Review

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What is the impact of circulating histones in COVID-19: a systematic review

https://doi.org/10.1515/cclm-2022-0574
Received June 13, 2022; accepted July 8, 2022;
published online July 19, 2022

Abstract: The infectious respiratory condition COVID-19 manifests a clinical course ranging from mild/moderate up-to critical systemic dysfunction and death linked to thromboinflammation. During COVID-19 infection, neutrophil extracellular traps participating in cytokine storm and coagulation dysfunction have emerged as diagnostic/prognostic markers. The characterization of NET identified that mainly histones, have the potential to initiate and propagate inflammatory storm and thrombosis, leading to increased disease severity and decreased patient survival. Baseline assessment and serial monitoring of blood histone concentration may be conceivably useful in COVID-19. We performed a literature review to explore the association among increased circulating levels of histones, disease severity/mortality in COVID-19 patients, and comparison of histone values between COVID-19 and non-COVID-19 patients. We carried out an electronic search in Medline and Scopus, using the keywords "COVID-19" OR "SARS-CoV-2" AND "histone" OR "citrullinated histones" OR "hyperhistonemia", between 2019 and present time (i.e., June 07th, 2022), which allowed to select 17 studies, totaling 1,846 subjects. We found that substantially elevated histone values were consistently present in all COVID-19 patients who developed unfavorable clinical outcomes. These findings suggest that blood histone monitoring upon admission and throughout hospitalization may be useful for early identification of higher risk of unfavorable COVID-19 progression. Therapeutic decisions in patients with SARS-CoV-2 based on the use of histone cut-off values may be driven by drugs engaging histones, finally leading to the limitation of cytotoxic, inflammatory, and thrombotic effects of circulating histones in viral sepsis.

Keywords: citrullinated histone; COVID-19; heparins and heparinoids; histone; NETosis; NETs; SARS-CoV-2; systematic review; thrombo-inflammation.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiopathogenetic cause of the worldwide infectious disease named Coronavirus disease 2019 (COVID-19), generating dramatically progressive respiratory manifestations up to multi-organ failures (MOF) and even death [1, 2]. An important strategy of innate immune system is represented by the formation of extracellular traps (ETs), which can be released by several immune cells (e.g., monocytes/macrophages, and neutrophils) extruding into the extracellular microenvironment cell-free DNA, histones, constitutive proteins/enzymes and proteolytic enzymes (such as neutrophil elastase, and myeloperoxidase),

forming a peculiar tridimensional web-like structure trapping pathogens [3]. Most of the NET proteins (around 70%) consists of extracellular histones. Histones (i.e., positively charged multifunctional nuclear proteins) can be released through finely regulated mechanisms (as in ET formation) or through unregulated processes (as in cell damage) and contribute to the neutralization of pathogens, including viruses [4]. Physiologically, the nucleosome consists of core histones H2A, H2B, H3, and H4, bound to DNA, and an additional linker histone H1, located between two nucleosomes, furtherly compacting chromatin into 30 nm fibers. N-terminal tails of core histone frequently undergo to post-translational modifications, which are involved in transcriptional activation, gene silencing, chromatin assembly and DNA replication [5].

Extracellular histones exert cytotoxic effects through membrane disruption, increase of intracellular calcium, Toll-like receptor activation, inflammasome and complement activation. They have been described as important damage associated molecular patterns (DAMPs) in a plethora of human diseases, including inflammatory diseases, in sepsis, autoimmune diseases, myocardial infarction and in COVID-19 [3, 4]. In fact, histones were recently identified in body fluids and tissues as biomarkers for predicting outcomes of several human diseases [6]. In particular, recent evidence demonstrated that ETs and histones are linked to cardiovascular events (e.g., myocardial infarction, and stroke, as well COVID-related cardiac manifestations) [7]. Circulating extracellular histones are regarded as one of the most important biomarkers of illness severity in patients with sepsis [6, 8–11].

Among the laboratory findings in COVID-19 diagnosis and prognosis [12, 13], both neutrophil- and monocyte-extracellular traps, have emerged as diagnostic and prognostic marker in COVID-19 [14–16], actively participating in both cytokine storm and coagulation dysfunction [17–19].

Among the biomolecular compounds of NETome, histones have been characterized for their potential to initiate and propagate inflammatory storm and thrombosis when not physiologically and finely regulated, leading to both increased disease severity and decreased patient survival [11, 20, 21].

