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UNRAVELING MONOCYTE AND PLATELET MORPHO-FUNCTIONAL ALTERATIONS IN SEPSIS: EMERGING BIOMARKERS FOR EARLY DIAGNOSIS

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Contents

Preface

Sepsis represents one of the leading causes of morbidity and mortality worldwide, due to its unpredictable onset, complex pathophysiology, fast progression, and difficult diagnosis. During my three-years PhD research, I focused my project on identifying reliable biomarkers that could improve the early recognition and cellular and biochemical understanding of this multifaced syndrome.

Among these, Monocyte Distribution Width (MDW) represents a promising early marker of massive infection-related immune activation measuring monocyte anisocytosis. This study aims to lay the foundation for a deeper understanding of this biomarker, by exploring the mechanisms involved in the morphological and functional changes of monocytes during sepsis. Monocytes are not the only major actors in this PhD thesis; complementary analyses of neutrophils are also included for a broader cellular and immunological perspectives. These were obtained through the study of Cell Population Data (CPD), which provided additional insights into innate immune responses. Also platelets are key players in thrombo-inflammation, and some platelet indices are examined for their critical contribution in the early detection of sepsis.

Particular attention was also given to the role of extracellular histones, which are increasingly recognized as drivers of the inflammatory and procoagulant responses observed in sepsis. Histones are normally confined in the nucleus but during cell death, like necrosis, or during a process defined as NETosis, they are released in the extracellular space where they act as Damage-Associated Molecular Pattern Proteins (DAMPs), triggering a host defence response. Extracellular histones play cytotoxic effects on endothelial cells, promote platelet activation, monocyte morpho-functional alterations, Neutrophil Extracellular Traps (NETs) formation, inflammatory mediators' release, and other biomolecular effects that amplify the thrombo-inflammatory environment characterizing severe sepsis.

This PhD dissertation, structured in ten chapters, integrates original research articles with an introduction and a conclusive discussion, to provide a comprehensive exploration of the immunological characteristics of sepsis, hoping to contribute to its earlier recognition and improved management.

Chapter 1

General Introduction

Sepsis

Definition

Sepsis represents a critical medical emergency, one of the main causes of morbidity and mortality worldwide (1). The term “sepsis” was used for the first time in Homer’s poem about 2700 years ago, and it originates from the Greek language with the means of “decomposition”. Centuries later, Hippocrates and Galen also wrote about sepsis, but it was only with the “germ theory” in the 1800s that the involvement of microorganisms in this unknown and frightening syndrome was understood. Hugo Schottmüller, in 1914, introduced one of the earliest definitions of sepsis, writing that “the presence of sepsis occurs from a focus from which pathogenic bacteria invade the bloodstream, constantly or periodically, causing both subjective and objective symptoms”. This is a modern and forward-looking definition for its time, emphasizing the role of the microorganism and introducing a wide variety of signs and symptoms (2). The definition of sepsis has undergone a continuous process of revision, reflecting advances in clinical knowledge and diagnostic criteria over time. In 1991, the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) established the first consensus definition of sepsis, as Systemic Inflammatory Response Syndrome (SIRS) in response to a confirmed or suspected infection. SIRS is identified when patients exhibited at least two of the following criteria (Table 1): a body temperature above >38°C or below <36°C, heart rate exceeding >90 beats for minute, respiratory rate over 20 in one minute or $PaCO_2$ below 32 mmHg, and important leucocytes abnormalities including leucocytosis (>12,000/mm^3), leukopenia (<4000/mm^3), or increased band form (>10%) (3, 4). Severe sepsis is diagnosed when the inflammatory response is complicated by organ dysfunction, reduced tissue perfusion, or low blood pressure. If hypotension remains unresponsive to appropriate fluid resuscitation, the condition progresses to septic shock. (4, 5).

Table 1. SIRS Criteria (3).

