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XXXV cycle

Circulating **H**istones **I**nduce **I**nflammatory **R**esponses in **M**onocytes: **E**ffects of **H**eparins as **H**istone-**N**eutralizing **A**gents

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CONTENT

STRUCTURE OF THE THESIS

The thesis structure consists of a description of the objectives, followed by an introduction and a series of chapters reporting data from published and submitted research articles.

The first section, "Introduction," was developed to discuss the relatively new process of ETosis, focusing attention on the harmful functional features of histones, and providing the emerging rationale for their use as potential biomarkers of disease and therapeutic targets.

Chapters 1 presents submitted data highlighting the inflammatory responses promoted by histones in a whole blood assay model and how these could be modulated by using heparin variants as histone-neutralizing agents; chapters 2 and 3 are referred to papers that have already been published, reporting an overview on the most recently published papers on the role of circulating histones in COVID-19 infection and the results of the morphological changes occurring in monocytes after histone treatment, respectively. Chapter 4 lists papers that have already been published but are unrelated to the topic of the thesis.

At the end of the Introduction a list of all abbreviations used can be found. The references of the Introduction and Table 2 can be found at the end of the thesis.

AIMS OF THE THESIS

The dangerous impact of free and/or circulating histones on human inflammatory and immunological responses, coagulation system alterations, and endothelial functions, has recently emerged as relevant topic for the scientific community due to the novelty of the extracellular effects of histones (the most common are summarized in **Table 1**) and to their potential use as therapeutic targets [1].

One of the most important cellular processes involving the extracellular release of histones is related to the extrusion of Extracellular Traps (ET) from White Blood cells [2], in a process collectively named ETosis. ETosis is considered as both a cell death mechanism (along with apoptosis and necrosis) and an alternative immune system defense mechanism against infections carried out by different types of white blood cells. When not finely regulated, ETosis is associated with a massive extrusion of net-like structures, whose main components are represented by histones. As a results, the blood levels of extracellular histones increase, thus predisposing their harmful interaction with several cell types and biomolecules during several human diseases [3], such as thrombosis, auto-immune disorders, systemic lupus erythematosus, classical and viral sepsis [4, 5].

In this view, several *in vitro* and *in vivo* investigations highlighted the potential of extracellular histones as predictors or biomarkers for the aforementioned pathologies.

In line with this, the aims of this PhD project were:

I) to evaluate the ability of histones to modulate the inflammatory responses activated by White Blood Cells, in a whole-blood model assay. The rationale for this choice is based on the wide spectrum of histone interactions with all blood components and

plasma proteins, in order to provide an improved and complete picture of what happens *in vivo.*

II) to investigate the ability of four commercially available anticoagulant drugs (Enoxaparin, Unfractionated Heparin, Sulodexide, and Fondaparinux) to modulate the histone-induced inflammatory profiles in the same whole-blood model assay.

III) to test the capability of extracellular histones to induce morphological changes in the monocyte population through the use of MDW (Monocyte Distribution Width) a novel FDA-approved, and EC-marked early sepsis indicator of monocyte heterogeneity upon massive inflammatory, calculated using Volume, Conductivity, and Scatter technologies (VCS), which can detect qualitative aspects of monocytes and supply useful information about morphological reactions of the cells to environmental factors. This cell population data parameter is calculated along with routine complete blood cell count, detecting the volumetric changes of circulating monocytes, using Automated hematology analyzers.

Table 1. *The most common effects of circulating histones and their pathophysiological consequences*

HISTONE-INDUCED EFFECTS	CONSEQUENCES
CYTOTOXIC	• Cell membrane disruption • Calcium influx and overload • Toll-like receptor (e.g., TLR2, 4, and TLR9) signalling activation • Complement activation
PRO-INFLAMMATORY	• NLP3 inflammasome activation • NF-kB activation • MyD88 pathway stimulation • Cytokine storm phenomenon
PRO-COAGULANT	• Platelet aggregation and activation • TF expression • Activated thrombin generation • Phosphatidylserine exposure
OTHER	• Impaired fibrinolysis • Heterocomplexe formation with other biological molecules (e.g., HMGB1, and LDL)

Abbrevations: *HMGB1, High Mobility Group Box 1; LDL, Low Density Lipoproteins; NLP3, NLR family pyrin domain containing 3; NF-kB, nuclear factor-kappa-light-chain-enhancer of activated B cells; MyD88, myeloid differentiation primary response 88.*

INTRODUCTION

1.1 A brief overview of the NETosis process

Neutrophils are the most abundant polymorphonuclear leukocytes (60-70%) involved primarily in host responses to pathogens. Neutrophils have a very short lifespan in the bloodstream, once activated they exert their effects and finally die. Neutrophil death can occur through various processes **(Figure 1)** as apoptosis, which prevents the extracellular release of potentially harmful cell components, necroptosis, pyroptosis, and NETosis [6].

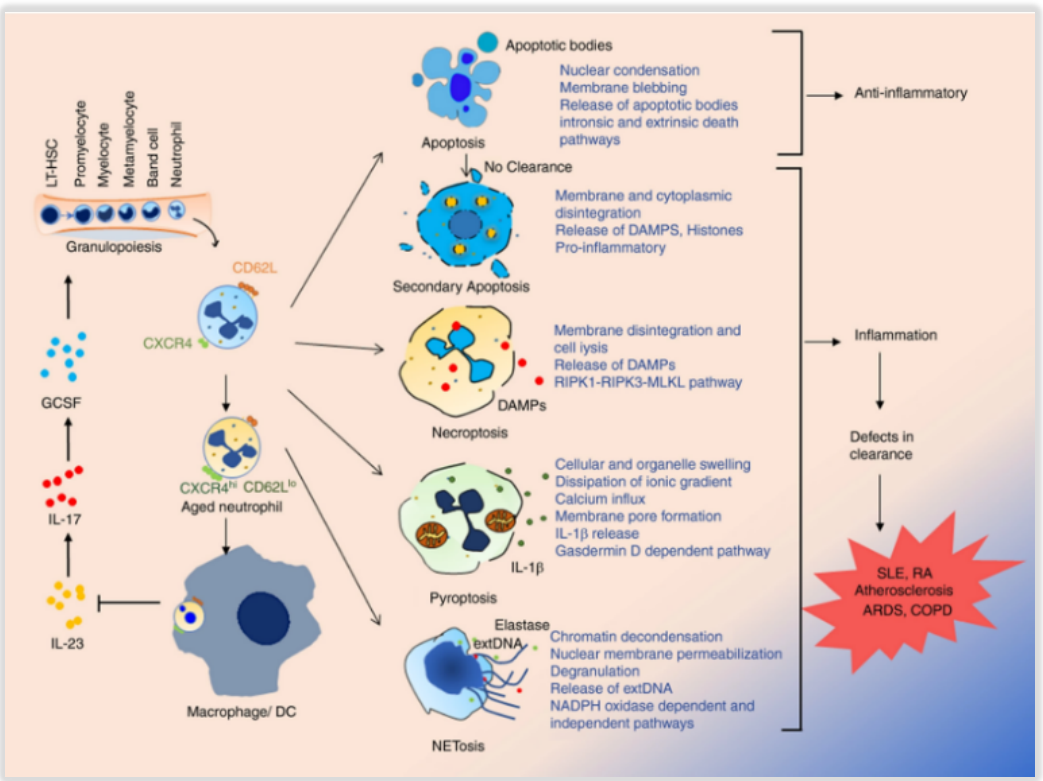

Figure 1. *Neutrophil homeostasis and major death pathways under steady-state and inflammation [6]*

Several pieces of evidence suggest that neutrophils, in addition to their protective role, may have a potentially dangerous effect in some clinical settings.

Experimental and clinical data show that neutrophil absence or decrease leads to frequent infections and autoimmune disorders, on the other hand, their massive activation and dysregulated or delayed clearance leads to induction and exacerbation of an inflammatory response and tissue injury [7].

As a result, the regulation of neutrophil homeostasis is a fundamental process that results in a delicate balance between neutrophil formation, recruitment, activation status, and removal from circulation [8] via various death pathways. Non-apoptotic pathways of neutrophil death can be associated with the extracellular release of intracellular contents, which can impair the resolution of the initial insult. These cell contents extruded into the extracellular environment can act as danger signals, inducing a hyperinflammatory response. In this context, the NETosis pathway has recently sparked considerable interest.

Takei et al. [9] described NETosis for the first time in 1996. Afterwards, Brinkmann and colleagues, in 2004, improved NETosis characterization describing it as a unique neutrophil cell death mechanism in which fibers of decondensed chromatin decorated with granular, nuclear, and cytosolic proteins, named neutrophil extracellular traps (NETs), are extruded into the extracellular space upon neutrophil activation [10]. Using this suicidal strategy, neutrophils capture and neutralize pathogens as well as viruses [10, 11]. However, since its discovery, the idea that NETosis is an exclusive mechanism of cell death has been challenged. Compelling *in vivo* and *in vitro* studies revealed that neutrophils put in place a stimulus- and time-dependent antimicrobial defense mechanism that does not result in cell death [12].

To date, two controversial types of NETosis have been described: *suicidal* or *classical NETosis* and *vital NETosis. Suicidal NETosis*, the lytic form of NETosis, occurs through a stepwise progression of cell morphological changes. This process is completed within 120-240 minutes of stimulation and begins with the segregation of the chromatin into its constitutive parts and the loss of the nuclear lobular structure. At this point, the nuclear envelope disappears, and nuclear material mingles with cytosolic contents. Fuchs and colleagues proposed that this mixing occurs through a charge interaction between the positively charged granular and cytoplasmatic proteins and the DNA [13]. At later time point, the cell membrane disintegrates after pore formation, and dying cells release intracellular meshwork structures into the extracellular space **(Figure 2A)** [14].

The *vital NETosis* **(Figure 2B)** is the alternative pattern of NETosis that happens more quickly (5-60 minutes after cell insult) and does not result in membrane rupture and cell death. Through *vital NETosis,* neutrophils preserve their viability and functionality, such as chemotaxis, phagocytosis, and leukocyte recruitment. In contrast to *suicidal NETosis*, which is a cellular response to specific cues, activation of this NETosic pathway appears to be mediated by multiple types of pathogen stimuli recognized by toll-like receptors (TLRs) or complement receptor for C3 protein [12].

It is worth noting that in some circumstances [15], viable neutrophils can also undergo an additional form of NETosis characterized by the activation of different biomolecular pathways upstream, and the release of mitochondrial DNA downstream [16].

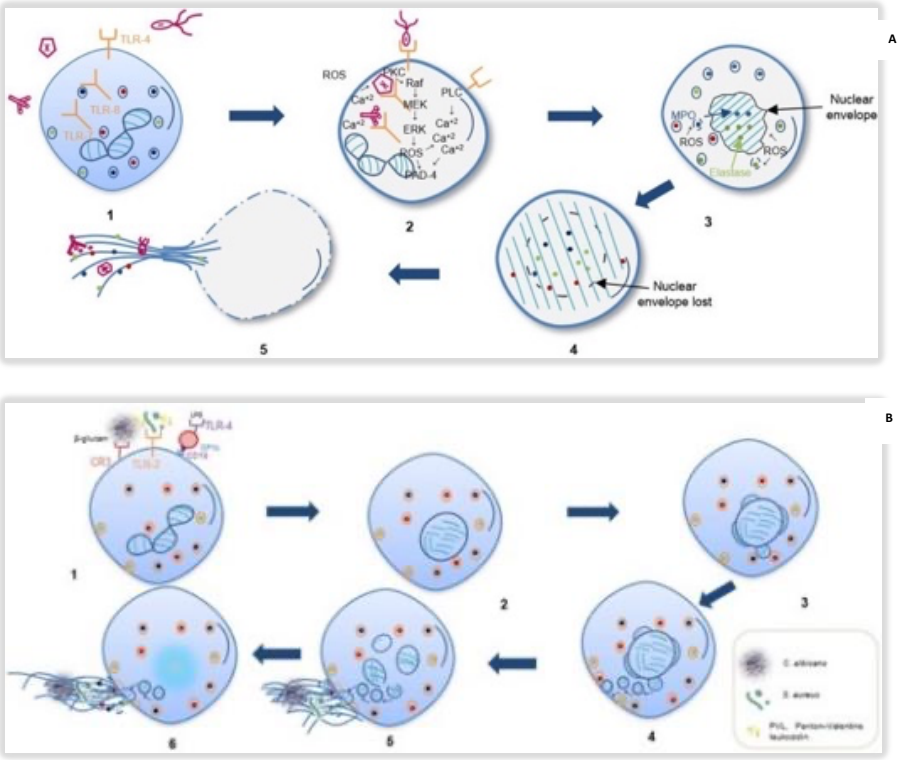

Figure 2. *(A) Sequential step of suicidal NETosis; (B) Sequential step of vital NETosis [14]*

1.1.1 Stimuli triggering NETosis

Quiescent neutrophils become fully activated in response to a variety of stimuli and eventually acquire a NETosic phenotype characterized by the production and extrusion of NETs [17]. In this regard, several inducers were tested *in vitro*, which often led to very conflicting results [18]**.** Indeed, given the different signaling pathways that may be involved upstream, these triggers can induce the stimulation of lytic or non-lytic NETosis and therefore determine a heterogeneous composition of NETs extruded. For instance, current researches describe phorbol 12-myristate 13-acetate (PMA), and *Pseudomonas aeruginosa* as lytic inducers of NETosis, while hypochlorous acid, *Staphylococcus aureus*, *Candida albicans*, and calcium ionophore (CI) as non-lytic triggers of NETosis [10, 19-23]. In this

context, intriguingly is the behavior of lipopolysaccharide (LPS). In whole blood *in vitro* assays, it has been demonstrated that LPS can induce lytic or non-lytic release of extracellular traps, depending on both its bacterial origin and the presence or absence of serum and/or platelets [24]. Among these, PMA and CI have generally been recognized as the most potent NETosis stimulants. PMA is a biochemical stimulus that acts specifically activating protein kinase C (PKC). Most literature data report that PMA has a 100% success rate and timing of action ranging from 10 minutes to 24 hours. A feature of PMA-induced NETosis is chromatin decondensation and subsequent generation of NETs only following activation of autophagy and superoxide production [25]. On the other hand, cellular exposure to CI provokes a dose and time dependent extrusion of DNA-NET through the opening of calcium channels with subsequent fluctuations in intracellular concentrations of Ca^{2+}.

1.1.2 The biomolecular mechanisms underlying NETosis

The biomolecular mechanisms employed by cells to produce and release the web-like structures into the extracellular space during NETosis process are very complex and not all fully understood. To date, among these mechanisms, the production of reactive oxygen species (ROS) has aroused more interest. Several studies have shown a differential ROS involvement by different stimuli, allowing the classification of NETosis in ROS (or nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, NOX)-dependent and -independent **(Figure 3)** [26-28].