Although COVID-19 is a predominantly pulmonary disorder [22], it is also associated with end organ injury, systemic dysfunction, thrombosis, and ischemia [23, 24]. Elegant evidence has shed lights on the roles and functions of neutrophil- and monocyte-extracellular traps as proinflammatory biocompounds, suggesting histones serve as harmful signaling molecule able to activate cytokine storm through Toll-like receptors [25]. So, the baseline assessment and serial monitoring of blood histone concentrations may be at least theoretically, clinically useful in COVID-19. Nonetheless, the association between blood histone values and clinical outcome remains not fully understood in patients with SARS-CoV-2 infection, but strongly suggests histones as enhancer of COVID-19 severity [26].

Here we report the results of a systematic review aimed at exploring the possible association between increased blood histone levels, disease severity and mortality in COVID-19 patients, including a comparison of histones values between COVID-19 and non-COVID-19 patients.

Search strategies and selection criteria

We carried out regular and systematic screening by electronic search in PubMed (https://www.ncbi.nlm.nih.gov/) and Scopus (https://www.scopus.com/search/form.uri?display=basic#basic), using the keywords “COVID-19” OR “SARS-CoV-2” AND “histone” OR “extracellular histone” OR “circulating histone”, between 2019 and present time (i.e., latest search date: May 05, 2022), restricted to articles published in English. The reference list of all articles was also reviewed for identifying other potentially eligible studies. All resulting items were screened (title, abstract and full text, when available or necessary) by two of the authors (DL and FM), to capture observational, cross-sectional or prospective studies reporting data on histone values at admission (or at the earliest time point during hospitalization) in COVID-19 patients with or without severe disease, as well as in non-survivors vs. survivors. Severe disease was clinically defined as patients needing intensive care unit (ICU) admission, mechanical ventilation, hospitalization, pneumonia, or onset of critical symptoms and/or shock and/or presence of organ failure. All studies fulfilling these criteria were then included in a systematic literature review. Disagreements between authors with respect to study eligibility were resolved by discussion and consensus. Results of the review were organized into summary of finding tables.

Results

Studies identification and characteristics

A total number of 361 studies were initially identified by our search criteria, 173 of which were excluded for duplication among the two databases, whilst 171 were also excluded because they failed to report histone values.

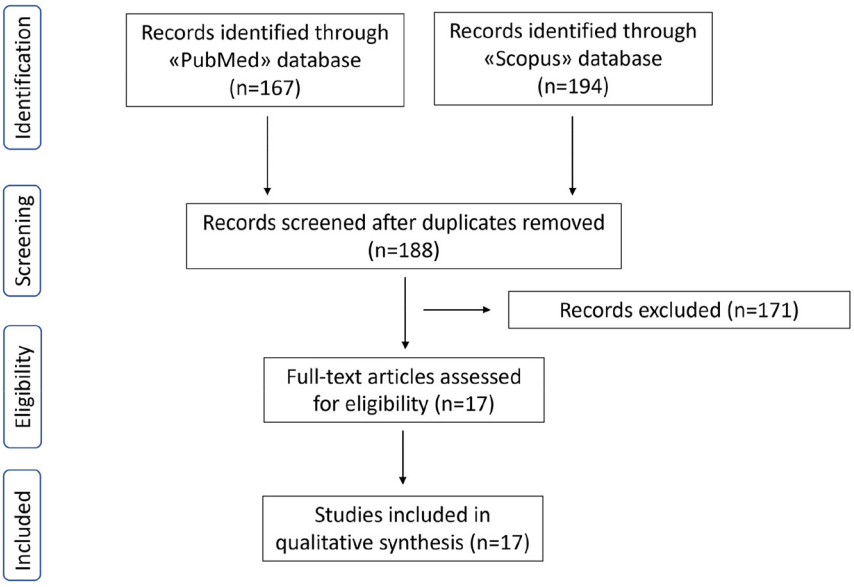

Figure 1: Preferred reporting items for systematic reviews and meta-analyses (PRISMA) diagram.

17 studies, totaling 1,781 patients, were finally included in our systematic review [19, 20, 27–41]. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) diagram is shown in Figure 1.

The selected studies were characterized as observational studies, prospective observational study, cross-sectional consecutive study or registered *a priori* clinical trial study. All COVID-19 patients were hospitalized and the diagnosis was confirmed by FDA-approved RNA testing (i.e., RT-PCR assays using nasopharyngeal swab, pharyngeal swab, bronchoalveolar lavage), and clinically confirmed by chest X-ray and/or CT-scan, according to the guidelines and severity classification of the World Health Organization. Several studies also included non-COVID hospitalized patients, but all patients were compared to healthy control subjects analyzed in a routine diagnostic setting.

The analyses of extracellular histones were performed through different methods, i.e. using semiquantitative western blotting [28, 32–34], qualitative-semiquantitative cyto-histochemistry by confocal microscopy technology [20, 27, 30, 38, 40], and quantitative ELISA assays for citrullinated or unmodified histones [19, 29, 31, 35–37, 39, 41].