SIRS Criteria (2 of the following)	Value
Heart rate	>90 bpm
Respiratory rate	>20 breaths in 1 min
Temperature	(>38°C or <36°C)
White blood cells count	(>12,000/mm^3, <4,000/mm^3, or >10% bandemia

In 2001, a second international consensus reviewed these definitions (Sepsis-2) and broaden the list of diagnostic criteria. However, no major modifications were introduced and SIRS criteria continued to be used in clinical practice for more than twenty years (6). Nonetheless, emerging evidence suggests limitations in this definition. For example, critically ill patients fulfil the SIRS criteria without having sepsis, while other patients with sepsis do not meet the SIRS threshold, raising concerns about the sensitivity and specificity of SIRS parameters (7). A new international task force formed by the SSCM and the European Society of Intensive Care Medicine (ESICM), redefined in 2014 the existing definition of sepsis and septic shock. They introduced substantial changes and these recommendations, published in 2016, are referred as Sepsis-3. This consensus removed the terms "Severe sepsis" and "SIRS", and, according to Sepsis-3 criteria, sepsis is actually defined as a "*Life-threatening organ dysfunction caused by a dysregulated host response to infection*" (3, 6, 7). Organ dysfunction is clinically identified by an increase in the Sequential Organ Failure Assessment (SOFA) score by ≥ 2 points, which has demonstrated stronger predictive value for in-hospital mortality compared to the SIRS-based approach. To assess the severity of the organ failure, the SOFA score evaluates dysfunctions across respiratory, cardiovascular, hepatic, coagulative, renal, and neurological systems (Table 2) (3).

Table 2. SOFA Score (3)

SOFA Score					
Organ System	0	1	2	3	4
Respiratory PO_2/FiO_2, mmHg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation platelets, $\times 10^3/mm^3$	≥150	<150	<100	<50	<20
Liver, bilirubin, mg/dL	<1.2	1.2-1-9	2.0-5.9	6.0-11.9	>12
Cardiovascular	MAP ≥70 mm Hg	MAP <70 mm Hg	Dopamine <5 or dobutamine (any dose) *	Dopamine 5.1-15 or epinephrine ≤0.1 or norepinephrine ≤0.1*	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1*
Central nervous system, Glasgow, Coma Scale	15	13-14	10-12	6-9	<6
Renal creatinine, mL/d	<1.2	1.2-1.9	2.0-3.4	3.5-4.9	>5.0
Urine output, mL/d				<500	<200

*Catecholamine doses are given as μg/kg/min for at least 1 hour.

FiO_2 fraction of inspired oxygen; MAP, mean arterial pressure; PO_2, partial pressure of oxygen.

Septic shock, following the Sepsis-3 consensus, is considered a subset of sepsis in which vasopressors are required, and serum lactate is higher than 2 mmol/L (4). The SSCM and the ESICM also proposed the quick SOFA (qSOFA) method, which is faster and easier to use than the SOFA method. It is composed of only three parameters and does not require laboratory testing (Table 3). A qSOFA score of two or more is associated with an increased risk of poor outcomes due to diffuse organ failure (3, 4).

Table 3. qSOFA criteria (3).

Criteria	Points
Respiratory rate ≥22/min	1
Change in mental status	1
Systolic blood pressure ≤100 mm Hg	1

The current definition of sepsis emphasizes the central role of the immune system, highlighting that it develops from an excessive and uncontrolled immune response which leads to immunosuppression (8).

Concluding, sepsis is characterized by the host's inability to maintain immune homeostasis in response to an infectious agent, resulting in an overwhelming inflammatory response followed by a state of immune suppression. This unbalanced reactivity and compromised capacity to counteract pathogens contributes to organ failure and, in the worst cases, death too (5).