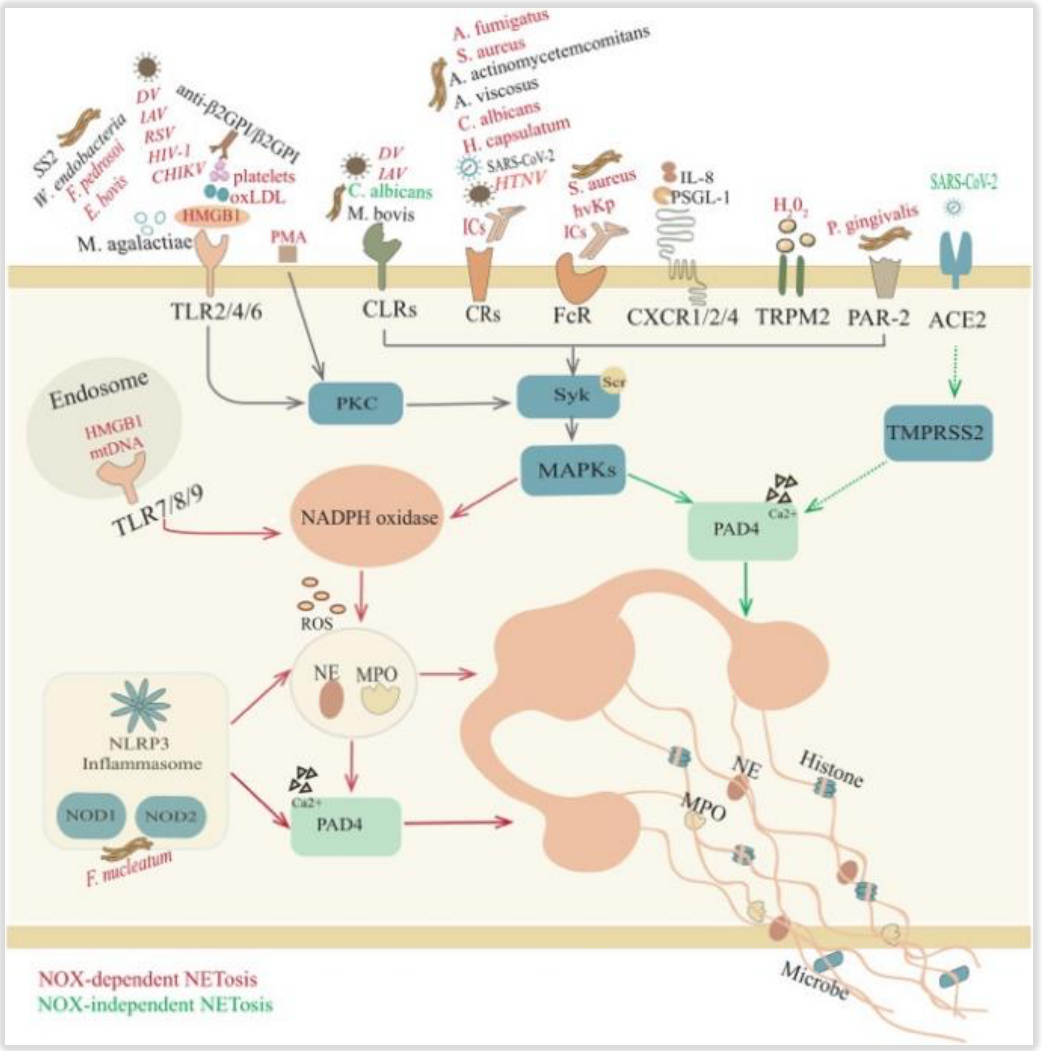

Figure 3. *Overview of NETosis [29]*

In general, lytic NETosis requires the activation of PKC, which, in turn mediates the assembly and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and promotes intracellular calcium fluxes. Activated NOX determines the production of superoxide anion, which, in turn is converted in hydrogen peroxide. In this oxidative environment, myeloperoxidase (MPO) dissociates form the azurosome (a protein complex found in azurophilic granules) [30] and catalyzes the formation of hypochlorous acid as well as other oxidant molecules. Activated MPO

promotes the release of neutrophil elastase (NE) from the azurosome and its translocation to the nucleus. Simultaneously, MPO migrates from the cytosol to the nucleus where, in combination with NE, it contributes to chromatin decondensation through histones cleavage (major components of extracellular traps). Also involved in this cascade of events is peptidyl arginine deaminase 4 (PAD4) [31], which, after moving to nucleus, catalyzes histones citrullination, leading to a weakening of the bond between DNA and histone proteins. At this point a series of changes in cell membrane occurs, culminating in cell death and the extracellular ejection of fibers of decondensed chromatin decorated with cytosolic and granular proteins [32].

Furthermore, in the above-described mechanism, it is noteworthy to underline the involvement of other kinases acting on the NADPH complex, such as c-Raf, MEK, ERK, and Akt [33], and the contradictory role of PAD4 in histone deamination especially when PMA is used as a NETosis inducer [23, 34].

The alternative mechanism that happens independently of NOX activity is faster than the NOX-dependent release of extracellular traps, which instead is slower. Nevertheless, it is not entirely excluded that ROS play a significant role in even this pathway of NETosis. In this regard, it has been reported that neutrophils from chronic granulomatous disease (CGD)-affected individuals, with unfunctional NOX, did not have the potential to form NETs, highlighting the importance of ROS [35]. As a result, additional sources, such as mitochondrial oxidative phosphorylation complexes, must produce enough ROS to trigger the NETs generation in the absence of functioning NOX. According to Douda et al., calcium ionophore stimulation can induce NETosis without the involvement of NADPH oxidase because it results in the generation of mitochondrial ROS (mtROS) through the involvement of the calcium-activated SK3 channel (small conductance calcium-activated potassium channel 3) [23]. This signaling pathway does not require the phosphorylation and

activation of kinases, as PKC, MAPK/ ERK, and results in the production and extrusion of ETs within 15 minutes of cell stimulation.

Furthermore, viable neutrophils can also form and release extracellular traps into the extracellular environment via a ROS, NOX, kinases, MPO, and NE independent mechanism. This model of NETosis is triggered by specific recognition of harmful cues by toll-like receptors and complement receptor for C3 protein, resulting in a movement of vesicles containing nuclear DNA, histones, and granular and cytosolic proteins, from inside the cell to the extracellular space [14].

The complex mechanism of NETosis is therefore currently understood to entail three different biomolecular pathways: conventional NETosis, mediated by NADPH-derived ROS, *vital NETosis* supported by mtROS, and NETosis unaffected by ROS and NADPH oxidase.

1.1.3 Beyond NETosis

Growing evidence has shown that many phenotypic subsets of cell types besides neutrophils can perform a mechanism similar to NETosis, coining a more generic term, namely "ETosis" **(Figure 4)**.

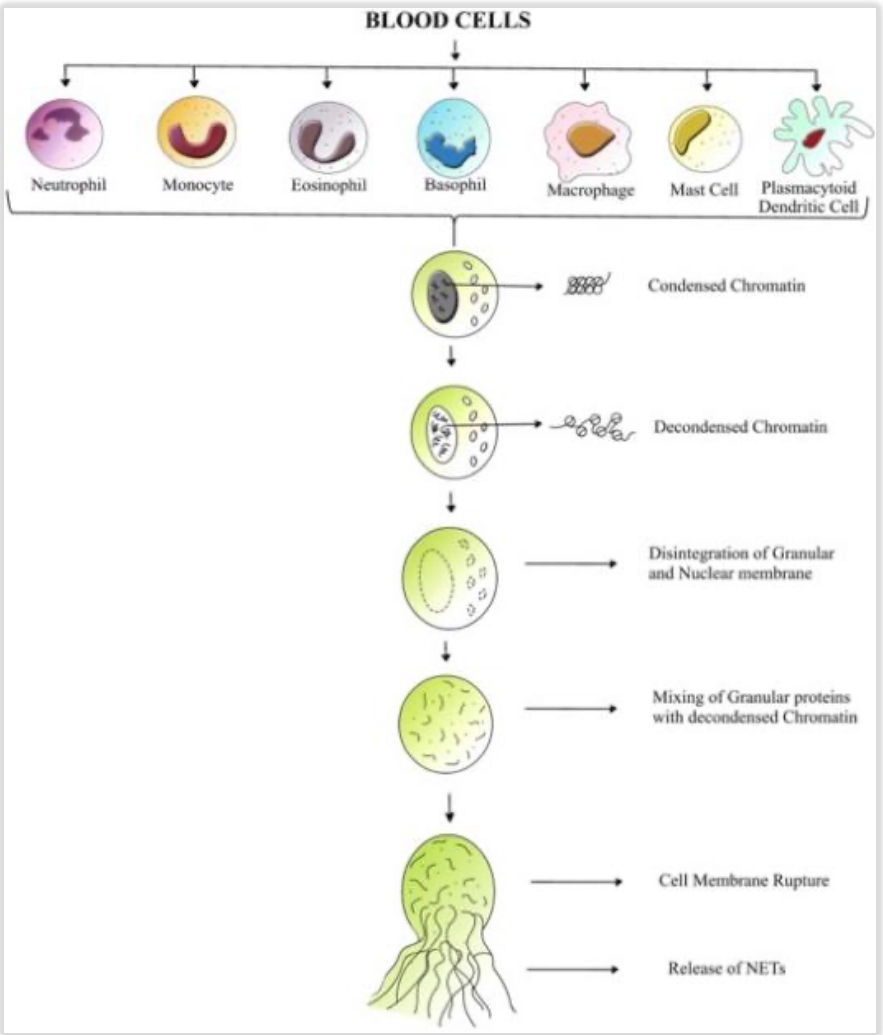

Figure 4. *Mechanism of extracellular trap formation in blood cells [36]*

Indeed, it has been demonstrated that eosinophils, mast cells, basophils, plasmacytoid dendritic cells, monocytes, and macrophages

can perform ETosis via various signaling pathways, depending on the type and degree of insult [36].

ETosis in eosinophils, also known as "EETosis", requires ROS and calcium, and it is characterized by the loss of the nucleus' bi-lobular shape, the breakdown of the nuclear membrane, and finally the disruption of the cell membrane with the EETs spilling into the extracellular space. Furthermore, stimulated eosinophils, like neutrophils can extrude extracellularly both nuclear and mitochondrial DNA, but unlike them, the granules are released as untouched structures [37].

Regarding extracellular traps released by mast cells (MECTs), few data have been published. For instance, it has been recently reported that the release of MECTs via ROS-dependent mechanisms results in an indirect antibacterial activity similar to that of NETs [36].

Recently, the plasmacytoid dendritic cells' spectrum of action was also widened at antifungal properties. Loures et al. have demonstrated that after *Aspergillus fumigatus* infection these cells liberates mesh-like structures (pETs) consisting mainly of DNA and citrullinated histone H3 that inhibit hyphae production by these species of fungus [38].

In vitro studies have demonstrated that activated basophils can form and release in the extracellular environment the so-called "basophil extracellular traps" (BETs) containing mitochondrial DNA (but not nuclear DNA), as well as granule-derived proteins. It has been observed that BETs generation occurs dependently on mtROS, allowing basophils to exhibit also antibacterial properties [39]. However, although some works have indicated that mtROS levels promote the basophil non-viral defense mechanism through the production of BETs, the details of how this occurs, and the nature of granular proteins are still not fully understood.

Another intriguing mechanism has also been discovered in monocytes, which has been well described in animal models and

poorly investigated in humans. Granger et al. have used PMA, calcium ionophore A23187, and two biological inducers to stimulate monocytes obtained from human blood samples, to provide a more thorough explanation of ETosis in human monocytes. The author demonstrated an ETs production and extrusion pattern in monocytes similar to that found in neutrophils, supported by ROS generation and unrelated to MPO activity [40].

Regarding the production of ETs by macrophages (METs), some reports suggest that this mechanism mirrors NETosis [36], albeit other research points out the discrepancies among these processes, such as the role of ROS [41]. In this context, another intriguing aspect concerns the different state of macrophages polarization (M1, pro-inflammatory; M2, anti-inflammatory), which might affect the METosis process. A single study is available, and it reports that THP-1 cells, after differentiation with PMA and in response to NETosic components, polarize towards M1 rather than M2, resulting in extracellular DNA release (probably of METosis origin) [42].

1.2 Characterization of Extracellular Traps

Over the years, proteomic approaches have made it possible to characterize the protein composition of extracellular traps. In this field, one of the early studies resulted in the identification of approximately 24 different proteins with nuclear, cytosolic, and granular origins [43], and several of these were showed to be shared by neutrophils, monocytes, and monocyte-derived macrophage ETs. With growing interest in the complex and fascinating world of ETosis, additional proteomic investigations have led to the discovery of over 270 proteins inside extracellular traps (particularly NET). Although the comparison of ETome between NOX dependent and -independent

ETosis showed some differences, S100A8/A9, also known as calprotectin protein complex with antibacterial and antifungal properties, catalase, elastase, cathepsin G, MPO, lactotransferrin (LTF), gelatinases, and histones are the components most commonly mentioned. It is noteworthy that most of data arise from *in vitro* studies using non-physiological stimuli like PMA and calcium ionophore. Additionally, it was found by bioinformatics studies that these NET-derived proteins' externalization was linked to particular biological processes that varied depending on the nature of inducing stimulus, such as gene silencing and pentose phosphate shunt, following stimulation with PMA and calcium ionophore, respectively [44].

Fousert and colleagues [45] recently published an exhaustive review highlighting the possibility that these ETs components, including cell free DNA, are recognized as autoantigens, thus initiating autoimmune diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis, and anti-neutrophil cytoplasmatic antibodies-associated vasculitis) **(Table 2)**.

Besides the protein components in ET, another key element is DNA. Indeed, according to the canonical description of extracellular traps, these structures consist of a backbone of extracellular DNA decorated with nuclear and granular-derived proteins.

Surprisingly, some observations have recently shed light on naRNA (NET-associated RNA) as a new abundant nuclear-derived component of NETs. When cells are stimulated, naRNA, which is normally confined inside of cells in association with LL37 (antimicrobial peptide), is extruded, amplifying NETosis [46] and promoting an inflammatory response in endothelial cells [47], via TLR8 (in human) and Tlr3 (in mouse) pathways.

Table 2. *Neutrophil extracellular traps (NETs)-associated molecules that are known autoantigens in various autoimmune diseases (modified) [45]*

Which Autoantigens Are Found on Neutrophil Extracellular Traps (NETs)?		To Which Autoimmune Disease Are These Autoantigens Associated?	
α-enolase	3,6	SLE	1
Annexin A1	3,6	SLE	2,14,20
		RA	14
Apolipoprotein A1	6	SLE	35
Bp	38	AAV	38
C1q	22,29	SLE	30
Catalase	6	SLE	24
		RA	24
Cathelicidin	6	SLE	2
Citrullinated histones	4,7,19,23,28,29	RA	19
		SLE	40
dsDNA		SLE	15,40
Histones	6	SLE	13
HMGB1	36,39	SLE	36,39,40
LAMP-2	33	AAV	16
LL37	6,10-11,21,29,37	SLE	11
		Psoriasis	9
MMP8	6	RA	25
MMP9	5,6	SLE	5
MPO	12,18,26,32,34,41	AAV	27,31
PR3	12,18,34	AAV	33
Properdin	38	AAV	38
TF	8,17	SLE	8

Abbrevations: Bb: complement factor B, C1q: complement component 1q, dsDNA: double-stranded DNA, HMGB1: high mobility group protein B, LAMP-2: Lysosomal membrane 2 protein, LL37: cathelicidin antimicrobial peptides, MMP8: matrix metalloproteinase 8, MMP9: matrix metalloproteinase 9, MPO, myeloperoxidase, PR3: proteinase 3, TF: tissue factor, AAV: anti-neutrophil cytoplasmic antibodies (ANCA) vasculitis, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus.

1.2.1 ET-derived histones

Histones, the major protein component of extracellular traps, have received more attention than any other ETosis-derived products.

Histones are normally bound to DNA to ensure the structure, stability, and functionality of the eukaryotic genome. Indeed, DNA is packaged within chromatin inside the cell nucleus via specific electrostatic interactions with histone and non-histone proteins. To form the nucleosome, 145-147 bp of DNA are wrapped around a histone core composed of histone tetramer H3-H4 and two histone dimers H2A and H2B. During various cell death pathways, including ETosis, this nuclear structure can be released into the extracellular space. In this context, MPO and elastase are crucial for the disassembly of the nucleosome and subsequent release of histones extracellularly [48]. Histones' post-translational modifications, such as those mediated by PAD4, are another step implicated in the generation of free histones during ETosis. The importance of this histone modification is related to the loss or reduction affinity for DNA. PAD4 is a nuclear enzyme that targets several sites in H3 and H4 histones, catalyzing the conversion of arginine residue to citrulline, resulting in a positive charge loss [49]. In this way, histones dissociate from DNA and emerging in the extracellular environment support the host's defenses against pathogens infection. However, when their release exceeds the body's ability to degrade them, they can become dangerous stimuli for the host.