All reported blood histone values were measured upon hospital admission or at the earliest time point immediately after hospitalization. Only in the study of Shaw et al. [34] were patients categorized according to their histone concentrations, highlighting a cut-off discriminating the prognostic value >30 μg/ml as a non-survivor index of patients with elevated risk of death. For a better evaluation and comprehension of the data complexity, we divided the studies according to the methodology used for detecting histones, i.e. as extracellular circulating proteins in plasma or body fluids or as intracellular proteins localized in cells and tissues, and finally discussing from a biologic and clinical biochemistry point of view their concentrations found in patients with severe disease vs. non-severe COVID-19 patients, compared to healthy control subjects.

Noteworthy, after a careful evaluation of all studies, the main difference among studies is also related to a inhomogeneity of histone types analyzed, with results obtained on unmodified histones (mainly type H3 and H4) and citrullinated histone H3 and H3 variants; nevertheless, all results give significant evidence on the novel roles and functions of histones in SARS-CoV-2 infection, confirming that the intricate NETosis mechanisms are based on distinct biological processes, including both neutrophil extracellular traps and leukotoxic hypercitrullination, as multifaceted aspects of an outstanding and nature's virtually perfect biological process involved in both physiology and pathology of human diseases [42–44]. For these reasons, we analyzed all the references that reported data on all isoforms or variants of histones involved in COVID-19.

Qualitative evidence of histones by microscopy and cytometry approaches

As summarized in Table 1, immunofluorescence staining of both NETs and citrullinated histone H3 were found consistently higher in autopsy lung specimens of COVID-19 patients, shedding light for the first time on the role of NET complexes and in particular linking the high levels of citrullinated histone H3 inside the neutrophil traps

Table 1: Immunofluorescence and flow cytometry evidence of histone in COVID-19.

Authors	Sample analyzed	Subjects and patients	Histone type	Method
Middleton et al. [27]	Lung autopsy	3	Citrullinated H3	Immunofluorescence
Blasco et al. [30]	Coronary thrombi aspirates	5	Citrullinated H3	Immunofluorescence
Veras et al. [20]	Blood mononuclear cells	65	Citrullinated H3	Confocal microscopy
	Tracheal aspirates			
Godement et al. [38]	Whole blood	58	Citrullinated H3	Flow cytometry
	Bronchoalveolar fluid	2		
Aymonnier et al. [40]	Blood mononuclear cells	14	Citrullinated H3	Confocal microscopy
	Tracheal aspirates	6		

infiltrating the lung tissue of COVID-19 non-survivors [27]. Citrullinated histone H3 positive neutrophils were colocalized with platelet-derived factors and inflammatory cytokines in pulmonary microthrombi, suggesting their potential role in COVID-related thrombo-inflammation.

Moreover, intracoronary microthrombi aspirates from COVID-19 patients were found to contain, as hallmarks of NETS, intensely higher amounts of citrullinated histone H3 than in patients with myocardial infarction and without SARS-CoV-2 infection [30].

Inflammasome activation in neutrophils and monocyte/macrophage cells collected from patients with COVID-19 revealed that more than 40% of nuclei were positive for citrullinated histone H3, demonstrating a significant correlation ($p=0.0062$) between inflammasome formation and nuclear histone citrullination. Moreover, patients with COVID-19 also had a significant increase in plasma and tracheal aspirates of citrullinated H3 levels [40].

With a similar approach, Veras et al. [20] described that NET concentration was found increased in plasma and tracheal aspirates. Moreover, Citrullinated H3 were localized by confocal microscopy in both tracheal aspirates and lung autopsies only in COVID-19 patients.

In the prospective observational NETCOV2 study the authors revealed via whole blood flow cytometry that consecutive patients admitted to intensive care unit for COVID-19 showed a significantly higher staining of circulating leukocytes with poly-citrullinated histone H3 compared to healthy controls ($p=0.0046$). Interestingly, the lack of decrease of the leukocyte levels of citrullinated H3 inside NETs suggested a discrimination of the non-survivor COVID patients [38].

Semi-quantitative histone evaluation by western blotting methods

The content of histone H3 (epigenetically or post-translationally modified protein) was extracellularly quantified in COVID-19 through Western blotting technique (Table 2). Firstly, both plasma and sputum samples of patients with moderate and progressive/severe COVID-19 contained significantly higher levels of histone H3 compared to healthy subjects or patients with mild COVID-19 infection [28]. Moreover, 73% of patients examined showed the citrullinated isoform of histone H3 and its presence in sputum was associated to a worsening condition of the lung.