Incidence, management and clinical presentation

Worldwide, the number of cases of sepsis and severe sepsis is 31.5 and 19.4 million per year, respectively (9). In 2020 Rudd, Kristina E et al. published in The Lancet the first global study about sepsis incidence, investigating 195 countries between 1990 and 2017. They concluded that 48.9 million cases of sepsis and 11 million sepsis-related deaths occurred in 2017. Notably, more than half of these cases were related to children and neonates. These results show a higher incidence than in previous studies due to the inclusion of low- and middle-income countries (10). Despite this evidence, data would still appear to be underestimated due to infectious disease like HIV, malaria, and respiratory infections that are common in resource-limited territories, contributing to sepsis rate. On the other hand, in developed countries there is an increase in the incidence of sepsis, but this is a result influenced by greater clinical attention and new diagnostic criteria. Therefore, the incidence varies based on which definitions and panel of biomarkers are used. Studies have shown that applying different methods to the same dataset can produce different results. Furthermore, financial incentives linked to diagnostic coding could influence the reported incidence, potentially leading to an overestimation of cases. Many studies excluded non-hospital cases, which contributes to a general underestimation. Finally, although sepsis is a serious health problem, the epidemiological data currently available is lacking in both consistency and geographical distribution (5). Certain is that sepsis represents one of the ten leading causes of death in high-income countries, resulting in a significant economic burden. In the USA, the annual cost of treating septic patients increased from $15.4 billion to $24.3 billion between 2003 and 2007 (4).

The risk of sepsis does not affect all age groups in the same way, showing a dual pattern: it is highest in infants and elderly, and lowest in young adults. Older adults are particularly susceptible due to the age-related decline in immune system function. The gender can also influence the incidence, females generally have lower rates of sepsis, possibly due to the protective effects of oestrogen on immunity and cardiovascular health. In contrast, male hormones may inhibit immune responses, increasing susceptibility. The influence of race on sepsis risk has been explored, but the findings remain inconclusive and elusive due to variations in diagnostic methodology and the challenges associated with studying different populations. The presence of comorbid conditions, such as renal disease, cancer, diabetes, chronic lung disease, heart failure and immunosuppression, increases the probability of developing sepsis. Finally, genetic variations can influence how individuals respond to infections or treatments related to sepsis by affecting drug metabolism or response (4).

In 2002, the ESICM, the SCCM and the International Sepsis Forum proposed the Surviving Sepsis Campaign (SSC), the first international guideline for the management of this complex syndrome. These guidelines have been revised several times, most recently in 2016, introducing a structured treatment protocols known as “sepsis bundles” (Table 4), aimed at promoting timely diagnosis and intervention (11). A fundamental early step in suspected sepsis or septic shock is the collection of microbiological samples before the antibiotic administration, as early antimicrobial therapy may reduce culture sensitivity and lead to false negative (12). These samples should be collected within 45 minutes of suspected sepsis to maximize diagnostic performance, and culture should be obtained from fluids like blood, urine, cerebrospinal fluid, respiratory secretions, wounds, or others (4, 11, 13). However, a standard culture method requires between 48 and 96 hours to obtain results, significantly delaying the initiation of the therapy (13). In this context, rapid diagnostic techniques such as Polymerase Chain Reaction (PCR) and Fluorescent In Situ Hybridization (FISH) have been shown to be very useful. PCR amplifies specific microbial DNA sequences and can detect resistance gene, and FISH uses fluorescent probe targeting ribosomal RNA (14). Despite their fast processing, these molecular tests require microbial growth in culture and are not useful for patients who have already started antibiotic therapy (15).This problem is solved by the mass spectrometry technologies like Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF MS) that allow identification of pathogens directly from positive culture (16). The most common pathogens implicated in sepsis are Gram-negative bacteria, first Escherichia coli, Gram-positive bacteria, especially Staphylococcus aureus, and fungi in immunocompromised patients (13).

Table 4: Sepsis bundles (4)

Surviving Sepsis Campaign Care Bundles	
To be completed in 3h	Measure lactate level Collect blood samples for cultures Administer broad spectrum antibiotics Administer fluid (crystalloid 30 mL/kg) in presence of lactate >4 mmol/L or Hypotension
To be completed in 6h	Administer vasopressors to maintain a mean arterial pressure >65 mmHg Measure central venous pressure (CVP) and central venous oxygen saturation in case of persistent hypotension or lactate >4 mmol/L Re-measure lactate if initial values were elevated

Despite advances in sepsis management, the early identification of infection and continuous monitoring of patients' conditions still remains a critical problem in clinical practice. Although various biomarkers are frequently used to support clinical decision, no one currently offer sufficient specificity to reliably distinguish sepsis from other inflammatory conditions. Nevertheless, they are commonly used to monitor the infectious process or to help rule out infections. The role of biomarkers in the context of sepsis is explored in a dedicated paragraph "Biomarkers of Sepsis".