Histone cytotoxicity appears to be mediated by their charge; in fact, it has been suggested that through electrostatic interactions, they attach to and damage pathogen membranes [50], interfere with prokaryotic DNA [51], and bind to and affect human neighboring cells and circulating immune cells. Several intriguing studies on the dark side of histones have been undertaken, revealing their capability to serve as damage-associated molecular pattern (DAMPs). DAMPs are molecules released by dying cells in response to stress or tissue injury and serve as "signal 0" for the innate immune system, driving the inflammatory responses. DAMP-like histones can cause collateral cell damage by interacting with one or more pattern recognition receptors

(PRRs), besides the complement and platelet activation. Particularly, it has been observed that the stimulation of TLRs signaling pathway, a class of PRR associated to cell membrane, especially TLR2, TLR4 and TLR9, plays a role in the upstream ETosis process. Histones-TLR2/TLR4 interaction induces the pro-inflammatory cytokines secretion in cooperation with myeloid differentiation primary response 88 (MyD88) signaling pathway. This latter stimulates nuclear factor-kappa B (NF-kB) complex, which, in turn promotes the production and release of some cytokines, such of IL-6 and TNF [52]. Furthermore, aside from the classical ligand-receptor interaction, extracellular histones can act, for instance by activating the nucleotide-binding domain, leucine-rich repeat containing protein 3 (NLRP3) inflammasome, which, in turn stimulates the immune system to produce IL-1β and IL-18 [53].

1.2.2 Extracellular histones in the pathogenesis of various diseases

Regardless the signaling pathway and cell types from which extracellular histones originate, mounting evidence demonstrates their strong contribution to the initiation and worsening of tissue injury and inflammation reactions **(Figure 5)** [4].

In 2009, Xu et al. [54] demonstrated for the first time that high levels of circulating histones were correlated to organ failure and death in sepsis patients. This observation has fueled supposition that extracellular histones may also affect other human diseases. To support these speculations, some observational studies have shown an increase in serum concentrations of histones in cardiovascular diseases, such as thrombosis, atherosclerosis, disseminated intravascular coagulopathy, systemic lupus erythematosus, traumas,

autoimmune disorders, multiple organ failure, and more recently COVID-19 infection. The role of histones in the aforementioned pathologies has been reconducted to their cytotoxicity, the ability to induce endothelial dysfunction, alter the coagulation system, and promote a systemic inflammatory state linked to the cytokine storm phenomenon.

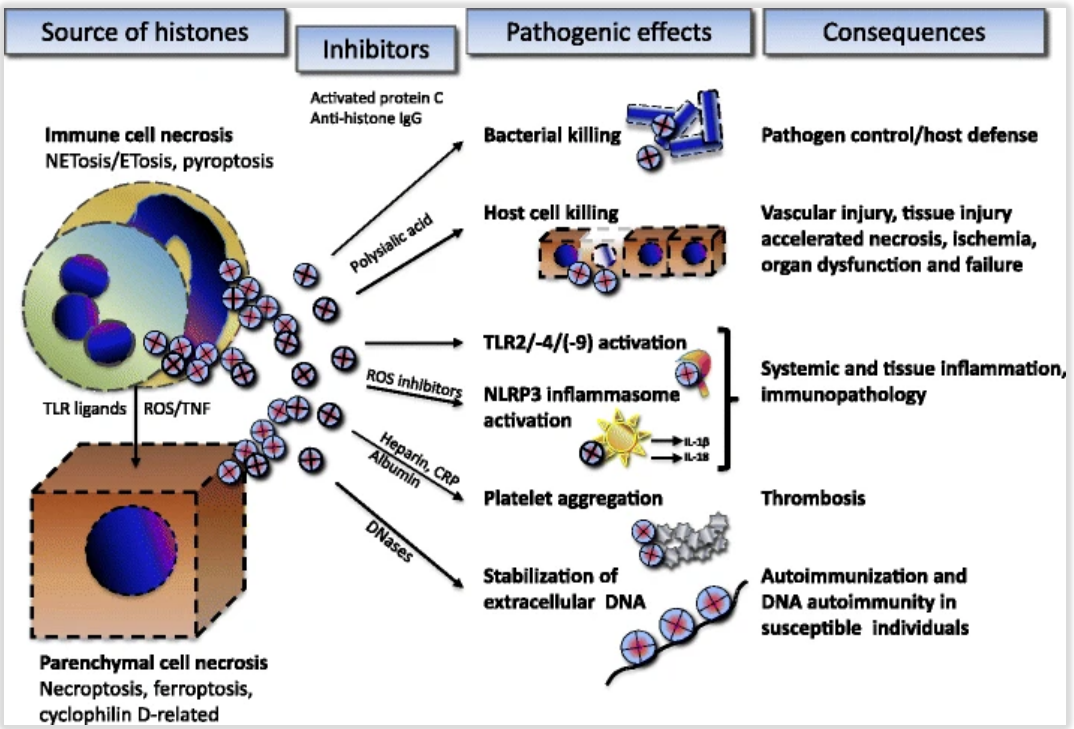

Figure 5. *How histones trigger tissue injury and inflammation [4]*

Given the crucial role in maintaining vascular haemostasis, several diseases can be linked to endothelial dysfunction. In this context, extracellular histones alter the pro- and anticoagulant properties of vascular endothelium by dose-dependently increasing tissue factor (TF) expression via TLR2 and TLR4 pathways, and subsequent NF-kB pattern activation [55]. Moreover that, it has been shown that histones can act on the protein C-thrombomodulin (TM)

system, thus influencing the coagulation dynamics. Indeed, both protein C and TM have anionic domains capable of electrostatic interaction with cationic compounds (as histones), namely an N-terminal carboxyglutamic acid domain and an O-linked chondroitin sulfate moiety, respectively. In physiological conditions, thrombin binds to TM, losing its procoagulant activity and activating protein C. Activated protein C (APC) in turn proteolytically cleaves activated V and VII factors, resulting in the anticoagulant effect. The final effect of ternary histone-TM-protein C complex is the suppression of protein C activation and the enhancement of the procoagulant system [56].

Once in the extracellular space, circulating histones also show cytotoxic effects, depending on their type (linker histone H1, and core histones H2A, H2B, H3, and H4) and concentration. These cellular damages have been extensively studied in *in vitro* and *ex vivo* experimental models, using for example endothelial, epithelial, mesenchymal, and monocytic cells. Regarding cytotoxic effect dependent on histone sort, histones H2B, H3 and H4 were found to be more cytotoxic than the other subtypes. Furthermore, it has been reported that H3 and H4 are major mediators of endothelial cell damage [57], whereas H1 specifically acts on leukemia cells and cortical neurons [4].

In addition, histone cytotoxicity has also been linked to their ability to bind to phosphatidylserine, altering the plasma membrane permeability to cations, upset the cellular calcium balance, ultimately resulting in cell death [58].

1.2.2.1 Circulating histones in sepsis and COVID-19 infection

As mentioned above, extracellular histones are able to contribute to adverse immune and inflammatory responses in sepsis with the development of multiorgan dysfunction and fatal disease outcomes. In septic patients, elevated plasma levels of circulating histones, primarily subtype H4, have been linked to worsened illness. Histones cytotoxicity, endothelial activation and dysfunction, platelets activation, and inflammation via TLR4 signaling pathway have all been linked to a septic response impairment.

Increased extracellular histone concentrations, for example, are frequently associated with a decrease in circulating platelet levels in severe sepsis due to their direct binding, recruitment of adhesion molecules, and finally, platelet aggregation. As a result, thrombocytopenia develops, along with impaired microcirculation, endothelial dysfunction, and organ injury overall increasing the risk of unfavorable clinical outcomes [59].

Furthermore, upon sepsis condition, histone-mediated immune cell activation with an increase in the secretion of inflammatory mediators has also been reported. In particular, the extracellular excessive release of histones could represent an additional trigger for cytokine storm activation, mainly characterized by high expression of TNF-α, IL-1β, IL-6, IL-8, and weak INF-γ induction, which, in turn can play a crucial role in the exacerbation of inflammation itself. This histone-mediated side effect could be attributed primarily to TLRs pathways. In fact, histones are known to bind to TLR2 and TLR4, and subsequent activation of MyD88 trigger inflammation. Other studies have instead reported the involvement of the NLRP3 inflammasome in the alteration of the inflammatory response mediated by histones. Activated NLRP3 promotes the release of critical pro-inflammatory cytokines, such as IL-1β, and IL-18, and the recruitment of other

immune cells, resulting in the further extrusion of extracellular traps, as well as histones, thus triggering a vicious loop [60].

The recent COVID-19 (Coronavirus disease 2019) pandemic has profoundly expanded and changed the scientific and medical literature. Although the pathogenesis of COVID-19 remains not fully understood, many have become interested in the association between COVID-19 infection and sepsis. Data obtained from hospitalized critically ill patients, and autopsy studies, showed some similarities with sepsis and septic shock, leading to the introduction of a new term namely "viral sepsis". Low levels of lymphocytes, reduction in platelets count, overproduction of cytokines, chemokines, tissue and growth factors (so-called cytokine storm), increased D-dimer values, and multi-organs failure are some shared signs between these two septic conditions [61]. However, notable dissimilarities have also emerged between classical and COVID-19 sepsis. For instance, the quick release of cytokines in bacterial-induced sepsis compared to the relatively slow timing of cytokine response in viral sepsis, acute clinical onset versus frequently chronic onset course are the most usually encountered. Another significant discrepancy between COVID-19 sepsis and non-COVID-19 came from monitoring some immunothrombosis-related parameters. According to Cani et al. protein C and cell-free DNA levels may be used as indicators of mortality risk in classical sepsis, whereas soluble thrombomodulin and citrullinated histones in viral sepsis [62].

In keeping with these speculations, therefore, the novel and unexpected importance of histones have emerged in the setting of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. ETosis circulating markers, such as histones, have been correlated with inflammation, endothelial dysfunction, and altered hemostasis in COVID-19 patients **(Figure 6)**, as well as with poor disease outcomes [63, 64].

Shaw and colleagues have for the first time suggested that extracellular histones deriving from dying immune cells, such as macrophages and neutrophils, were potential mediators of coagulopathy and mortality in COVID-19 infection. They found, through a translational investigation, that the severity of COVID-19 infection increased with histones concentration, also suggesting a new stratification approach of ill subjects (*mild= 2.6 µg/ml, moderate = 10.5 µg/ml, critical = 20.0 µg/ml, and non-survivors = 29.6 µg/ml*) [65].

A further link between Coronavirus disease 19 and extracellular histones (both citrullinated and unmodified) might be represented by the onset of multi-organ dysfunction/failure promoted by hyperinflammatory status, as a result of the stimulation of inflammasome and TLRs pathways [5].

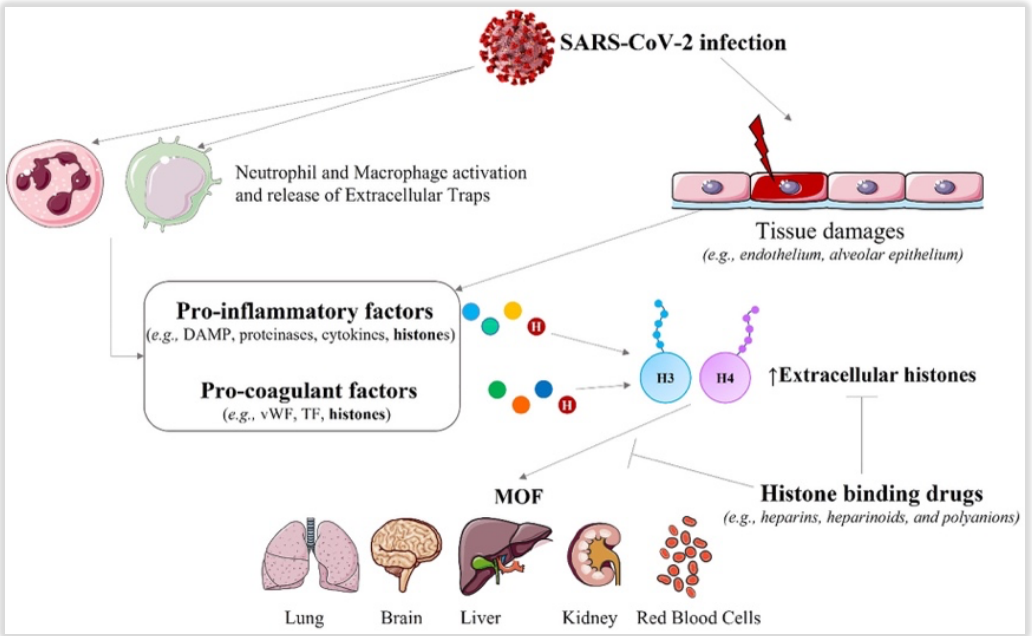

Figure 6. *Schematic overview of extranuclear and extracellular release of histones induced by COVID-19 infection, through direct NET/MET processes and tissue damages [5]*

Altogether these clinical and laboratory findings have, therefore, highlighted the importance of histones as prognostic biomarkers in sepsis and COVID-19 disease.

1.3 Monocyte Distribution Width

Sepsis and COVID-19 share a further diagnostic and prognostic laboratory marker, namely Monocyte Distribution Width (MDW), beyond extracellular histones.

Numerous studies have demonstrated that the imbalance between pro- and anti-inflammatory mechanisms, which characterize both these conditions, might be mirrored by the morphological alterations in monocytic cells. Monocytes and monocyte-derived macrophages are known regulators of cellular immunity, inflammatory responses, as well as plasma coagulation system, all of which influenced by extracellular histones. These cells under hyperstimulation suffer considerable functional and morphological alterations, establishing themselves as the cornerstone of histone-mediated harmful effects.

Volumetric increase in immune cells can reflect an early manifestation of acute and severe infection, providing a window into the hyperinflammatory microenvironment.

From this point of view, it has been suggested that monocyte morphological changes can be identified through the measurement of MDW calculated using an automated analyzer based on VCS (Volume, Conductivity, and Dispersion) technology [66] that exploits three independent energy sources simultaneously. In particular, accurate cell volume and the degree of size variations are measured using direct current impedance, the conductivity of the internal cell composition is determined using radio frequency measurement, and

cytoplasmatic granularity and nuclear structure are obtained using a laser beam to measure light scatter [67, 68]. The processing of data related to these Volume, Conductivity, and Dispersion parameters thus provides the final value.

MDW is referred to as a hematological laboratory parameter obtained quickly by a routine blood draw, indicating the dispersion of the volume of monocytes in whole blood around the population means. This survey tool allows to quantitatively detect the morphological alterations in both immature and reactive cells, similarly as a qualitative microscopic assessment of a peripheral blood smear.

Recently, MDW index emerged as a reliable EC-marked and FDA (Food and Drug Administration)-cleared early sepsis indicator [69]. The cut-off point of monocyte distribution width as predictor of sepsis was validated at 20.0 units (U), although it may vary based on the anticoagulant used in the sampling, 20.0 U for K_2-EDTA and 21.5 U for K_3-EDTA. In this regard, it is worth pinpointing that the FDA recommends collecting blood samples for MDW testing in EDTA dipotassium blood tubes [70] for two reasons: first, the higher potassium concentration in K_3-EDTA compared to K_2-EDTA can cause shrinkage of erythrocytes, and second, it can affect the mean corpuscular volume [71]. It should be noted that patients without sepsis may have a high baseline MDW resulting in a false-positive, therefore, the data should be interpreted in association with other clinical and diagnostic findings. On the other hand, values of MDW less than or equal to the cut-off point should not exclude the diagnosis of sepsis. Furthermore, based on the cut-off setting of 20.0 U, MDW sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios were established and are provided in **Table 3** for informational purpose.

Currently, the MDW used in tandem with other biomarkers such as C-reactive protein (CRP) and procalcitonin (PCT), together

with parameters such as routine complete blood count (CBC), and risk scoring systems like systemic inflammatory response syndrome (SIRS) and quick sequential organ failure assessment (qSOFA) have improved the early diagnose of sepsis [66, 72, 73]. For example, it has been showed through a cohort study, that the measurement of MDW in combination with WBC counts allowed both to discriminate sepsis from other forms of acute disease and to early detect sepsis, with a greater accuracy than MDW or WBC alone [74].