Already at admission to the intensive care unit the levels of histone H3 differed significantly among COVID-19 and non-COVID patients and healthy controls [33], suggesting a possible fast release of histone H3 during the earlier phases of COVID-19, also correlated with neutrophil counts and specific proteases. Moreover, the same research group successively identified that unmodified histone H3 increases during COVID-19 progression, significantly correlating with the increase of thromboembolic events, and that histone H3 may be partially or fully proteolytically cleaved, indicating (other than the post-transcriptional modification) also crucial proteolysis processing of native unmodified histones in COVID-19 patients [32]. The finding of native histone H3 in at least 50% of critically ill COVID-19 patients has been linked to the harmful effect of extracellular histones, associated with cytokine storm, thromboembolic events, and adverse outcomes, highlighting a role of histone-mediated damage on the development of thrombo-inflammatory phenotype typical of severe COVID-19. Accordingly, the incidence of adverse events is increased in the non-cleaved histone H3 group of severe COVID-19 population, suggesting the proteolytic cleavage of histones as possible mechanism limiting the deleterious effects of extracellular release of histone during SARS-CoV-2 infection.

According to this evidence, Shaw et al. [34] confirmed the significantly higher levels of unmodified histone H3 in COVID-19 compared to healthy subjects but also associated with the increasing severity infection. In fact, they stratified the risk of adverse outcomes to the increase levels of

Table 2: Histone levels in COVID-19 patients obtained by western blotting techniques.

Authors	Sample	Subjects and patients	Histone type	COVID-19 profiles (% histone H3 pos)	p-Value
Busch et al. [28]	Plasma	228	H3 and citrullinated H3	Mild (0) Moderate (12.3) Severe (13.7)	0.02 overall
	Sputum	9		Citrullinated H3 (73) Sputum citrullinated H3 (100)	
Huckriede et al. [33]	Plasma	126	H3	Healthy controls (0) At admission [28]	0.046 overall
Huckriede et al. [32]	Plasma	143	H3 and cleaved H3	At admission [28] During stay at ICU [50] Cleaved isoforms [23]	0.001
Shaw et al. [34]	Plasma	113	H3	All samples (100) Healthy controls ~mild <3 µg/mL Moderate and critical <30 µg/mL Non-survivors >30 µg/mL Cleaved forms in critical/non-survivors [22]	<0.001 overall

circulating histone H3 and adopting 30 µg/mL as cytotoxic histone threshold at which they distinguished moderate/critical COVID-19 vs. non-survivors, correlating histone H3 levels to inflammatory and coagulative features. This study represents a milestone in documenting extracellular unmodified histone H3 as a driver for both inflammatory and coagulative damages in critical illness in SARS-CoV-2 infection and reinforcing the evidence of neutrophil and monocyte ETosis as the major source of circulating histone in COVID-19, reflecting the extent of cytotoxic effects of histones released by tissue and cells and associated with poor outcomes [7].

Quantitative ELISA assays of native unmodified and citrullinated histones

Among the studies performed through histone quantitative ELISA methods (Table 3), some utilized photometric enzyme immunoassay determining the cytoplasmic histone-associated DNA fragments (i.e., mono- and oligonucleosomes) with antibodies reacting with histones H1, H2A, H2B, H3, and H4. Accordingly, it was found that blood levels of histone-DNA complex in COVID-19 patients were dramatically higher than those reported in control subjects and this result was associated with the number of affected

Table 3: Histone concentrations in COVID-19 patients by ELISA assays.

Authors	Sample	Subjects and patients	Histone type	COVID-19 profiles	p-Value
Gueant et al. [31]	Serum	190	Histone-DNA complex	Healthy controls Ambulatory for screening Hospitalized	<0.0001 overall
Zuo et al. [29]	Serum	84	Citrullinated histone H3	Healthy controls At admission	<0.0001
Cavalier et al. [35]	Plasma	95	Histone H3.1 Histone H3R8 citrullinated	Healthy controls ICU hospitalized	<0.0001
Traby et al. [41]	Plasma	78	Citrullinated histone H3	Healthy controls At admission Severe conditions	<0.001 overall
Ng et al. [36]	Plasma	136	Histone H3citrullinated-DNA	Healthy controls At admission Non-survivors	0.0058 overall
Zuo et al. [19]	Serum	44	Citrullinated histone H3	Healthy controls Hospitalized with thrombosis	<0.01
Bouchard et al. [39]	Plasma	12	Histone-DNA complex	Healthy controls Hospitalized at ICU	0.012
Seery et al. [37]	Plasma	235	Citrullinated histone H3	Healthy children vs. COVID-19 MIS-C children	n.s. <0.001 overall

organs [31]. The authors suggested that histone-DNA provide a significant threshold for admission into intensive care units (during the first phases of COVID-19) revealing a key role in the severity of multiorgan manifestations in the later phases of infection.