The non-specific signs and symptoms of sepsis make early recognition of sepsis challenging. The type of symptoms (i.*e*, neurological, pulmonary, cardiovascular, renal, haematological, and hepatic) depends on which organ is most affected. Neurological signs include lethargy, confusion and delirium. The involvement of respiratory system is common, with an increase in breathing rate and possible respiratory failure, caused by damage to the lungs induced by inflammation. Cardiovascular effects include myocardial depression, with hypotension or shock, driven by inflammatory cytokines and oxidative stress. Renal failure is another critical complication, typically due to fluid loss and reduced perfusion. Moreover, sepsis can trigger Disseminated Intravascular Coagulation (DIC), which may present as either excessive bleeding or thrombosis depending on the balance between coagulation and fibrinolysis. Also liver dysfunction is frequent. Hepatic injury appears linked to reduced perfusion, leading to liver cell death (5). Effective sepsis management requires supportive treatment across all these systems, to reduce mortality, with three main priorities: controlling the infection, stabilizing circulation, and managing the whole-body response. A timely administration of a broad-spectrum antibiotic treatment is essential and should not be delayed while waiting for the microbiological results. Once the pathogen has been identified, the therapy should be adjusted to minimize the risk of toxicity, limit antibiotic resistance and reduce healthcare costs. Hemodynamic management consists in distinct phases, with fluid resuscitation and administration of vasoactive agents like norepinephrine. Respiratory support is frequently necessary, and lung-protective strategies are recommended. Despite improvements in this complex management, many survivors develop chronic critical illness (3, 5, 11). To improve the understanding of all these systems the following section explores the pathogenesis of sepsis.

Pathogenesis

As previously mentioned, bacteria are the most common microorganisms involved in sepsis. Viruses, fungi, and parasites can also cause sepsis, although less often, and in 30-50% of cases, the pathogen remains unidentified or unclear (17-19). All these microorganisms have different structures, but all share the expression of Pathogen-Associated Molecular Patterns (PAMPs) that allow them to be recognized by host immune system, triggering its activation. Once these PAMPs are detected and bound by immune cell surface receptors, a cascade of responses, that lead to the engulfment and destruction of invading bacteria as well as the removal of cellular debris from damaged tissues (19). As previously described, sepsis arises when this immune response becomes dysregulated, it is driven by the simultaneous recognition of both PAMPs and Damage-Associated Molecular Patterns (DAMPs), like histones, Adenosine triphosphate (ATP), uric acid, Deoxyribonucleic acid (DNA) or Ribonucleic acid (RNA), and High Mobility Group box (HMGB)-1 released by dead or damaged host cell (5) (2). They are detected by Pattern Recognition Receptors (PRRs) such as Toll-Like Receptors (TLRs), Nucleotide-binding oligomerization domain-like receptors (NOD)-like receptors (NLRs), Retinoic acid-inducible gene (RIG)-I-Like receptors (RLRs), C-type lectin receptors (CLRs) and cytosolic RNA and DNA sensors (19). After PAMP recognition, specific intracellular signalling start, leading to the activation of proteins such as Mitogen-Activated Protein Kinases (MAPKs), Janus Kinases (JAK), Signal Transducers and Activators of Transcription (STATs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) which drive the early transcription of inflammatory genes. They promote the expression of cytokines and chemokines, including Tumor Necrosis Factors (TNF), Interleukin 1 (IL-1), IL-12, IL-18 and Type I Interferons (IFNs), which in turn activate other mediators like IL-6, IL-8, IFNγ, and CC-chemokine ligand 2 (CCL2) but also CCL3, and CXC-chemokine ligand 10 (CXCL10) (5). In this scenario, also complement and coagulation pathways are activated, as well as the vascular endothelium (20). The complement system activation is a fundamental part of the innate immune system and initiates after the recognition of PAMPs and DAMPs, leading to the release of complement peptides, C3a and C5a. These molecules exert pro-inflammatory activity like recruitment and activation of leucocytes, endothelial cells and platelets (5, 19). C5a covers a central role binding to its receptors (C5aR1 and C5aR2) on innate immune cells promoting the recruitment of neutrophils, monocytes, and macrophages to the site of the infection. C5a also triggers the release of Reactive Oxygen Species (ROS) and proteolytic enzymes, which contribute to tissue damage (18), and stimulate the production of proinflammatory cytokines and chemokines amplifying the systemic inflammatory response (21). These effects contribute to the progression of sepsis and the inhibition of C5a has been shown to significantly improve outcomes in experimental animal models (22). Moreover, proteolytic enzymes are part of Neutrophil Extracellular Traps (NETs), web-like structures released by activated neutrophils with a role of defence. NETs immobilize invading microorganisms facilitating their clearance by immune cells, but when excessively extruded they can also damage tissue contributing to inflammation, thrombosis, and organ dysfunction, as in sepsis condition. Histones play a crucial role as components of NETs because, once in the extracellular space, they induce several pathological effects (19), as discussed in a the paragraph “Extracellular Histones:

definition, origin, and pathological effects”. Sepsis is also marked by a disruption of the coagulation balance, due to an excessive activation of procoagulant pathways alongside a suppression of anticoagulant mechanisms (19). There is a close relationship between coagulation and inflammation in sepsis, for example thrombin can stimulate the production of C3a and C5a, while C5a enhances Tissue Factor (TF) expression on endothelial cells. Moreover, coagulation factors can activate Protease-Activated Receptors (PARs) on immune cells amplifying inflammatory signalling (19).

Immune suppression

Sepsis-induced immunosuppression or “immune exhaustion” involves different cell types, and it’s implicated in the increased susceptibility of patients to secondary infections (18). Immunosuppression occurs to counteract the massive, uncontrolled and prolonged pro-inflammatory responses in septic patients. Anti-inflammatory mechanisms, in fact, attempt to regulate inflammation and maintain host homeostasis, but during sepsis this balance is broken (19). Immunosuppression is also characteristic of patients who survived sepsis, and the caption “Persistent Immunosuppression/Inflammation and Catabolism Syndrome” (PICS) is used to identify this condition. While the first and early inflammatory response is activated by PAMPs and DAMPs, as described before, PICS’ reason and mechanisms are unknown. DAMPs, continuously released by damaged tissues, or opportunistic infections have been proposed as potential driving mechanisms of PICS (5). Immunosuppression is characterized by several mechanisms, including the increased release of anti-inflammatory cytokines, the expansion of regulatory cells, and the loss of immune effectors cells (23). The most abundant cytokines released are IL-4, IL-10, and IL-37. IL-4, released by activated T cells and mast cells, leads during sepsis condition to the differentiation of naïve T cells to Th2 cells. Moreover, both IL-4 and IL-10 counteract the release of pro-inflammatory cytokines, like IL-2 and IFN-γ, through the inhibition of the differentiation of CD4+ T cells into Th1 cells (23). IL-10 covers the role of immunosuppressive cytokine also promoting the proliferation of immunosuppressive cells like Regulatory T cells (Tregs) and Myeloid-derived suppressor cells (MDSCs) (23). The immunosuppressive role of Tregs involves multiple mechanisms, including the release of anti-inflammatory cytokines (e.g. IL-10 and TGF-β), the upregulation of inhibitory receptors on effector cells, the epigenetic regulation of Foxp3, and metabolic shift toward oxidative phosphorylation. MDSCs inhibit both innate and adaptative immune responses, with an increase rate correlating with impaired T cell proliferation and reduced IL-2 production. MDSCs presence, if prolonged, may contribute to chronic immune dysfunction and high risk of secondary infections (23-25). In addition, sepsis alters the function of Professional Antigen-presenting Cells (APCs) downregulating the expression of HLA-DR, this reduced expression is consistently associated with immune dysfunction and poor prognosis (5, 23). The loss of immune effector cells can occur with different types of cell death, such as immunocyte apoptosis, immunocyte pyroptosis but also autophagy and ferroptosis. All these mechanisms contribute to sepsis-induced immunosuppression because of the reduction of immune cells (Figure 1) (18, 19, 23).