Table 3. *Performance of MDW* *(https://www.beckmancoulter.com/download/file/wsr262828/C21894AC?type=pdf)*

MDW Cut-off at 20.0						
	Sensitivity	Specificity	Predictive Values		Likelihood Ratios	
			Positive	Negative	Positive	Negative
Estimate	0.740	0.720	0.365	0.927	2.646	0.361
Lower 95% Confidence Interval	0.694	0.699	0.332	0.912	2.406	0.304
Upper 95% Confidence Interval	0.782	0.741	0.399	0.940	2.911	0.428

Since MDW is linked to whole monocytic population volume modifications upon excessive immune activation, its promising application in quick diagnosis/prognosis even in critical cases of COVID-19 has proposed **(Figure 7)** [75]. In fact, high MDW values have detected not only in sepsis due to bacteria, but also in viral sepsis, e.g., SARS-CoV-2 infection [73]**.** Many authors reported higher MDW values in COVID-19 patients than non-COVID-19 patients, and an incrementally trend of these values in correlation with the increase of disease severity. Therefore, from a laboratory perspective, while awaiting the results of cellular and humoral biomarkers, the quicker assessment of MDW enables prompt prediction of illness outcomes, thereby, whenever possible, averting the onset of multi-organ failure development and ensuing death.

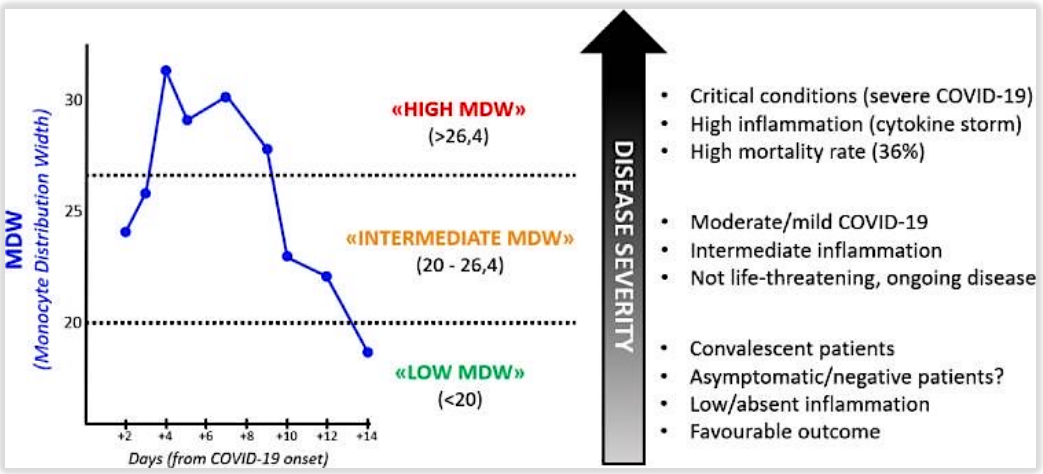

Figure 7. *A working model for MDW use in COVID-19 patients [75]*

1.4 Pleiotropic effect of heparins and heparinoids

Based on the circumstances, the ETosis machinery can perform contradictory actions as two sides of the same coin. Biomolecules making up the extracellular traps represent a supplementary immune weapon to disable pathogens, which must, however, necessarily be limited and regulated to avoid adverse consequences to the host. Over the years, in fact, it has been demonstrated that both regulated expulsion of ETs and immune inflammatory response are body's beneficial mechanisms. On the other hand, if ETosis as well as extracellular histones ejection is excessive and uncontrolled, they themselves become dangerous cues, triggering or worsening various human diseases. Therefore, pharmacological inhibition of the pathological effects of histones became one of the enticing approaches to attenuate and/or abolish their harmful consequences.

To this purpose, various inhibitors of histone-mediated inflammation, endothelial dysfunction, and altered coagulation

dynamic have been studied. Most of these studies have been based on histone charge shielding through the electrostatic interaction with negatively charged molecules, including albumin [76], polysialic acid [77], prostaglandin E_2 [78] activated protein C [79] heparin and heparinoids [80, 81]**.** The mechanism of action of these physiological and synthetic molecules is not yet completely clarified, but presumably might be traced to an interference with the crosstalk between histones and their targets, thus blocking the activation upstream of the signaling pathways linked to cellular responses.

Physiologically, heparin is synthesized in the Golgi organelles of certain cell types and represent a common human and animals tissue component. During the biosynthesis process the linear polysaccharide backbone are enriched from N-deacetylated, N-sulfonated, and O-sulfonated sugars that following epimerization afford the highly sulfated molecule [82]**.** Heparin belongs to glycosaminoglycan (GAGs) family, whose high sulfation heterogeneity is almost always responsible of several biological functions ranging from mechanical support to regulation of inflammation and coagulation, e.g., through the specific binding with antithrombin III (AT) and heparin cofactor II. The latter prompted the discovery of heparin-like drugs, the most used forms since more than 100 years in the treatment and prophylaxis of thrombotic disorders, such as venous and arterial thrombosis, pulmonary embolism, and rethrombosis after thrombolysis [83]. Three forms of heparin are currently available and FDA-approved: unfractionated heparin (UFH) with an average molecular weight of 16 kDa, low-molecular weight heparins (LMWHs) with an average molecular weight of 3.5 – 6.0 kDa, and ultralow molecular weight heparins (ULMWHs) with an average molecular weight < 2.0 kDa. LMWH is produced by controlled chemical or enzymatic depolymerization of UFH and exhibits a similar antithrombotic mechanism. UFH enhances the action of antithrombin by binding it, causing the inactivation of Factor Xa, and preventing the

generation of thrombin and fibrin, whereas LMWH preferentially binds to Factor Xa, and exhibits a lower capability to inhibit the conversion of prothrombin into thrombin. Also, unlike UFH, LMWH has fewer thrombotic complication such as lower risk of heparin-induced thrombocytopenia (HIT), and bleeding [84]**.** ULMWH is a newest type of heparin produced via chemical synthesis, characterized by a lower risk of bleeding, similar or better effectiveness, longer half-life (17-21 hours vs 4-7-hours, and 45 minutes, for LMWH and UFH, respectively) than other currently available antithrombotic drugs, but its high cost limits its common use. The antithrombotic effect of ULMWH is due to the potentiation of the antithrombin action by about 300-fold, but its small size prevents its direct effect on thrombin.

Besides the interactions with the coagulation system other biological activities have been attributed to heparins, including anti-inflammatory, antiviral, anti-complement and restoration of vascular endothelial barrier [85, 86] **(Figure 8)**. A range of studies have demonstrated these additional beneficial properties in various clinical setting, including burns, asthma, ulcerative colitis [83], cancer [87], neurodegenerative diseases [88] diabetic complications [89], sepsis [90], and COVID-19 [91].

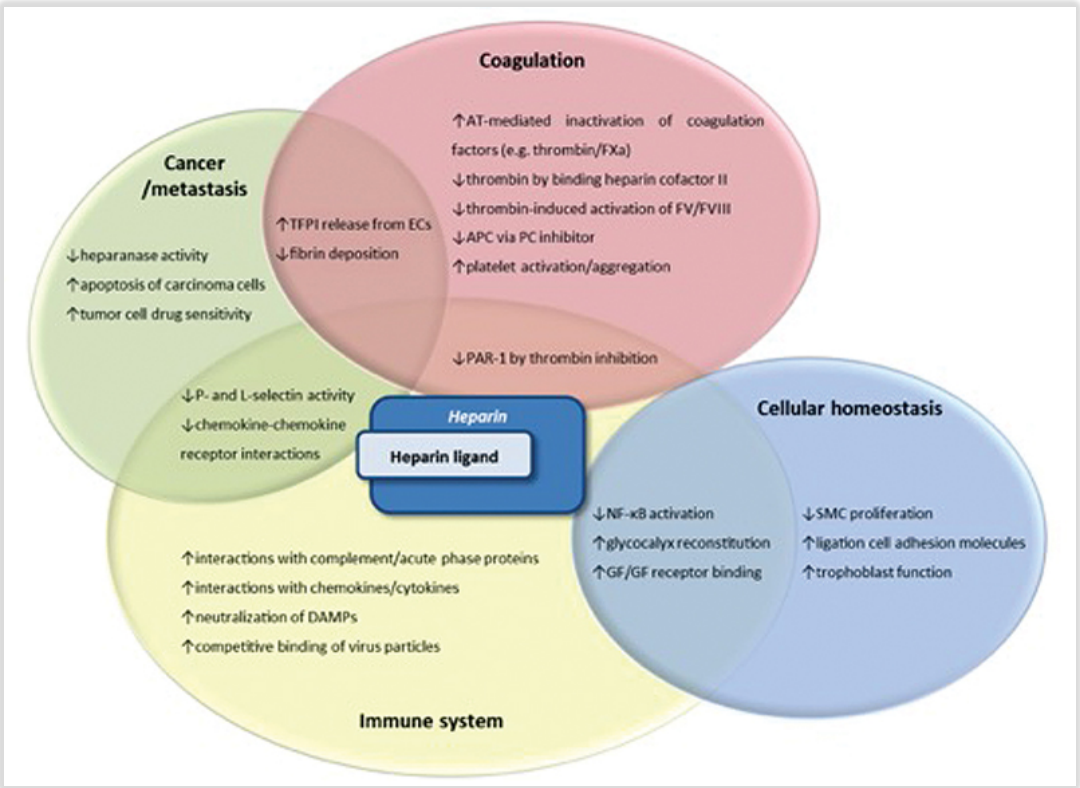

Figure 8. *Heparin–ligand interactions in physiological and pathological states [86]*

An area that received a certain attention is related to the capability of heparins to exert anti-inflammatory effects regardless their anticoagulant and antithrombotic properties. Therefore, numerous efforts have been directed towards the production of heparin-like molecules able to attenuate the inflammatory response avoiding the risk of bleeding, since as reported, the anti-inflammatory effect is displayed at high concentration of heparin. For this purpose, several processes of chemical modification have been described, for example, to reduce the affinity with Factor Xa, to increase the selective interaction for heparin-binding proteins, or to directly affect the gene expression of pro-inflammatory cytokines and chemokines.

The mechanism driving the anti-inflammatory effect of heparins is not fully understood. It has been proposed that their negative charge, sulfation degree, and molecular size potentially play

a pivotal role in the non-specific interaction with several proteins like extracellular histones. In fact, heparin and heparin derivatives have recently emerged as promising pharmacological strategies to reducing adverse clinical consequences promoted and/or accelerated by circulating histones.

Sharma et al. have observed the ability of LMWH, UFH, and fondaparinux (synthetic heparin derivate) to neutralize the cytotoxic and pro-coagulant effect of extracellular histones by binding them with size-dependent binding affinity. Various heparin types with a MW > 1.7 kDa abolish the harmful effect of histones with a different effectiveness, also inhibiting the pro-coagulant action of monocytic cells [92]**.** A heparin variant selectively desulfated (AADH) produced from UFH, is demonstrated able to form complexes with histones, inhibiting their toxic effect on endothelial cells accompanied by reduction of the secretion of pro-inflammatory cytokines, both *in vitro* and *in vivo* mouse models.

Recently, another exhaustive study reported the capability of heparins to attenuate the pro-inflammatory activity of circulating histones dependently on specific structural features, especially the sulfation degree. The findings of a decreased profile of pro-inflammatory mediators, such as IL-6, IL-8, TF, and C3a in an *ex vivo* whole blood experimental model after histone stimulation, simulating the *in vivo* setting of sepsis [93] is the basis for the suggestion that heparins may serve as anti-histone agents. Another potential mechanism underlying the anti-inflammatory effect of heparins could be attributed to the blocking of the NF-kB signaling pathway, stimulated upstream by extracellular histones after interaction with TLR2/TLR4 receptors. NF-kB is an inducible transcription factor involved in the regulation of transcription of several genes, whose products can play a crucial role in the amplification of inflammatory reactions and endothelial dysfunction when excessively released. Therefore, inhibition or down-regulation of the NF-kB by heparins,

e.g., UFH and LMWH, could mitigate the endothelial cytotoxicity following histone-induced hyperinflammation [94].

Collectively, given the ability of heparin and heparinoids to target and hamper multiple pathways related to circulating histones, they could represent an important therapeutic approach to improve the clinical course of human life-threatening conditions.

SDX is a highly purified mixture of GAGs derived from porcine intestinal mucosa that contains 80% fast-moving heparin (FMH) and 20% dermatan sulfate (DS). The average molecular weight of fast-moving heparin fraction is 7 kDa, whereas the DS moiety has a molecular weight of 25 kDa. Over the years, glycosaminoglycan Sulodexide has been tested in various clinical conditions, demonstrating to have pleiotropic activities by acting on different biological targets [95]. Many studies have shown that SDX exhibits anticoagulant and profibrinolytic effects, making it an ideal drug in the blood coagulation setting. These pharmacological properties are mediated by DS and FMH Sulodexide components, which simultaneous act on ATIII and HCII. The FMH fraction shows similar mechanism of action to LMWH, by specifically interacting with ATIII. The DS moiety on the other hand is responsible for the selectively interaction and activation of HCII, increasing its activity by about 1000-fold [96]. As a result, thrombin inhibition or production, as well as the extension of thrombin clotting time and the activated partial thromboplastin time (aPTT), increases the efficacy of SDX in the prophylaxis and treatment of vascular disorders.

Sulodexide has been demonstrated to have other pharmacological qualities aside from those anticoagulant and pro-fibrinolytic, such as antioxidant, anti-ischemic, anti-apoptotic, and antiproliferative activities, as well as endothelium protective effect, anti-atherosclerotic, anti-inflammatory, and anti-proteolytic capability [95, 97, 98].

SDX's anti-inflammatory efficacy has been demonstrated in multiple *in vitro* and *in vivo* studies, which have underlined a significant reduction in pro-inflammatory cytokines, chemokines, and colony stimulating factors in macrophages, together with a dose-dependent suppression of ROS generation [99, 100].

Another notable functional property of this glycosaminoglycan-based drug is the ability to inhibit the matrix metalloproteinases family, particularly the expression of the MMP-9 precursor (pro-MMP-9), resulting in an endothelial protective effect [101]. MMPs are well-known proteolytic enzymes which degrade several extracellular matrix constituents, e.g., collagen, elastin, and proteoglycans, and hence play a crucial role in endothelial integrity loss.

Furthermore, it is also important to note the first beneficial effects of SDX, namely its capacity to promote lipoprotein lipase release and reduce the levels of circulating lipids. For example, it has been reported that SDX is effective in decreasing very low-density lipoprotein levels, by reducing their uptake or increasing their hepatic metabolism [96].