Recently, cell-free histone levels (associated with DNA fragments) were found significantly elevated in the plasma of severe COVID-19 compared to plasma from healthy controls. In fact, histones in plasma from COVID-19 patients may activate endothelial cells leading to a dysregulated thrombin generation by releasing von Willebrand factor and P-selectin from Weibel-Palade bodies, inducing the translocation of sphingomyelinase resulting in tissue factor decryption and releasing procoagulant microparticle, finally participating in the formation of structurally abnormal clots [39, 45].

As constitutive part of NETs, citrullinated histone H3 received particular attention and several studies focused on ELISA quantitative determination in COVID-19 patients. In detail, six studies performed the analyses on the citrullinated form of histone H3, with a total of 516 patients measuring via commercially available kits, and affirmed the roles and functions of histones in initiation and progression of COVID-19 infection [19, 29, 35–37, 41](Table 3).

These studies highlighted that COVID-19 samples showed higher levels of citrullinated histone H3 compared to non-COVID patients, but interestingly without any association with cell-free DNA. Moreover, a significant positive correlation between Citrullinated H3 and platelet counts was found ($p<0.0001$), suggesting its ability to induce the activation of intrinsic pathway of coagulation and that COVID-19 infection may have multiple biomolecular pathways of histone release not only due to NETosis processes [29]. In fact, citrullinated histone H3 was found significantly elevated in blood from COVID-19 patients diagnosed with a thrombotic event as compared with matched controls [19]. For the first time, NET processes in COVID-19 were hypothesized as mechanistically associated with the following different biomolecular pathways: (a) activation DNA of the extrinsic pathway of coagulation by NET DNA; (b) initiation of the intrinsic pathway by Tissue Factor release upon histone binding to platelets; (c) proteolysis of tissue factor inhibitors. These mechanisms link biocompounds of NETs and enhanced coagulation turnover in the COVID-19 prothrombotic state [19].

As expected by a plethora of data, plasma/serum levels of citrullinated histone H3 are well correlated with NET markers, with higher concentrations already at the time of admission, with increased levels associated with increased respiratory support with further significant association with poor clinical outcome and/or short-term mortality, suggesting their roles as prognostic markers, with an observed decline to levels found in healthy subjects at 4 months post-infection [36].

According to the recent evidence that during COVID-19 several pathways of histone release may be available [26], a multicentric study highlighted that both circulating histone H3.1 variant and citrullinated histone H3R8 were found at significantly higher levels in patients diagnosed with COVID-19 compared to healthy controls and non-COVID patients. These data showed also that the levels of both nucleosomal histones were higher in COVID-19 patients with more severe disease requiring intensive care due to catastrophic cytokine storm, suggesting also a threshold value of 1.25 μg/mL for H3.1 variant for stratifying/predicting the risk for non-survivors [35].

An interesting observational study in children with COVID-19 and multisystemic inflammatory syndrome (MIS-C) highlighted that plasma concentrations of citrullinated histone H3 were similar in COVID-19 and healthy children, without capability to distinguish disease severity in children with COVID-19 infection, even if some NET markers and citrullinated histone H3 levels were found significantly higher in MIS-C affected children [37].

Finally, a very recent study demonstrated that plasma samples from patients infected by COVID-19 contained higher levels of both extracellular vesicles and citrullinated histone H3 compared to control subjects, with higher levels in patients with severe disease, suggesting that both proteins expressed on extracellular vesicles and citrullinated histone H3 may represent biomarkers of COVID severity and progression, and associated with COVID-related coagulation activation, endothelial inflammatory processes and multiorgan injuries [41].

Impact of circulating histones on COVID-19 mortality

Among the studies describing the risk of death in COVID-19, four studies compared blood histone values in COVID-19 survivors vs. non-survivors (357 total patients, 55 [15.4%] died). In all such studies performed with either flow cytometry approach [38], immunofluorescence microscopy [27], quantitative ELISA assay [36] and/or semi-quantitative Western blotting technique [34] non-survivors displayed significantly higher levels of both unmodified and citrullinated histone H3 compared to survivors, demonstrating that patients with high circulating histone levels on admission had a higher risk of mortality. This was also supported also by the multivariate analyses demonstrating that histones were independently associated with mortality after

Table 4: Histone concentrations according to the severity of COVID-19 disease.

