, CRP, D-dimer and procalcitonin), as reported previously in other COVID-19 cohorts[14, 15]. Also, lymphopenia, which is highly prevalent in severe COVID-19[16], was not observed in AIS patients with COVID-19. Moreover, COVID-19+ AIS patients showed trends towards exhibiting increased neutrophil and NLR values, as previously reported[14]. These findings generally support the notion that neutrophil mediated inflammatory mechanisms may be involved in AIS occurring in the setting of COVID-19. However, absence of significant correlation between NETosis and inflammation parameters suggests that there is no direct causal link between these two factors and this association is probably more complex than anticipated.

IL-8 has been implicated as an important role player in pathogenesis of COVID-19 mediated tissue damage. Plasma IL-8 levels correlate with disease severity and death and inhibition of IL-8 signaling ameliorates severity of lung destruction and pulmonary microthrombosis in mice[9]. C5a, a component of the complement system, and its receptors play a critical role in the genesis of the COVID-19-associated hypercoagulable state. Disruption of C5a receptor signaling attenuates the thrombogenicity in COVID-19 and the C5-inhibiting monoclonal antibody eculizumab ameliorates findings of severe COVID-19[10, 17, 18]. Both IL-8 and C5a is known to be critically involved in NETosis[9, 10]. Thus, we looked for a possible association between these two

factors and NETosis in AIS patients. As a matter of fact, we found the previously proposed link between IL-8 and NETosis in COVID-19 patients without AIS. However, this association was abolished in AIS patients with or without COVID-19. Therefore, our results suggest that stroke might be activating alternative NETosis inducing pathways thus eliminating the regulating impact of IL-8 on NETosis induction. Overall, our results argue against involvement of IL-8 and C5a in COVID-19 associated NETosis or AIS. NETosis and subsequently increased vascular thrombosis may be caused by several different immunological factors such as IL-1 and IL-6[19, 20]. Conceivably, SARS-CoV-2 might be inducing the prothrombotic cascade directly through activation of the ACE-2 receptor without using any other inflammation mediators[21].

A drawback of our study was the low number of patients in the groups, which reduced the statistical power and the generalizability of the results. A second limitation was the low number of investigated NETosis parameters.

In brief, our results provide, for the first time, a preliminary support for the role of NETosis in COVID-19 associated ischemic stroke. Increased levels of H3Cit in COVID-19 related AIS suggest that NETosis may cause susceptibility to arterial thrombosis. However, H3Cit levels do not correlate with clinical severity measures and inflammation parameters arguing against the prognostic biomarker value of NETosis factors. Investigation of a wider panel of NETosis factors, evaluation of neutrophil activity with functional assays and histological investigation of NETosis in thrombi of COVID-19 patients in future studies may provide a more mechanistic representation of the NETosis-AIS interaction. These efforts might in due course emphasize NETosis factors as drug development targets in infection-associated AIS cases.

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