ABBREVIATIONS

NET	neutrophil extracellular trap
TLR	toll-like receptor
C3	complement component 3
PMA	phorbol 12-myristate 13-acetate
CI	calcium ionophore
LPS	lipopolysaccharides
PKC	protein kinase C
ROS	reactive oxygen species
NOX	nicotinamide adenine dinucleotide phosphate oxidase
MPO	myeloperoxidase
NE	neutrophil elastase
PAD4	peptidyl arginine deiminase 4
ERK	extracellular signal-regulated kinase
Raf	rapidly accelerated fibrosarcoma
MEK	mitogen-activated protein kinase
Akt	protein kinase B
CGD	chronic granulomatous disease
mtROS	mitochondrial ROS
SK3	small-conductance calcium-activated potassium channel
ETs	extracellular traps
EETs	eosinophil extracellular traps
MECTs	mast cells extracellular traps
pETs	plasmacytoid dendritic cells extracellular traps
BETs	basophil extracellular traps
METs	macrophage extracellular traps
S100A8/A9	calprotectin complex
LTF	lactotransferrin
naRNA	NET-associated RNA
DAMPs	damage-associated molecular patterns
PRRs	pattern recognition receptors
NF-kB	nuclear factor-kappa-light-chain-enhancer of activated B cells
IL	interleukin
TNF	tumor necrosis factor
NLRP3	NLR family pyrin domain containing 3
COVID-19	coronavirus disease-19
SARS-CoV-2	severe acute respiratory syndrome coronavirus-2

TM	thrombomodulin
APC	activated protein C
INF- γ	interferon-γ
TF	tissue factor
MyD88	myeloid differentiation primary response 88
MDW	monocyte distribution width
CRP	C-reactive protein
PCT	procalcitonin
CBC	complete blood count
WBC	white blood cells
SIRS	systemic inflammatory response syndrome
qSOFA	quick sequential organ failure assessment
GAGs	glycosaminoglycans
AT	antithrombin
UFH	unfractionated heparin
LMWH	low molecular weight heparin
ULMWH	ultralow molecular weight heparin
AADH	antithrombin affinity depleted heparin
HIT	heparin-induced thrombocytopenia
DS	dermatan sulfate
ATIII	antithrombin III
HCII	heparin cofactor II
FMH	fast-moving heparin
aPTT	activated partial thromboplastin time
MMPs	matrix metalloproteinases

CHAPTER 1

Heparins and Heparinoids modulate histone-induced inflammation

(Submitted)

HEPARINS AND HEPARINOIDS MODULATE HISTONE-INDUCED INFLAMMATION

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Abstract

Recent reports have indicated that circulating histones act as damage-associated molecular pattern (DAMP) proteins in the extracellular space, thus mediating the development or worsening of various human pathologies such as cardiovascular diseases, cancer, COVID-19, and sepsis. Several agents have been proposed to prevent their harmful effects, including heparins and related compounds. We now report a dose-dependent inflammatory response induced by histone and its modulation by four common heparin/heparinoid treatments in an *ex vivo* human whole-blood model.

Keywords: Histone; Heparin; Heparinoid; Inflammation; Cytokine

Abbreviations: COVID-19, Coronavirus Disease 2019; DAMP, Damage-Associated Molecular Pattern; UFH, unfractionated heparin; LMWH, low molecular weight heparin; ULMWH, ultra-low molecular weight heparin; NET, Neutrophil Extracellular Trap; SDX, Sulodexide; GAG, *Glycosaminoglycan;* ATIII, antithrombin III; HCII, heparin cofactor II; FXa, activated factor X; TLR, Toll-like Receptor; IL, Interleukin.

Intranuclear histones act as DAMPs when extracellularly released during various hyperinflammatory conditions. In fact, during both passive and active cell death processes, such as NETosis, the nucleosome disassembly leads to the release of DNA and histones into the circulatory system and other body fluids. Detailed investigations revealed that the excessive release of histones activates immunoinflammatory responses, contributing directly and indirectly to the pathogenesis of various infectious and non-infectious human diseases. This

detrimental effect can be mediated by activating separately or simultaneously receptor-dependent (mainly Toll-like receptors) and receptor-independent signaling pathways.

In particular, the interaction between free histones and TLRs causes aberrant stimulation of immune cells, resulting in the induction of the cytokine storm phenomenon, which is associated with systemic collateral damage (1).

Moreover, histones exert a procoagulant effect by stimulating platelet activation, promoting prothrombin auto-activation and phosphatidylserine exposure in red blood cells and monocytes, increasing tissue factor activity in monocytes, promoting the release of von Willebrand factor from endothelial cells and impairing the physiological anticoagulant pathways by inhibiting thrombomodulin-dependent protein C activation and by interfering with antithrombin-mediated neutralization of thrombin, thus emerging as critical players in immunothrombosis (2).

Several *in vitro* studies have shown that histone-related cytotoxicity can be neutralized by electrostatic interaction between positive-charged histones and negative-charged molecules, including activated protein C, C-reactive protein, albumin, and heparin (3). In this respect, several findings demonstrated that heparins and heparinoids could exert a beneficial role by inhibiting histone cytotoxicity (3-6), with heterogeneous effects among heparin/heparinoid variants. Heparin belongs to the sulfated glycosaminoglycans family and is one of the most used anticoagulant and antithrombotic drug, with emerging non-anticoagulant properties (6), including its ability to bind histones (4).

The purpose of this study is to investigate the ability of a histone mixture (including H1, H2A, H2B, H3, and H4 subunits) to promote the release of a wide panel of inflammatory mediators from whole blood cells, and how this response could be counteracted by heparin variants.

We selected Unfractionated heparin (UFH), Enoxaparin as low-molecular-weight heparin (LMWH), Sulodexide, and fondaparinux as heparin variants. UFH consists of a heterogeneous mixture of GAGs that binds Antithrombin (ATIII) and inhibits clot formation. LMWHs are a class of heparin derivatives

produced through controlled UFH depolymerization. In contrast to UFH, LMWHs primarily inhibit FXa and show the ability to modulate the release of Tissue Factor Pathway Inhibitor, profibrinolytic mediators, and adhesion molecules. Fondaparinux is a synthetic pentasaccharide belonging to the ULMWH class able to bind ATIII with high affinity (5). SDX is a highly purified mixture of GAGs extracted from porcine intestinal mucosa that contains 80% fast-moving heparin and 20% dermatan sulfate. SDX exhibits a wide range of biological effects on the vascular system, including anticoagulant and profibrinolytic properties by acting on ATIII and HCII (7).

On these bases, this study was designed to investigate the ability of histones to promote inflammatory responses in an *ex vivo* human whole blood model, and to characterize the role of heparins and heparinoids in attenuating the release of cytokines.

To this end, human whole blood samples from healthy subjects (anticoagulated in citrate tubes) recruited among the staff of the University of Urbino were diluted in RPMI 1640 according to Hogwood et al (4). Diluted whole blood was treated with increasing doses of a histone mixture (0, 7, 25, 50 μg/ml; Roche Diagnostics) under sterile conditions, up-to 24h. The ability of heparins to modulate the histone-mediated inflammatory reaction was investigated by adding to diluted whole blood a histone 50 μg/ml + heparins mixture (UFH 0.25 IU/ml, SDX 0.12 LSU/ml, Enoxaparin 1 IU/ml, or Fondaparinux 2 μg/ml) incubated 50 min, +4 °C. The histone-heparin mixture was added to whole blood both entire and as supernatants obtained after centrifugation (20,000 x g, 5 min). All treatments were maintained for 24h, at 37 °C, 5% of CO_2. After incubations, all samples were centrifuged (2,500 x g, 10 min, +4 °C) to obtained platelet poor plasma.

Plasma samples were assayed for the analysis of a panel of 27 inflammatory mediators (Pro Human Cytokine 27-plex Assay, Bio-Rad) through multiplex immunomagnetic assay technique (Bio-Plex, Bio-Rad), according to manufacturer's instructions. Statistical analyses were performed using GraphPad Prism 9.0. Values are expressed as mean ± standard error (SEM) and p values <

0.05 were considered significant. Significant differences between controls and treatments were determined using one-way ANOVA followed by post-hoc test.

We showed that histone treatments induced a significantly increased release of almost all parameter analyzed. In particular, we observed that the lowest dose of 7 μg/ml of histones significantly up- regulated the release of IL-7, IL-12(p70), IL-13, eotaxin, GM-CSF, and VEGF. The dose of 25 μg/ml was able to significantly enhance the release of almost all parameters, and the highest dose of histones (50 μg/ml) very significantly increased the release of all parameters (excepted for IL-12(p70) and RANTES). This trend highlighted a dose-dependent release mechanism for several cytokines, including IL-1β, IL-2, IL-6, IL-17, IFN-γ, TNF-α, IL-1ra, IL-4, IL-5, IL-9, IL-10, IL-8, eotaxin, MCP-1, MIP-1α, MIP-1β, G-CSF, bFGF, and PDGFbb (Fig. 1A).

Comparing the cytokine profile observed in HIS 50 μg/ml treated samples, with those observed in samples treated with a combination of histones and heparin variants, we showed that heparin variants exhibit a different ability to modulate the inflammatory responses promoted by histones (Fig. 1B). We highlighted that the mixture histone+heparin variants was associated with a significant increase of IL-4 ($p=0.01-0.05$), IL-5 ($p<0.05$), 12p70 ($p<0.05$), and G-CSF ($p<0.05$) by UFH, of IL-4 ($p<0.05$) and TNF-α ($p=0.0001-0.001$) by Enoxaparin, and of IL-4 ($p<0.0001$), IL-5 ($p=0.0001-0.001$), IL-7 ($p=0.0001-0.001$), IL-12p70 ($p<0.0001$), IL-13 ($p<0.0001$), eotaxin ($p=0.001-0.01$), and G-CSF ($p=0.001-0.01$) by Fondaparinux *vs.* HIS 50. On the other hand, a significant decrease of IL-6 ($p<0.05$), IP-10 ($p=0.001-0.01$), and PDGF-bb ($p=0.001-0.01$) by UFH, of IL-6 ($p=0.001-0.01$), IL-10 ($p<0.05$), and PDGF-bb ($p=0.0001-0.001$) by Sulodexide, and of IP-10 ($p=0.001-0.01$) and PDGF-bb ($p=0.0001-0.001$) by Enoxaparin *vs*. HIS 50 was observed (Fig. 1B).

The use of supernatants obtained after histone+heparins mixture centrifugation as triggers revealed a general reduced secretion of inflammatory molecules induced by UFH, Sulodexide, and Enoxaparin, in contrast with a general but not significant increase of cytokines induced by Fondaparinux. In particular, it emerged that Sulodexide was able to reduce all cytokines considered (excepted

for IP-10 which was significantly increase *vs*. HIS 50; p=0.0001-0.001), with a significant inhibiting effect on IL-10, bFGF, GM-CSF, PDGFbb, MIP-1β and TNF-α ($p<0.001$), IL-1β, IL-1ra, IL-2, IL-6, IL-7, IL-9, IL-13, MIP-1α, and VEGF (p=0.001-0.01), and G-CSF and IFN-γ ($p<0.05$) (Fig. 1B).

Other heparin variants exhibited a certain grade of selectivity, with a significant reduction of IL-1β ($p<0.05$) and IL-6 (p=0.001-0.01) levels induced by UFH, and a significant decrease of IL-6 and MCP-1 ($p<0.05$) promoted by Enoxaparin *vs.* HIS 50 (Fig. 1B).

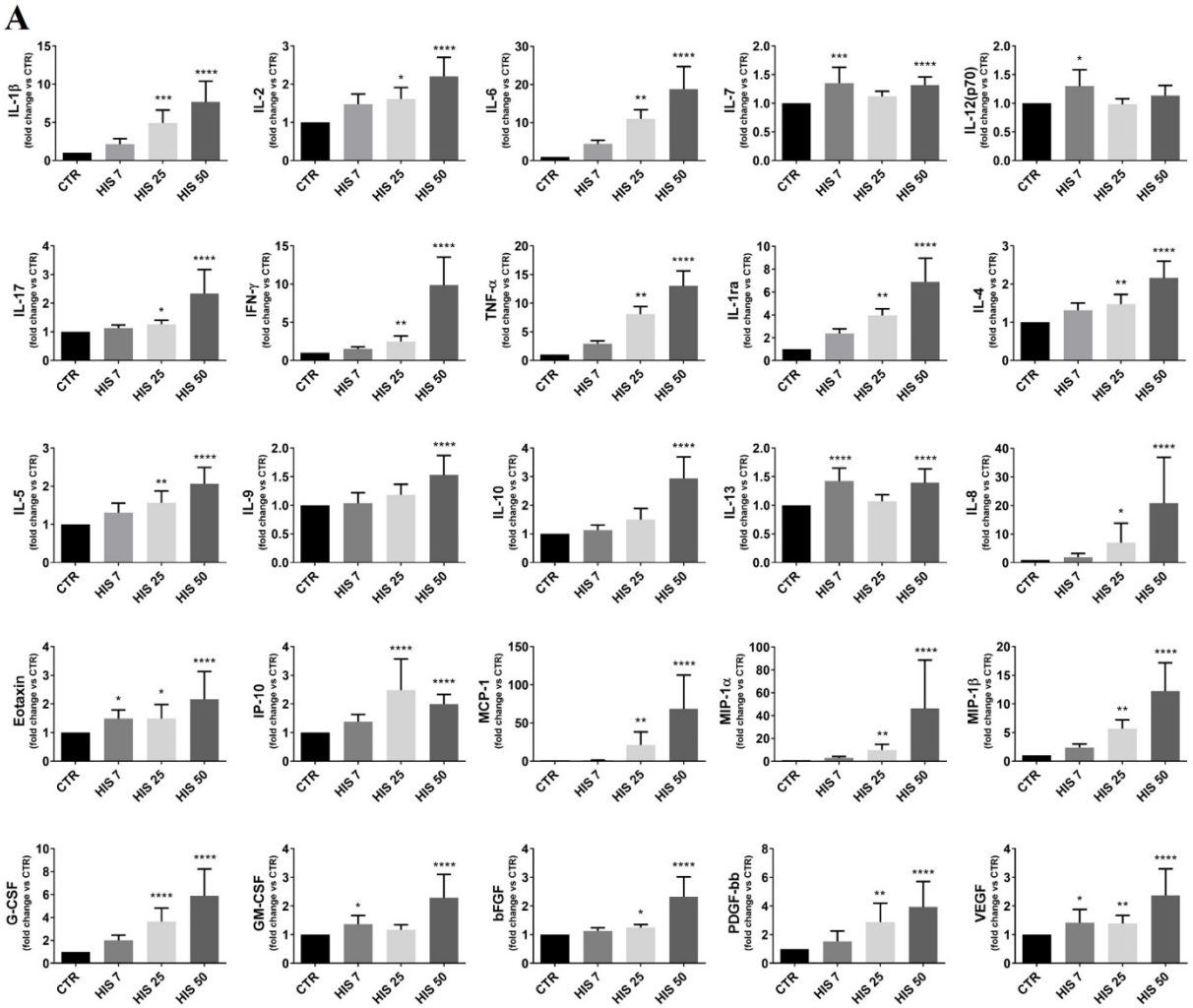
In agreement with literature data (1, 8, 9), our results demonstrated that histone treatment was able to promote a significantly increased release of cytokines, most with a dose-dependent mechanism, highlighting its immunostimulatory effect on blood cells. The release of a wide panel of cytokines is in agreement with the ability of histones to activate TLR, NF-kB, and inflammasome signaling pathways, finally resulting in an enhanced cytokine secretion (1, 10); in this perspective, histone can contribute to trigger or exacerbate the cytokine storm associated with multiple human diseases, including COVID-19 and sepsis (11).

Noteworthy, we demonstrated that histone-induced inflammation could be attenuated/modulated by heparin variants, highlighting a different ability of each heparin variant to counteract histone's effect. We hypothesize that these differences depend on the diverse heparin sulfation, size, and complexity of the GAG mixture, and are independent of the anticoagulant property, according to other reports (5, 12). Moreover, heparins also exert anti-inflammatory properties by down-regulating MAPK, NF-kB, and c-Jun signaling pathways (12), highlighting that their effects could both depend on their charge-mediated binding of histone, with different affinity for heparin variants and different histone subunits, and on their intrinsic anti-inflammatory ability. Moreover, heparins show the ability to directly bind cytokines and chemokines, providing a further mechanism to decrease the trafficking of lymphocytes, neutrophils, and eosinophils to the site of inflammation and the release of other pro-inflammatory molecules by leukocytes (13).

Our results indicate that in the absence of histone stimulation, most parameters were not significantly affected by heparins (data not shown), whereas heparins were able to down-regulate the inflammatory reactions sustained by histone, both when used as co-treatment, and mainly when added to samples after *in vitro* reaction. This result indirectly shows that heparins could bind histone subunits, thus reducing the release of inflammatory mediators.

In conclusion, we report that histones can dose-dependently induce the cytokine storm in our *in vitro* human whole blood experimental model and that heparins may provide potential non-anticoagulant benefits in several diseases characterized by a dysregulated immune and inflammatory response.