Authors	Sample	Patients	Histone type	COVID-19 profiles	p-Value
Godement et al. [38]	Serum	3	Citrullinated histone H3	Survivors vs. day-28 mortality	0.001
Middleton et al. [27]	Lung autopsy	58	Citrullinated histone H3	Survivors vs. non-survivors	0.0004
Ng et al. [36]	Plasma	136	Histone H3 citrullinated-DNA	Survived vs. deceased	0.0058
Shaw et al. [34]	Plasma	113	Unmodified histone H3	Survivors vs. non-survivors	<0.001
			Cleaved histone H3		0.002

adjustments for some parameters or categorical variables (Table 4). In particular, a median range of 331 ng/mL (interquartile range of 200–575 ng/mL) of citrullinated histone H3 [36] and a median of 29.6 μg/mL (interquartile range of 11.2–60.0 μg/mL) of unmodified histone H3 [34] were identified as characteristics of COVID-19 non-survivors, significantly higher than those found in COVID-19 survivors (median of 176 ng/mL and 10.5 μg/mL, for citrullinated and unmodified histone H3, respectively).

Discussion

The worldwide pandemic of SARS-CoV-2 has attracted tremendous attention among the scientific communities pushinger researchers to work towards the identification of both pathogenic biomolecular pathways and clinical laboratory biomarkers with possible diagnostic and prognostic applications, allowing to identify triggers for developing novel targeted therapeutic treatments [1, 13, 46]. In this view, substantial efforts were focused on the role and function of NETosis and NET-related biocompounds in COVID-19 initiation and progression [14–16]. Among the molecules involved in NETosis, histones released as unmodified and/or citrullinated proteins received particular attention due to their well-known roles in organ injuries and systemic diseases [11, 21].

Discovered more than one hundred 30 years ago, histones are well recognized highly conserved cationic intranuclear proteins supporting the chromatin structure and regulating gene expression [6]. Histones may be released in the extracellular space during several acute and chronic diseases, functioning as damage-associated molecular pattern molecules (DAMPs) and triggering the activation of multiple signaling pathways [5, 11, 21]. Moreover, their roles in functions in sepsis are well-established, contributing significantly to cellular injury and inflammation [9, 10].

As crucial components of NETs, circulating histones, as mediators of epithelia/endothelial cell damages, have emerged as diagnostic/prognostic markers of NETosis in SARS-CoV-2 infection [2, 14, 16, 20, 47], participating in COVID-19 related immunothrombosis and thromboinflammation [18] and significantly enhancing the severity of SARS-CoV-2 [26]. It has also been hypothesized that circulating histones may participate in COVID-19 multi-organ failures through the activation of inflammasome, Toll-like receptors and cytokine storm [7, 25, 40] (Figure 2).

However, it is important to note that the concentration of this biomarker should be considered a direct measure of tissue and cell injuries in sepsis [8–10, 48]. Additionally, hyperhistonemia during sepsis can be down-regulated by some medications (e.g., heparins and histone-neutralizing agents) [10, 49, 50]. Viral sepsis induced by SARS-CoV-2 leads to the commonly observed hyperinflammation and hypercoagulability among hospitalized patients (e.g. hypoperfusion or hypoxemia, acute or renal impairment, acute heart failure, acute pulmonary decompensation, micro- and macro-thrombosis, cytokine storm, and sepsis), paving the way for the use of anti-inflammatory and anticoagulant (and anti-histone?) agents, including various heparins and heparinoids [7, 46, 51–55].

In COVID-19 the early identification of hyperhistonemia, which has been associated with an higher mortality risk [34, 36], will thus provide some novel unexpected insights on whether circulating histone monitoring may be helpful in patients with SARS-CoV-2 infection [7].

Severe COVID-19 illness frequently develop a systemic disease, leading to multi-organ failures and death in the most severe cases; currently, COVID-19 treatment guidelines suggest that patients with severe COVID-19 should be treated identical to those with septic shock [56], even if to-date these laboratory biomarker indications have not been directly related to blood histone values [12, 13, 57].

The results of our systematic literature review clearly and surprisingly demonstrate that COVID-19 patients with worse outcomes have higher histone values than those with better disease course, independently of any epigenetic/post-translational modifications of circulating histones released by monocyte and neutrophil extracellular traps. However, careful analysis of the included studies reveals a more complex and variable biochemical picture, due to the variability of technologies used to monitor and assay the