In the frame of our study, we recently demonstrated that histones are able to significantly increase the Monocyte Distribution Width (MDW) (14), which is an index of monocyte heterogeneity, whose levels correlate with monocyte activation. In this respect, it is well-known that the inflammatory responses obtained from whole-blood assay reflect mainly the monocyte reactions (15), and further studies from our groups are ongoing to evaluate the ability of heparin variants to modulate MDW and the associated inflammatory phenotype. Despite the limited number of samples and conditions, our preliminary results sustain the critical involvement of histone-induced hyperinflammation in the pathogenesis of numerous human diseases (11), making them promising diagnostic markers potentially modulated by heparins.


A
IL-1β
IL-2
IL-6
IL-7
IL-12(p70)
IL-17
IFN-γ
TNF-α
IL-1ra
IL-4
IL-5
IL-9
IL-10
IL-13
IL-8
Eotaxin
IP-10
MCP-1
MIP-1α
MIP-1β
G-CSF
GM-CSF
bFGF
PDGF-bb
VEGF
(fold change vs CTR)
CTR
HIS 7
HIS 25
HIS 50

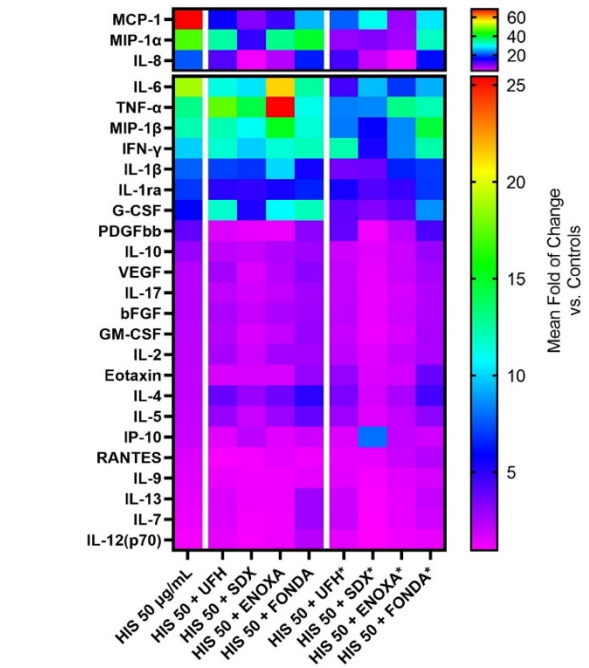

B
MCP-1
MIP-1α
IL-8
IL-6
TNF-α
MIP-1β
IFN-γ
IL-1β
IL-1ra
G-CSF
PDGFbb
IL-10
VEGF
IL-17
bFGF
GM-CSF
IL-2
Eotaxin
IL-4
IL-5
IP-10
RANTES
IL-9
IL-13
IL-7
IL-12(p70)
HIS 50 µg/mL
HIS 50 + UFH
HIS 50 + SDX
HIS 50 + ENOXA
HIS 50 + FONDA
HIS 50 + UFH*
HIS 50 + SDX*
HIS 50 + ENOXA*
HIS 50 + FONDA*
Mean Fold of Change vs. Controls

Fig. 1. Histone-mediated cytokine release and heparin-induced modulation. (A) Cytokines released after stimulation of whole blood samples with increasing doses of histones (7, 25, 50 µg/ml). Values are expressed as mean ± SEM of the fold of change *vs*. untreated controls (Statistical test: One-way ANOVA; **** = p < 0.0001; *** = p: 0.0001-0.001; ** = p: 0.001-0.01; * p: 0.01-0.05). (B) Heat-map showing the different effects of heparins + histones compared to samples treated with the highest dose of histones (50 µg/ml). Columns 2-5 (HIS 50 + heparin variants) are referred to treatments with the mixture obtained without centrifugation; Columns 6-9 (HIS 50 + heparin variants*) are referred to treatments with the supernatants obtained after mixture centrifugation. The scale on the right indicates the mean fold change of cytokines levels in treated samples *vs*. untreated controls.

CRediT authorship contribution statement
Rosanna Maniscalco: Investigation, Methodology, Writing – original draft. **Daniela Ligi:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **Chiara Della Franca:** Investigation. **Ferdinando Mannello:** Conceptualization, Writing – review & editing. All authors contributed to the revision of the manuscript and have read and approved the final version.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that may appear to or directly influence the work reported in this paper.

Data availability
Data will be made available on reasonable request.

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CHAPTER 2

Do Circulating Histones Represent the Missing link among COVID-19 Infection and Multiorgan Injuries, Microvascular Coagulopathy and Systemic Hyperinflammation?

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Opinion

Do Circulating Histones Represent the Missing Link among COVID-19 Infection and Multiorgan Injuries, Microvascular Coagulopathy and Systemic Hyperinflammation?

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Abstract: Several studies shed light on the interplay among inflammation, thrombosis, multi-organ failures and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Increasing levels of both free and/or circulating histones have been associated to coronavirus disease 2019 (COVID-19), enhancing the risk of heart attack and stroke with coagulopathy and systemic hyperinflammation. In this view, by considering both the biological and clinical rationale, circulating histones may be relevant as diagnostic biomarkers for stratifying COVID-19 patients at higher risk for viral sepsis, and as predictive laboratory medicine tool for targeted therapies.

Keywords: histone; COVID-19; coagulopathy; cytokine storm; inflammation; multiorgan injury; neutrophil extracellular trap; heparin; heparinoids; laboratory medicine

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Several studies shed light on the crucial interplay among inflammation, thrombosis, cardiovascular diseases, multi-visceral manifestations, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, namely linking the roles of neutrophil extracellular traps (NETs), nucleosomes, histones, cytokines, and the coagulation cascade [1–5]. A possible novel association of coronavirus disease 2019 (COVID-19) with cardiovascular risk, heart attack, and stroke has also been underpinned [6,7], thus suggesting an urgent need to explore the biomolecular characteristics of such an increased risk of developing cardiovascular events in patients with SARS-CoV-2 infection [8,9].

To this end, the cellular and biomolecular microvascular mechanisms of coagulopathy will yield crucial information on COVID-19-dependent thrombotic-derived systemic manifestations (i.e., thrombo-inflammation), thus paving the way for a more appropriate and targeted therapeutic strategy [10,11].

Recent exhaustive meta-analyses and critical literature reviews of hematologic, biochemical, and immunological biomarkers abnormalities associated with COVID-19 [12–15] revealed some paradigmatic patterns of laboratory biomarkers in patients with severe or fatal COVID-19, thus highlighting the role and function of well-known plasma biomarkers (e.g., cardiac troponins, C-reactive protein, cytokines and a plethora of metabolites), but also focusing attention on the pivotal role of excessive NET formation during COVID-19 progression, a process significantly contributing to the immuno-thrombotic state.

Over the past decades, several studies revealed a pathogenic role of NETs besides COVID-19 [4,16], encompassing various human diseases such as thrombo-inflammatory states, sepsis, trauma, lung, kidney, and nervous system injuries, cancer, and atherosclerosis, etc. [17–20]. The biomolecular characterization of NETs (NETome) identifies their

main composition as an extracellular network of DNA, oxidant, and proteolytic enzymes of both cytosolic and granular origin, such as neutrophil elastase (NE), Myeloperoxidase (MPO), peptidyl arginine deiminase 4 (PAD4), cathepsin G, gelatinase, lysozyme C, leukocyte proteinase 3, lactoferrin, defensins, calprotectin, cathelicidins, HMGB1, actin and histones [3,21]. The release of these mediators, when not physiologically and finely regulated, has the potential to initiate and propagate inflammation and thrombosis, thus leading to both increased disease severity and shortened patient survival [18].

A plethora of recent studies underlining the roles and functions of extracellular histones as biomarkers for predicting outcomes of several human diseases have also been published [22,23]. Recent evidence especially demonstrates that the levels of NETs and histones may predict the cardiovascular risk [7], wherein circulating histones may function as signaling scaffold at the culprit site of myocardial infarction and stroke [24–28], also in COVID-19 patients with cardiac manifestations.

Interestingly, a recent study had first demonstrated that circulating histones play a crucial role in COVID-19-associated coagulopathy and mortality [29]. This outstanding and elegant research sheds light on the significant correlation between plasma histone levels and severity of COVID-19 infection, highly associated with severe coagulopathy, inflammation, and cardiac injury. In particular, the plasma levels of cardiac troponin were found to correlate with histone levels and were found to be significantly higher in COVID-19 patients who died compared to those who survived (median circulating histone levels in non-survivors vs. survivors: 29.6 μg/mL vs. 8.6 μg/mL, $p = 0.002$) [29], thus confirming literature data on both cytotoxic effects of extracellular histones on cardiomyocytes [25], and non-necrotic cardiac troponin release in COVID-19 patients [30].

Histones (i.e., positively charged multifunctional nuclear proteins) are key components in chromatin functions, which bind to the nucleosomal core particle around the DNA entry and exit sites. These intriguing molecules may be significantly released in body fluids during several targeted organ injuries (e.g., thrombosis, cancer, sepsis, etc.), thus mediating inflammatory pathways and coagulative cascade crucially linked to severity and mortality of many human pathologies [23,31].

All these observations suggest that injuries to the heart tissue caused directly by SARS-CoV-2 and/or indirectly by the release of histones SARS-CoV-2-related can be underlying causes of heart diseases (e.g., myocarditis and myocardial ischemia) in COVID-19 [25,29,32,33].

Our focused literature overview suggests that circulating extracellular histones may be significantly linked to cardiac injuries (at both cell- and tissue-level) [32], which are frequently reported in COVID-19 patients [33]. Thus, the well-known pro-inflammatory, pro-coagulant and cytotoxic functions of extracellular histones (released by NETs and nucleosome, acting as cytotoxic danger-associated molecular pattern, DAMP) [23,31] may represent an intriguing biomolecular mechanism that actively contributes to worsening the clinical course of COVID-19 and amplifying the risk of adverse outcomes [34].

In fact, histones exert endothelial and epithelial cytotoxicity interacting with both cell membrane phospholipids and cell membrane receptors (e.g., Toll-like Receptors, TLRs) and complement, thus promoting pro-inflammatory cytokine and chemokine release via MyD88, NFkB, and NLRP3 inflammasome-dependent pathway. Furthermore, histones could activate platelets, may bind red blood cells and increase their fragility, inducing phosphatidylserine exposure, finally promoting the development of micro-thrombi [29,31,34].

Moreover, they could be seen as a novel biomarker, which could assist risk stratification in patients with COVID-19 [29] and serve as a predictive factor for cardiac and lung injury/dysfunction, and ultimately are useful for individual management of the anticoagulant/anti-platelet therapy [35].

As concerns treatment options for COVID-19 coagulopathy [10], many studies have highlighted an urgent need to search pharmacological agents with endothelial-protective, histone-neutralizing properties and target histone removal in COVID-19 patients [36,37]. In particular, treatment of COVID-19 by heparins and heparinoids demonstrated their

beneficial roles through complex biomolecular networks, based on both non-anticoagulant and anticoagulant mechanisms [38–41].

A recent medical hypothesis has also suggested that polycations (e.g., histones secreted by neutrophils following COVID-19) may worsen viral infections, that may be mitigated/counteracted by administration of negatively charged polyanionic drugs (e.g., heparins and heparinoids) [42].

According to this interesting hypothesis, in a frame of our studies, we observed a significantly different in vitro modulation of whole blood histone-induced inflammation and coagulation by several synthetic and natural heparins and heparinoids (glycosaminoglycan-based commercially available drugs) frequently used for COVID-19 treatment [10,37,39] (as recommended by WHO; https://www.who.int/publications/i/item/clinical-management-of-covid-19, accessed on 5 February 2022), such as unfractionated heparin, low molecular weight heparins (LMWH), danaparoid, fondaparinux, and sulodexide.

In addition to their well-known roles and functions as anticoagulants and pro-fibrinolytic compounds, their peculiar high negative charge density allows them to bind and strongly interact with several proteins and proteinases, revealing anti-inflammatory, anti-complement, immunomodulatory and anti-viral activities, independently to anticoagulant properties [38–41,43–48]. In particular, we have preliminarily found that the heparin and heparinoid formulations possess significantly different anti-inflammatory abilities and capabilities to bind/precipitate histones, and so ultimately to prevent histone-mediated cytotoxicity (unpublished observations).

Moreover, a recent report demonstrated that other polyanions (such as oligonucleotides mixture defibrotide) [45] might act as histone-neutralizing agents, thus blocking their pathological effects and protecting the endothelium from thrombo-inflammation.

Noteworthy, no studies are available on the role of heparins/heparinoids as histone-neutralizing agents in COVID-19 patients, despite the discovery of a novel role for histones in COVID-19 patients [29]. Interestingly, several clinical trials (currently more than one hundred studies registered in https://www.clinicaltrials.gov/ct2/results?cond=COVID-19&term=Heparin&cntry=&state=&city=&dist=, accessed on 21 March 2022) are describing the use and/or potential benefit of heparins in COVID-19, focusing attention on the anticoagulant effects of heparins in COVID-19 patients but neglecting the well-known non-anticoagulant biochemical property of heparin to prevent histone cytotoxicity [49].

All these observations suggest the urgent need to clinically evaluate the beneficial role of histone-neutralizing therapy by focused trials involving the interesting roles of polyanionic compounds as potential additional strategy for protecting tissues and organs from inflammatory, cytotoxic and procoagulant effects of circulating histones, implicated in myriad NET- and histone-accelerated disease states, and in COVID-19 complications (Figure 1).

Finally, in full agreement with the "multifactorial" definition of COVID-19 association with cardiac injury and multi-organ thrombo-inflammation [9], besides the imaging abnormalities and the systemic metabolic perturbations (e.g., hyper-inflammation and immuno-thrombosis), we will need to focus translational research and clinical trials to novel emerging laboratory biomarkers, such as circulating histones, which may induce multi-organ deleterious effects, explaining SARS-CoV-2 tropism and helping to refine cardiovascular and systemic risk stratification along with clinical management of COVID-19 patients [29,50] (Figure 1).

This landscape encourages the search for other pharmacological agents [38,48], also with endothelial-protective and histone-neutralizing properties in COVID-19 patients (e.g., apolipoprotein A–I, activated protein C, thrombomodulin, recombinant anti-histone IgG, peptidylarginine deiminases inhibitors, etc.) [51–54], but also to develop a circulating histone sensitive assay for laboratory medicine for better stratifying the risk of COVID-19 patients (as well as for sepsis-affected patients) [2,23].

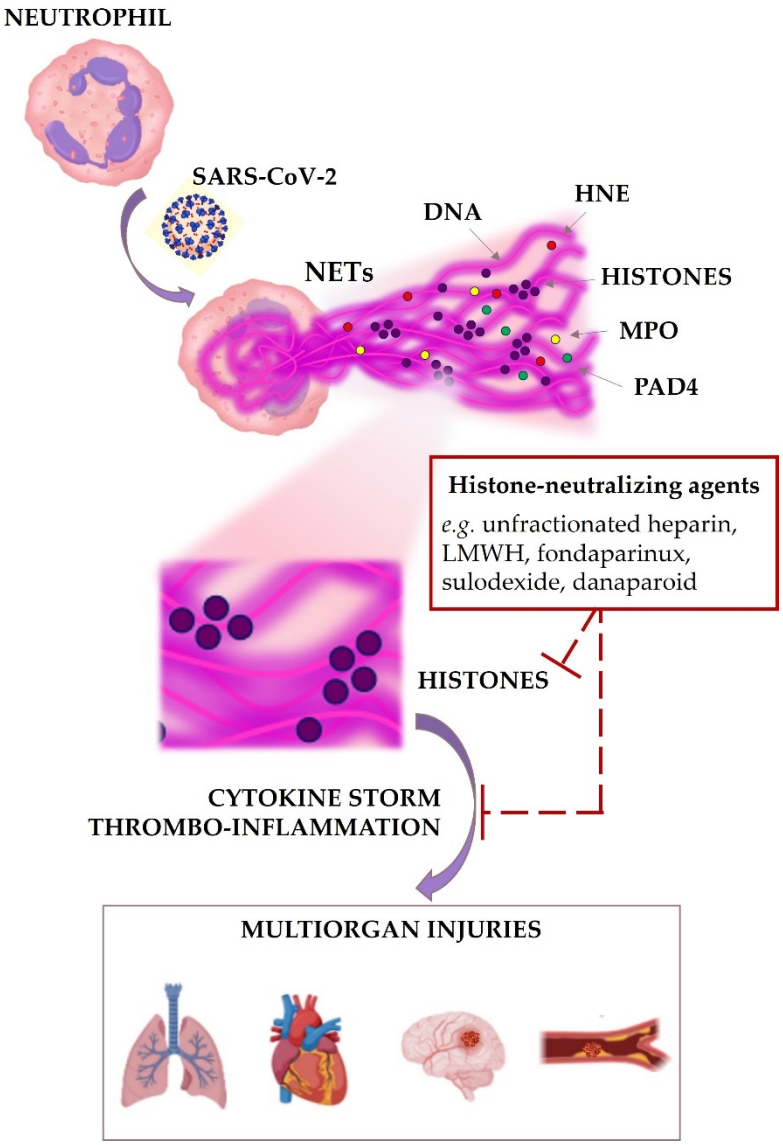

Figure 1. Histones as missing link among COVID-19 infection and multiorgan injuries. Among the main NET biomolecules, we focused attention on pathogenetic mechanisms of excess of histones potentially involved in multiorgan failure, coagulopathy, and systemic hyperinflammation during COVID-19 infection, and their possible therapeutic modulation by clinically used histone-neutralizing drugs (e.g., heparin and heparinoids). (NETs, neutrophil extracellular traps; DNA, deoxyribonucleic acid; HNE, human neutrophil elastase; MPO, myeloperoxidase; PAD-4, peptidylarginine deiminase-4; LMWH, low molecular weight heparins; HISTONES, positively charged multifunctional nuclear proteins).