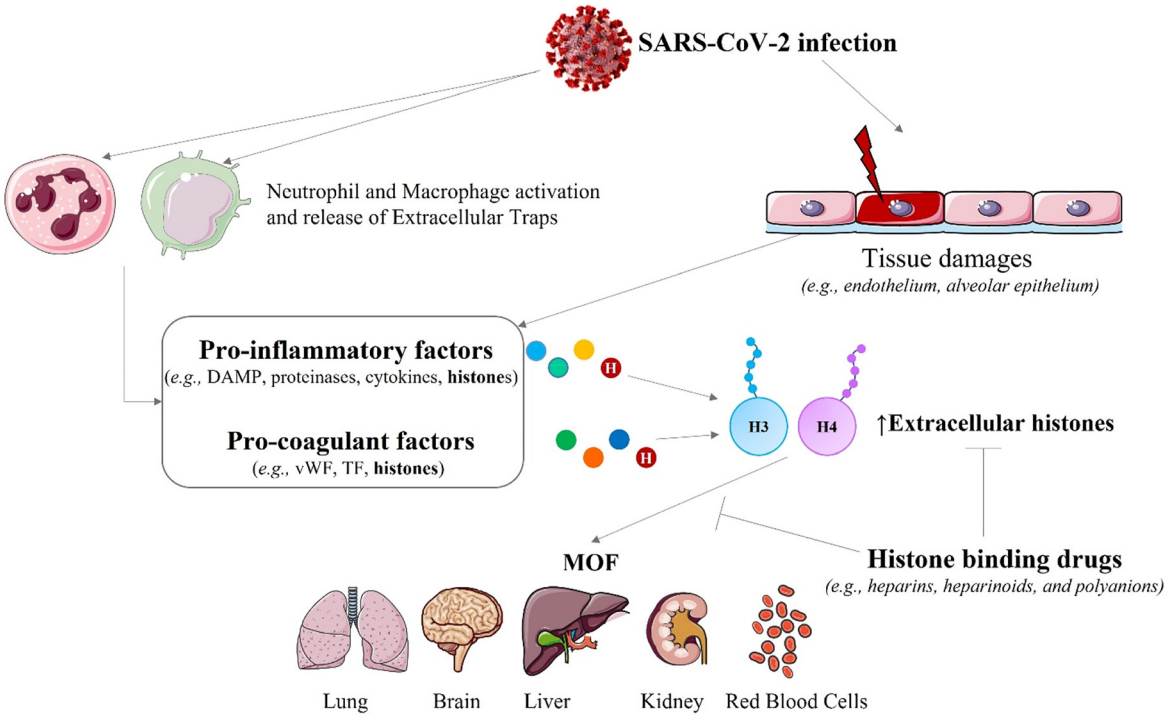

Figure 2: Schematic overview of the extranuclear and extracellular release of histones induced by COVID-19 infection, through direct NET/MET processes and tissue damages. Infected cell and tissues are stimulated to secrete both pro-inflammatory and pro-coagulant factors, including histones, as main effectors of multiorgan failure, severe clinical worsening, and death in COVID-19 patients.
SARS-CoV-2, severe acute respiratory syndrome-related coronavirus-2 or COVID-19; NET, neutrophil extracellular trap; MET, monocyte extracellular trap; DAMP, damage-associated molecular pattern; vWF, von Willebrand factor; TF, tissue factor; H3 and H4, histone subtypes; MOF, multi-organ failure/dysfunction.

concentrations of histones, both intracellularly and circulating in blood and body fluids. Nevertheless, despite the different methods applied, it is evident via a concordance of results that histones appear to play an important role in the initiation and progression of both inflammatory and thrombotic events characteristics of COVID-19, especially in patients with severe disease and in non-survivors.

It is important to underline that circulating histone concentrations were often absent/low in many non-COVID-19 and not significantly higher in mild COVID-19 patients, whereas patients who developed unfavourable clinical outcomes were identified and stratified at higher risk of mortality according to the histone levels in both blood and in body fluids.

Although the evidence on histones reinforces on the one hand the multifactorial pathogenesis of severe COVID-19 illness, on the other hand it provides novel evidence on the unexpected roles of histone in COVID-19 viral sepsis, similar to that obtained for classic bacterial/fungal sepsis, even though the increased circulating histone value in COVID-19 patients with unfavourable clinical progression is significantly different from those typically observed in severe sepsis. Our view is in full agreement with the recent paradigm about the overlap of some pathogenetic events between SARS-CoV-2 and sepsis, improving the knowledge of biomolecular events detectable in patients with classic and viral sepsis [58]. Recent evidence highlighted that the most important predictors of mortality for non-coronavirus disease pneumonia patients (classical sepsis) was protein C and cell-free DNA whereas for coronavirus disease 2019 patients (viral sepsis) are soluble thrombomodulin and citrullinated histones [59], suggesting that the pathophysiology and the complex network of immuno-inflammo-thrombosis differs between the classic and viral sepsis groups.

In keeping with these suggestions, the levels of histones at ICU admission differ from those detected during time course of COVID-19 infection; in fact, histone values mirrored organ dysfunction [31], and significant hyperhistonemia was prevalently found only before death (i.e., COVID-19 non-survivors) [34, 36], but still only present in less than half (i.e., 44.8%) of all patients who died with SARS-CoV-2 infection.

Noteworthy, we recently demonstrated that histones could act as modifiers of monocyte distribution width (MDW), a recent FDA-approved biomarkers for both viral and classical sepsis (Ligi et al., under review), highlighting the unexpected novel potential role of circulating histones in both thrombotic and inflammatory monocyte-related processes described both in Sepsis and COVID-19.