All these promising future approaches reinforce the indefeasible urgent need for widespread (universal) vaccination against COVID-19, the primary strategy for lowering severity, morbidity, and mortality rate, and for limiting the diffusion and effectively protecting against COVID-19 variants.

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CHAPTER 3

Circulating histones contribute to monocyte and MDW alterations as common mediators in classical and COVID-19 sepsis

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BRIEF REPORT

Open Access

Circulating histones contribute to monocyte and MDW alterations as common mediators in classical and COVID-19 sepsis

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Abstract

Objective: Histone proteins are physiologically involved in DNA packaging and gene regulation but are extracellularly released by neutrophil/monocyte extracellular traps and mediate thrombo-inflammatory pathways, associated to the severity of many human pathologies, including bacterial/fungal sepsis and COVID-19. Prominent and promising laboratory features in classic and viral sepsis emphasize monocyte distribution width (MDW), due to its ability to distinguish and stratify patients at higher risk of critical conditions or death. No data are available on the roles of histones as MDW modifiers.

Design: Comparison of MDW index was undertaken by routine hematology analyzer on whole blood samples from patients with COVID-19 and Sepsis. The impact of histones on the MDW characteristics was assessed by the in vitro time-dependent treatment of healthy control whole blood with histones and histones plus lipopolysaccharide to simulate viral and classical sepsis, respectively.

Measurements and main results: We demonstrated the breadth of early, persistent, and significant increase of MDW index in whole blood from healthy subject treated in vitro with histones, highlighting changes similar to those found in vivo in classic and viral sepsis patients. These findings are mechanistically associated with the histone-induced modifications of cell volume, cytoplasmic granularity and vacuolization, and nuclear structure alterations of the circulating monocyte population.

Conclusions: Histones may contribute to the pronounced and persistent monocyte alterations observed in both acute classical and viral sepsis. Assessment of the biological impact of circulating histone released during COVID-19 and sepsis on these blood cells should be considered as key factor modulating both thrombosis and inflammatory processes, as well as the importance of neutralization of their cytotoxic and procoagulant activities by several commercially available drugs (e.g., heparins and heparinoids).

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Keywords: Histones, COVID-19, Sepsis, Critical care, Monocyte distribution width, Monocyte, Biomarkers

Introduction

Histones are key components for chromatin physiological functions but extracellularly mobilized during pathological processes [1]. Acting as endogenous damage-associated molecular pattern molecules, histones mediate both inflammatory pathways and coagulative cascade linked to the severity of several pathologies, including Sepsis and COVID-19 [2–6]. In fact, histones interact with blood cells (e.g., monocytes and platelets) promoting cytotoxicity, inducing phosphatidylserine exposure, modulating Toll-like receptors, releasing pro-inflammatory cytokine/chemokines and activating the coagulative cascade [1].

Laboratory findings in COVID-19 diagnosis and prognosis [7, 8] highlighted that leukocyte extracellular traps (including histones, extracellular DNA, oxidant and proteolytic enzymes) emerged as diagnostic/prognostic markers in COVID-19 [3, 9], actively participating in both cytokine storm and coagulation dysfunctions [1]. Recently, circulating histones were emphasized as predictive biomarkers [1] in patients with severe COVID-19 "viral Sepsis" [10], acting as sub-lethal signaling molecules and inducing cytokine storm [9].

Among common laboratory biomarkers shared by SARS-CoV-2 and Sepsis infections [7, 11], the modification of the hematological parameter Monocyte Distribution Width (MDW) predicts both multiorgan failure and increased mortality rate in Sepsis conditions [11]. MDW index (FDA-approved, EC-marked early Sepsis indicator of monocyte heterogeneity upon massive inflammatory activation [11]) is further recognized as diagnostic/prognostic marker for COVID-19 severity and clinical outcomes, as a kind of novel viral Sepsis biomarker [12–14].

Researches had linked MDW index to both COVID-19 and Sepsis [11], as well as studies have associated Sepsis and COVID-19 to histone levels [6, 15]; curiously, no data are currently available on histones as MDW modifiers. These bases raised the possibility that histones may contribute to the activation and morphological dysregulation of monocytes in both COVID-19 and Sepsis infections [4, 16]. With our whole blood in vitro model, we investigated the ability of histones to modify monocyte morphology and MDW index. We further compared these in vitro modifications to those measured in COVID-19 and Sepsis patients.

Materials and methods

Healthy subjects were voluntarily recruited among staff at the Dept BiND of University of Palermo and Dept DISB of University of Urbino. MDW values and clinical data for both COVID-19 ($n=7$, age range 52–85 years) and Sepsis ($n=8$, age range 47–81 years) patients were extracted by data archives of the University Hospital of Palermo.

The cut-offs reported for both MDW index and histone values are in agreement with literature references (healthy control subjects [5, 6, 11, 12, 15]; COVID-19 patients [12, 14, 15]; and Sepsis patients [5, 6, 11]).

Our non-interventional in vitro study was in accordance to the Declaration of Helsinki principles, peripheral venous whole EDTA blood samples were collected from healthy volunteers ($n=6$, mean age 48.5 ± 15 years, range 31–63 years). Routine complete blood cell counts were performed on *UniCell DxH900 Hematology Analyzer* (Beckman Coulter). Automated slide preparation (*unit SP-100, DI-60 system workflow*, Sysmex) was used to obtain May-Grunwald-Giemsa-stained blood smears.

Statistical analyses

All statistical tests were performed using GraphPad Prism 9.0. Values are expressed as mean ± standard error (SEM) and p values <0.05 were considered significant. Unless otherwise specified, significant differences between groups were determined using one-way ANOVA followed by post-hoc test (i.e., Tukey's multiple comparison test). Regression analyses were performed through simple linear regression.

Results

We performed 93 MDW measurements on healthy blood samples before and after in vitro histone treatments. Firstly, based on the laboratory dataset on COVID-19 patients at hospital admission, we observed a mean MDW value of 25.58 ± 0.68, significantly higher compared to healthy subjects ($p<0.0001$) (Fig. 1A).

These finding are in agreement with literature observations (reviewed in Ligi et al.) [17], and are mainly linked to monocyte hyperinflammatory activation characterizing COVID-19 illness [16, 18].

In our series of classical Sepsis patients MDW levels were found significantly higher compared to the values observed in both healthy subjects and COVID-19 patients ($p<0.0001$) (Fig. 1A). Our results are in agreement with literature supporting the monocyte inflammatory processes in Sepsis patients caused by multiple bacteremia and associated with multiorgan failure and disease severity [6].

In our study, we treated in vitro whole blood samples with 100 µg/mL of a mixture of human histones to test

the impact of histone levels found in critical COVID-19; similarly, we tested 100 µg/mL of histone mixtures + 1 µg/mL of LPS for studying Sepsis condition.

We demonstrated that healthy whole blood treated for 3 h with histone and histone + LPS showed MDW levels significantly higher compared to controls ($p < 0.0001$) (Fig. 1A). In particular, we found that histone-induced MDW values overlapped those found in COVID-19 patients with moderate/critical infection. Likewise, histone + LPS treatment results in a MDW increase similar to that found in Sepsis-affected patients (Fig. 1C).

A time-dependent increase of MDW induced by histone treatments was revealed (Fig. 1B). Furthermore, significant linear regressions sustained the time-dependency of MDW changes induced by histone ($Y = 0.03751x + 18.06$, $r^2 = 0.6995$) and by histone + LPS ($Y = 0.06951x + 17.85$, $r^2 = 0.9317$).

In our time-course studies, MDW value of controls did not significantly change at RT within 3 h (Fig. 1B). Furthermore, after 30 min of histone treatment, we revealed a significantly different MDW compared to respective controls ($p = 0.0012$), whereas histone + LPS showed an extremely significant difference vs control ($p < 0.0001$) (Fig. 1B). At this short time of treatment, no difference was found between histone + LPS and histone alone (Fig. 1B). After 60 min of incubation, a significant difference between histone + LPS vs histone alone ($p = 0.0019$) and between histone versus controls ($p = 0.0065$) were observed (Fig. 1B). After 3 h of incubation, extremely significant differences ($p < 0.0001$) were found among all treatments and vs controls (Fig. 1B).

No statistical difference was found in MDW values obtained between in vitro histone treatment and in vivo COVID-19 "*viral Sepsis*" infection; as well, no statistical difference between in vitro histone + LPS and in vivo bacterial/fungal "*classical Sepsis*" was observed (Fig. 1A, C).

Discussion

For the first time, we demonstrated that histone concentrations similar to those found in critical COVID-19 condition [15], as well as in classical Sepsis [5, 6], are able to induce significant morphological modifications and a time-dependent increase of MDW value, related to monocyte heterogeneity and inflammatory activation [17], characteristics of both SARS-CoV-2 infection [18] and critical Sepsis condition [2, 11] (Fig. 1D).

Noteworthy, our in vitro whole blood experimental model demonstrated significant alterations of MDW values among treatments, but without significant modifications of both number and percentage of monocyte population up-to 3 h (data not shown). In full agreement with literature data, our results on histone-induced MDW modifications sustain the deleterious role of extracellular histones, which promote the monocyte-linked inflammatory processes, worsening the disease severity of both Sepsis and COVID-19 [2, 16].

The MDW index is based on specific positional parameters using simultaneously three independent energy sources: *direct current impedance,* to measure cell volume of cell types; *radio frequency opacity*, to characterize conductivity for internal composition of each cell; a *laser beam,* to measure light scatter for cytoplasmic granularity and nuclear structure [13]. The resulting MDW value quantitatively detects morphologic changes in reactive/activated monocyte cells, similarly to qualitative microscopic evaluation of a peripheral blood smear. In agreement with literature data, we found in healthy untreated

(See figure on next page.)

Fig. 1 MDW index modifications in whole EDTA blood samples collected from healthy subjects treated in vitro with 100 µg/mL of histone mixture and 100 µg/mL of histone mixture + 1 µg/mL of LPS, compared to COVID-19 and Sepsis profiles, and mechanistic network of histone actions in sepsis. Sepsis patients was categorized according to Sepsis-2/3 diagnostic criteria; $n = 8$, mean age 63 ± 13.2 years; median SOFA score of 3, range 2–7; no patient needed for mechanical ventilation or continuous renal replacement therapy. COVID-19 patients had mild/moderate SARS-CoV-2 infection; $n = 7$, mean age 68 ± 14.4 years; no patient needed for mechanical ventilation. Aliquots of 1 mL of whole blood from each volunteer were exposed to a mixture of commercially available histones (100 µg/mL) (Histone from calf thymus, Sigma), in absence or presence of 1 µg/mL of lipopolysaccharide (LPS) (from Escherichia coli O127:B8 strain, Sigma). The samples, maintained at RT, were analyzed for MDW at 30, 60 and 180 min after careful inversion avoiding sedimentation of blood cells, and processed within 4 h of collection. MDW and routine complete blood cell counts were performed on *UniCell DxH900 Hematology Analyzer* (Beckman Coulter). The choice of whole blood treatment with 100 µg/mL of a mixture of commercially available human histones is in agreement with the literature evidence suggesting that the concentration of 20 µg/mL of circulating histone H3 was detected in patients with critical COVID-19 [15] and that the same deleterious effects of histone H3 is obtained with five-fold higher concentrations of mixture of histones [3]. The MDW values, scatter plots and blood smears are representative of at least triplicate analyses. Values are plotted as mean ± SEM (**very significant = *p:* 0.001–0.01; ****extremely significant = $p < 0.0001$). **A** MDW modifications after histones and LPS + histone treatments for 3 h in control subjects compared with classical and viral Sepsis. **B** Time-dependent increases of MDW values (linear regressions: control subjects, $Y = 0.01029x + 17.45$ $r^2 = 0.4065$; histone 100 µg/mL, $Y = 0.03751x + 18.06$ $r^2 = 0.6995$; 100 µg/mL of histone mixture + 1 µg/mL of LPS, $Y = 0.06951x + 17.85$ $r^2 = 0.9317$). **C** Representative modifications of MDW, blood smears and scatter plots in both classical and viral Sepsis, compared to histone and histone + LPS whole blood treatments. **D** Schematic representation of a possible predictive/mechanistic network of how circulating histones commonly mediate monocyte alterations in both classic and viral sepsis (METosis, monocyte extracellular traps; TLR, Toll-like receptor; NLRP, NOD-like receptor protein; LPS, lipopolysaccharide; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; MyD88, myeloid differentiation primary response gene 88; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells)

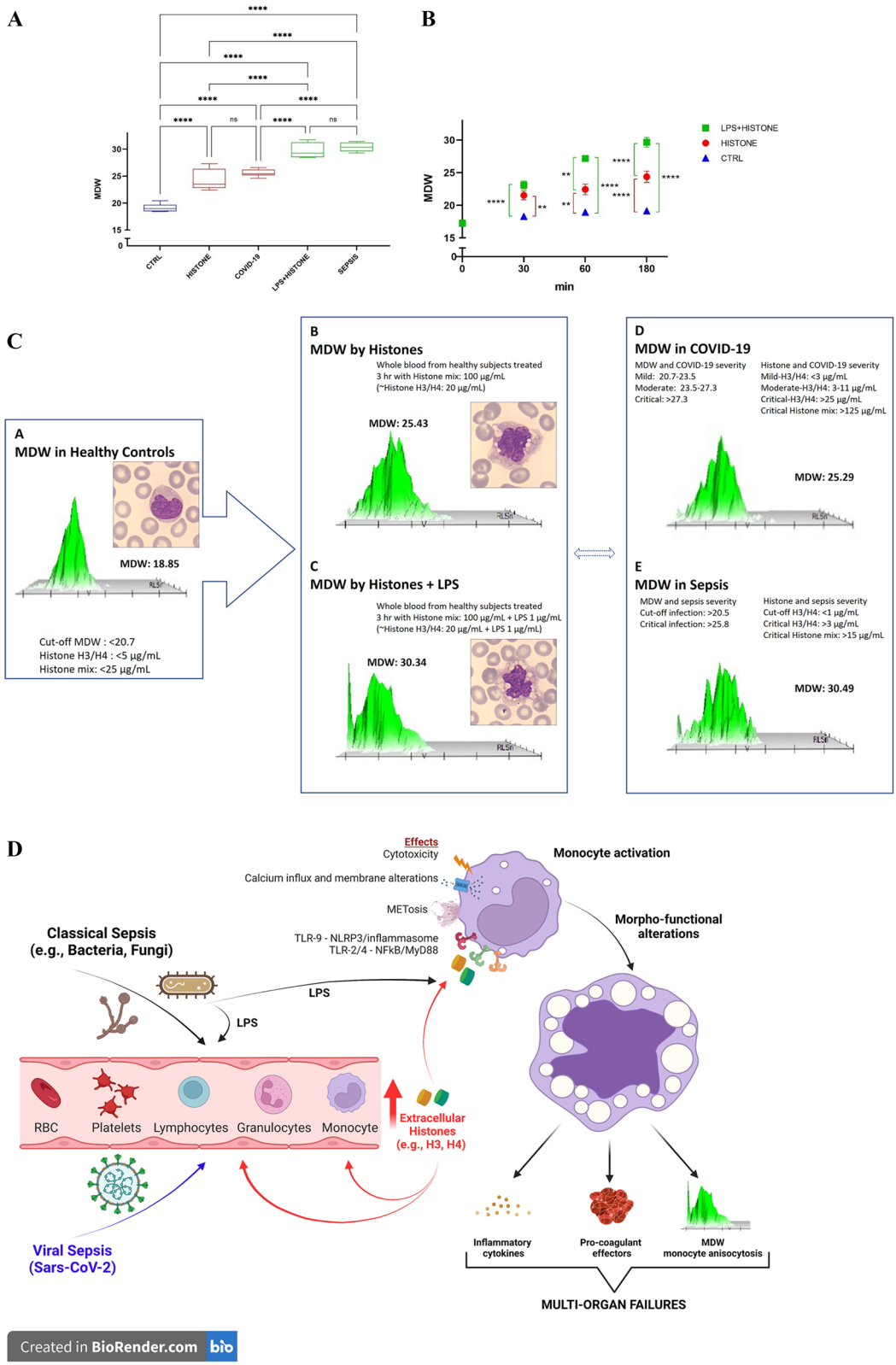

Fig. 1 (See legend on previous page.)

controls a MDW index < 20.7 associated with normal morphological features of monocyte in blood smears; moreover, the homogeneity of monocyte populations in controls is highlighted through the innovative scatter plot (Fig. 1C, inset A). Comparing COVID-19 data with in vitro results of histone, we found similar scatter plots and overlapping MDW values, suggesting a closely associated monocyte heterogeneity (Fig. 1C, inset B vs. D), significantly different from controls. Likewise, in vitro histone + LPS treatments revealed scatter plots and MDW values overlapping to the features of in vivo Sepsis (Fig. 1C, inset C vs. E), extremely different from control values.

Interestingly, the comparison among controls vs histones vs histone + LPS revealed a significantly different profiles of monocyte heterogeneity, scatter plots and MDW values, sustained also by enhanced volume, intracellular vacuolization and granularity, membrane alterations and nuclear structure changes, as observed through blood smears (Fig. 1C, inset A vs. B vs. C).

Being up to 30–50% of Sepsis as culture negative, MDW and histone assay may provide additional clinical laboratory tools defining the classical and viral Sepsis conditions. Since these assays could not be hindered by possible limitations/bias (e.g., hemodilution; monocytopenic conditions; neither classic nor viral Sepsis showed low monocyte counts), both parameters may be routinely determined [7, 11].

Moreover, recent observations suggest possible therapeutic approaches with polyanions (e.g., heparins, heparinoids) [10] as potential strategies for protecting tissues from histone-induced inflammation/thrombosis [5, 19].

A possible limitation of our study may be linked to the lack of our analyses of histones in COVID-19 and Sepsis patients, due to the retrospective nature of this study.

Finally, although our findings were obtained in vitro in healthy subjects, we demonstrated that histones significantly affect monocytes, mechanistically acting as endogenous MDW modifiers and mirroring MDW features clinically observed during Sepsis (Fig. 1D).

Evaluations of further cellular/biochemical targets of histones (e.g., inflammatory and proteolytic pathways, and circulating blood proteins) in whole blood model is currently ongoing.

Conclusions

We demonstrated that circulating histones represent one common mediator of monocyte alterations in both classic and viral Sepsis. We suggest MDW values and scatter plots as additional laboratory tools to simultaneously detect the monocyte volume, cytoplasmic granularity, and nuclear structure changes, paving the way for an early identification of enhanced monocyte heterogeneity in patients at higher risk of severe classical and viral Sepsis.

Abbreviations

COVID-19: Coronavirus disease 2019; ET: Extracellular trap; LPS: Lipopolysaccharide; MDW: Monocyte distribution width; MOF: Multiorgan failure; SARS-CoV-2: Severe acute respiratory syndrome-CoronaVirus-2.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13054-022-04138-2.

Additional file 1: **Table S1** Comparison of MDW characteristics between healthy subjects after 3 h of in vitro treatment and in vivo patients affected by classic and viral Sepsis. **Table S2** Time-dependent MDW modifications obtained in healthy whole blood samples after in vitro treatments.

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This work uses data obtained by healthy volunteer subjects (research staff and volunteer medical students) collected at the Dept of BiND and DISB, University of Palermo and Urbino, respectively, as part of their care and healthy bio-humoral checks. We are extremely grateful to the generosity of the participants for their individual contributions in these difficult times.

Authors' contributions

DL and FM designed the conceptualization of the current study; DL, BLS, LA,RVG, RM and CD performed experiments and data analysis; DL, BLS, RVG, LA, RM and CDF performed figure visualization; BLS, LA, RVG carried out data curation and retrospective extractions from laboratory archives/database; DL and FM had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis; DL and CDF performed statistical analyses; FM, DL and MC prepared and wrote original manuscript; FM, DL and MC edited and reviewed the final version; FM supervised the work. All authors have read, edited, and approved the final manuscript.

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Availability of data and materials

The dataset used in this paper are not publicly available since they are still under elaboration for publication by the Authors but are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of University of Palermo (protocol: 07/2019). This work uses data obtained by healthy volunteer subjects (research staff and volunteer medical students) collected at the Dept of BiND and DISB, University of Palermo and Urbino, respectively, as part of their care and healthy bio-humoral checks. The need for informed consent from individual patients was waived owing to the retrospective nature of the study. In fact, clinical data for both COVID-19 and Sepsis patients were retrospectively collected by review and extraction medical data and laboratory test results from electronic health records of University Hospital Lab Med Dept of Palermo. All investigations have been conducted according to the Declaration of Helsinki principles. In line with non-interventional retrospective design of this in vitro study, MDW assessments were performed on volunteers without clinical indications and no clinical decisions were made based on MDW values.

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

ADDITIONAL FILE

Table S1: Comparison of MDW characteristics between healthy subjects after 3 h of *in vitro* treatment and *in vivo* patients affected by classic and viral Sepsis.

	Mean±SD	Median	Min-Max	25°-75° percentile	CV%	p
CTRL 3h	19.13±0.77	18.99	18.42 – 20.45	18.50 - 19.68	4.0	---
HISTONE	24.37±1.97	23.52	22.43 – 27.31	22.82 - 26.36	8.1	p < 0.0001 (vs. CTRL)
COVID-19	25.58±0.68	25.43	24.60 – 26.56	25.16 – 26.19	2.6	p < 0.0001 (vs. CTRL) n.s. (vs. HIS)
LPS+HISTONE	29.68±1.51	29.27	28.44 – 31.74	28.50 - 31.27	5.1	p < 0.0001 (vs. CTRL) p < 0.0001 (vs. HIS)
SEPSIS	30.40±0.80	30.39	29.29 - 31.41	29.63 – 31.17	2.6	p < 0.0001 (vs. CTRL) n.s. (vs. LPS+HIS) p < 0.0001 (vs. COVID-19)

n.s. = Not significant

Table S2: Time-dependent MDW modifications obtained in healthy whole blood samples after *in vitro* treatments

	Mean±SD	Median	Min-Max	25°-75° percentile	CV%	p
CTRL 0 min	17.27±1.19	17.40	15.4 – 18.95	16.38 - 18.05	6.9	---
CTRL 30 min	18.28±0.97	18.23	16.64 – 19.38	17.71 - 19.19	5.3	n.s. (vs CTRL)
CTRL 60 min	18.93±0.52	18.93	18.42 – 19.42	18.45 - 19.40	2.8	n.s. (vs CTRL)
CTRL 180 min	19.13±0.77	18.99	18.42 – 20.45	18.50 - 19.68	4.0	n.s. (vs CTRL)
HISTONE 30 min	21.53±1.69	21.80	19.07 – 23.12	20.08 - 23.01	7.8	p < 0.0001 (vs CTRL)
HISTONE 60 min	22.46±1.65	22.95	20.09 – 23.84	20.74 - 23.69	7.3	p < 0.0001 (vs. CTRL) n.s. (vs. HIS 30 min)
HISTONE 180 min	24.37±1.97	23.52	22.43 – 27.31	22.82 - 26.36	8.1	p < 0.0001 (vs. CTRL) p = 0.0133 (vs. HIS 30 min) n.s. (vs. HIS 60 min)
LPS+HISTONE 30 min	23.11±1.27	23.33	21.49 – 24.27	21.80 - 24.19	5.5	p < 0.0001 (vs CTRL)
LPS+HISTONE 60 min	27.19±0.08	27.19	27.13 – 27.24	27.13 - 27.24	0.3	p < 0.0001 (vs. CTRL) p = 0.0129 (vs. LPS+HIS 30 min)
LPS+HISTONE 180 min	29.68±1.51	29.27	28.44 – 31.74	28.50 - 31.27	5.1	p < 0.0001 (vs. CTRL) p < 0.0001 (vs. LPS+HIS 30 min) n.s. (vs. LPS+HIS 60 min)

n.s. = Not significant

CHAPTER 4

Conclusions

Innate immune cells such as neutrophils, basophils, eosinophils, monocytes, and monocyte-derived macrophages are key players in the host's defense systems against invasive pathogens. One of the defense mechanisms put in place by these cells is the fascinating ETosis process [36]. During ETosis, stimulated cells release into the extracellular environment mesh-like structures called ETs, which consist of decondensed chromatin fibers decorated with nuclear, cytoplasmatic, and granular proteins [43]. Several pieces of evidence suggest that ETs can act as a double-edged sword. On the one hand, they participate in a wide range of host defense processes; on the other hand, ETs contribute to the onset or worsening of several human diseases, including cancer, thrombosis, systemic lupus erythematosus, diabetes, hyperinflammation, and rheumatoid arthritis [9-11, 45]. The major protein fraction of ETs is represented by histones. These markers when excessively or dysregulated released acquire an important pathophysiological significance by acting as damage-associated molecular pattern molecules. From a literature overview has emerged that elevated circulating histones levels correlate with increased severity of disease and shorter patient survival. In fact, extracellular histones by interacting with cell membrane phospholipids and cell membrane receptors exert an endothelial and epithelial cytotoxic effect, induce platelet activation and aggregation, erythrocyte fragility, and MyD88, NF-kB, and NLRP3-inflammasome pathways activation, finally promoting the massive release of proinflammatory mediators also known as cytokine storm phenomenon [4, 52, 53, 55, 56]. Concerning the pro-inflammatory effect of extracellular histones, we have demonstrated using an *ex vivo*

human whole blood assay that increasing concentrations of histone, similar to those found in COVID-19 patients [65], were able to promote a dose-dependent secretion by blood cell populations of a wide panel of proinflammatory biomarkers. In view of the negative contribution of free histones in the exacerbation of inflammatory responses, we then demonstrated the different ability of four heparin variants as UFH, Enoxaparin, Sulodexide and Fondaparinux, to counteract this deleterious role of histones. In agreement with literature data, we observed that heparins and heparinoids independently of their anticoagulant properties were able to attenuate or modulate the histone-induced cytokine storm, probably due to an electrostatic interaction between the negative charge of the heparins and the positive charge of histone proteins [81, 83, 90, 92, 93]. Furthermore, we hypothesized that the different anti-histone effect of heparins observed could be due to their different degree of sulfation, size and complexity of their glycosaminoglycan nature. The current understanding of the pivotal impact of free histones on adverse immune and inflammatory responses has aroused increased attention, especially in the context of recent COVID-19 and sepsis [5]. In fact, data obtained in both hospitalized COVID-19 and septic patients, have revealed elevated serum cytokine and chemokine levels, as well as the involvement of multiple organ systems. These findings prompted the scientific population to introduce the terms of viral sepsis and classical sepsis [61]. In this context, we demonstrated for the first time that these two life-threatening illnesses also share persistent and significant morphological alterations in monocytic cell populations mirrored to the increased values of MDW hematological parameter. In particular, we found in vitro that histones, similarly to what observed in *in vivo* critical viral as well as classical sepsis infection, significantly influence the morpho-functional features of circulating monocytes, acting as pivotal endogenous MDW modifiers. The association between blood histone values and adverse clinical outcomes in patients with SARS-

CoV-2 infection and classical sepsis strongly suggests histones as an enhancer of disease severity mainly due to the activation of inflammatory cytokine storm and the multiorgan failure. The evaluation of MDW blood monocyte morphological index [73, 75] could represent therefore an important early diagnostic and prognostic tool to distinguish and stratify patients at higher risk of critical conditions or death, reflecting the state of activation of innate immunity as a consequence of toxic histone stimulus. Furthermore, the assessment of MDW in correlation with circulating histone levels could represent a promising strategy for the more accurate choice of histone neutralizing therapy, e.g., heparins and heparinoids, dependently on disease progression, thus blocking or delaying the unfavorable disease progression.

CHAPTER 5

Additional Papers

This chapter consists of the papers published during my three years of research Doctorate program.

The arguments of these articles have no bearing on the topic of the thesis.

Ligi D, Maniscalco R, Mannello F. MMP-2 and MMP-9 in Human Peripheral Blood: Optimizing Gelatinase Calibrator for Degradome Research and Discovering a Novel Gelatinolytic Enzyme. J Proteome Res. 2020 Jan 3;19(1):525-536. doi: 10.1021/acs.jproteome.9b00261. Epub 2019 Oct 30. PMID: 31612719.

Ligi D, Maniscalco R, Mannello F. New Frontiers for an Old Drug: What Is New on the Pleiotropic Effect of Sulodexide in Chronic Venous Disease. J Cardiovasc Pharmacol. 2020 Mar;75(3):208-210. doi: 10.1097/FJC.0000000000000799. PMID: 32040037.

Sharebiani H, Fazeli B, Maniscalco R, Ligi D, Mannello F. The Imbalance among Oxidative Biomarkers and Antioxidant Defense Systems in Thromboangiitis Obliterans (Winiwarter-Buerger Disease). J Clin Med. 2020 Apr 7;9(4):1036. doi: 10.3390/jcm9041036. PMID: 32272606; PMCID: PMC7231233.

Giglio RV, Lo Sasso B, Agnello L, Bivona G, Maniscalco R, Ligi D, Mannello F, Ciaccio M. Recent Updates and Advances in the Use of Glycated Albumin for the Diagnosis and Monitoring of Diabetes and Renal, Cerebro- and Cardio-Metabolic Diseases. J Clin

Med. 2020 Nov 11;9(11):3634. doi: 10.3390/jcm9113634. PMID: 33187372; PMCID: PMC7697299.

Raffetto JD, Ligi D, Maniscalco R, Khalil RA, Mannello F. Why Venous Leg Ulcers Have Difficulty Healing: Overview on Pathophysiology, Clinical Consequences, and Treatment. J Clin Med. 2020 Dec 24;10(1):29. doi: 10.3390/jcm10010029. PMID: 33374372; PMCID: PMC7795034.

Fazeli B, Ligi D, Keramat S, Maniscalco R, Sharebiani H, Mannello F. Recent Updates and Advances in Winiwarter-Buerger Disease (Thromboangiitis Obliterans): Biomolecular Mechanisms, Diagnostics and Clinical Consequences. Diagnostics (Basel). 2021 Sep 22;11(10):1736. doi: 10.3390/diagnostics11101736. PMID: 34679434; PMCID: PMC853504

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Table 2

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