Taken together, these data provide novel insights into the biomolecular roles of polycationic histone proteins in SARS-CoV-2 infection and raising hope about the possible therapeutic effects of polyanionic compounds in protecting the tissues from thromboinflammation with further potential implications for a myriad NET- and histone-accelerated disease states.

It has been widely accepted that ET and extracellular histones have significant roles in immune defenses against several pathogens (e.g., viruses, bacteria, fungi) [3, 5], but at the same time their excessive release can be harmful to the host cells [6, 21]. During pathological conditions, as in COVID-19, an uncontrolled release of histones can contribute to damage tissues and organs, promoting the onset of complications as ARDS and immunothrombosis and, in severe cases, increasing the mortality rate [34]. Despite the limited number and the diversity of the summarized studies, the aim of this review was to provide scientific results supporting the possible role of excessive histone levels in unfavorable clinical outcomes in COVID-19 patients. In fact, we are aware that in COVID-19 literature the association between elevated levels of histone and unfavorable outcomes might not be "causative", but we believe that circulating histones should be taken into consideration among the biochemical critical players and as additional clinical biomarker of Sars-Cov-2 infection.

The novel and unexpected importance of histones and their impact on COVID-19 requires new strategies to address these changes in clinics and laboratory medicine. Classical sepsis and viral COVID-19 sepsis inflict significant damages on the organism, particularly via the thrombo-inflammatory processes [60], which in the early acute time may lead patients to a higher risk of severe/critical conditions up-to death, but in the medium-long term can cause "chronic" manifestations and health consequences for accumulating viral triggers [61].

Although the dynamics of this process seems related to unresolved inflammation [62] it requires further exploration, as it is essential to clarify under which biological circumstances (e.g., clinical feature and a plethora of epigenetic and biomolecular marks, including histones) it accelerates or becomes accentuated as multi-organ aggression [2], and whether its partial or complete reversion is feasible [63].

As COVID-19 (as viral sepsis) is multifactorial, the combination of both "old and new" biomarkers (summarized in some overviews) [12, 13, 56, 64] may be more accurate at measuring the residual healthspan. But in order to be useful, a novel biomarker needs to be optimized for the use in clinical practice, highlighting the possible mechanistic role of action involved in the intricate network of COVID-19.

In this view, neutrophil- and monocyte-extracellular traps, including the novel and unexpected roles of histone release, have emerged as key process and crucial biomarker in COVID-19 [5, 6, 11, 14, 16, 33, 65].

Summary and future perspectives

The results summarized in our systematic literature review suggest that monitoring blood and body fluids histone concentrations (both unmodified and citrullinated histone H3) in patients with SARS-CoV-2 infection upon admission and throughout hospitalization (especially in critical patients and/or those needing intensive care) may be a useful tool for early prediction of higher risk of unfavourable disease progression. Moreover, according to recent observations on possible therapeutic approaches with polyanionic compounds as a potential strategy for protecting the endothelium from inflammatory and thrombotic insult of circulating histones [7, 46, 50, 55, 66], future studies are needed to identify the mechanisms of histone-induced activation of both the inflammatory cytokine storm and the hypercoagulability events in COVID-19, searching and characterizing drugs able to directly engage histones [49, 66] and thereby block their deleterious effects on cells and tissues of several organs.

In the frame of the rapidly evolving knowledge about histones and monocyte involvements in COVID-19 [34, 67, 68], to search biochemical triggers and pharmacological targets, future studies should meet the current limitations focusing on three main areas:

(1) Establishment of ageing cohorts of COVID-patients and well-matched controls to account for differences in monocyte response to circulating histones on the basis of age and subtypes of monocytes [69, 70]. In fact, several evidence suggest different monocyte responses to COVID-19 and MIS-c according to age of this blood cell population [37, 71, 72].
(2) Large longitudinal studies with integrative novel biomarkers (epigenetic biomarkers in combination with wide panels of immunological and inflammatory markers) to accurately estimate biological ageing of blood cells triggered by histones and NET-associated

compounds for different clinical endpoints (survivors vs. non-survivors COVID-19 patients) [16, 40].

(3) Optimization of the biological features of circulating biomarkers (like circulating histones) for the use in routine clinical practice and for opening new frontiers in pharmacological targeted therapies (e.g., heparin and heparinoids, and drugs limiting the deleterious effects of histones) [7, 46, 49, 50, 55, 66].

Finally, in our opinion, the best strategy to deal with the complexity of the histone-mediated inflammatory and thrombotic processes at early and long-term phase in COVID-19 would require a multi-marker and multi-maker approaches.

Research funding: None declared.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Informed consent: Not applicable.

Ethical approval: Not applicable.

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