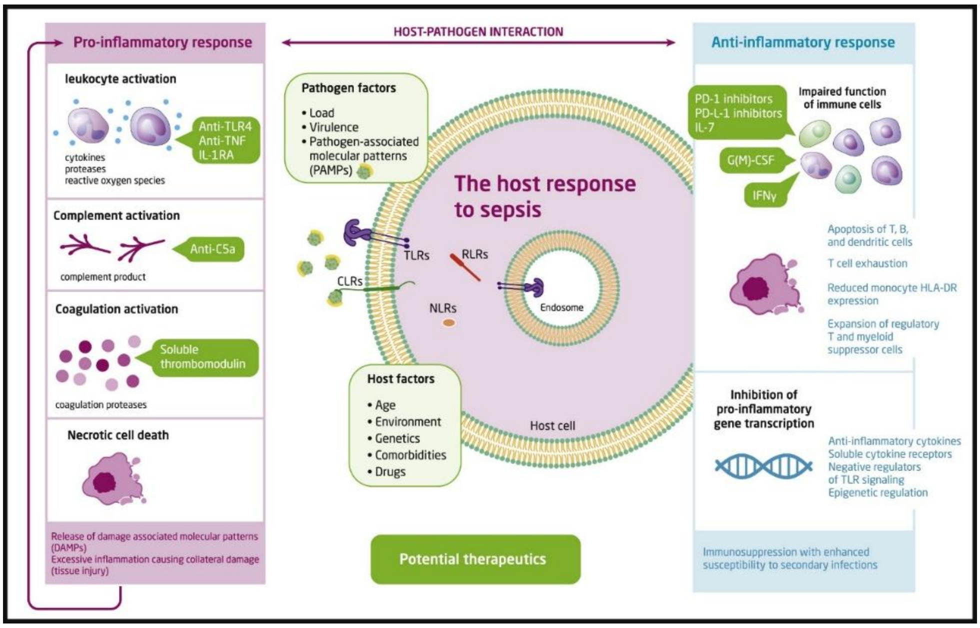

Figure 1 "Immunopathogenesis of sepsis": sepsis begin with the inflammatory response activated after the recognition of the invading pathogen by the immune system. The pro-inflammatory response consists in the release of pro-inflammatory mediators and in the activation of the complement and coagulative systems, resulting in cell death, DAMP release and the further exacerbation of pro-inflammatory pathways. The anti-inflammatory response consists in impaired funtion of immune cells with decreased capacity to produce cytokines. The green boxes highlitht theraputic strategiess targeting various stages of the septic inflammatory response (18).

Sepsis can be classified into immune endotypes, reflecting diverse inflammatory or immunosuppressive profiles with distinct outcomes and therapeutic implications. An "Endotype" consists in a biological subgroup of patients with a specific pathophysiological mechanism that distinguishes it from others. Transcriptomic profiling of blood leucocytes revealed different sepsis endotypes and this classification can be used to personalize the immunomodulatory therapies (19).

Coagulation Disorders

As briefly anticipated, coagulation abnormalities are common in sepsis patients, being observed approximately in 50-70% of the cases (26). These abnormalities originate from an increase in platelet reactivity for various reason (27), but they are also driven by the intense inflammatory response characterizing sepsis. For example, elevated levels of hormones in circulation (such as epinephrine and 5-hydroxytryptamine) can activate platelets, as well as high cytokine levels. Several pro-inflammatory cytokines, including TNF, IL-6, and IL-1, play a pivotal role in triggering coagulation activation (26). The endothelial injury caused by bacterial endotoxin during sepsis leads to an upregulation of TF, which activates Factor VII initiating the extrinsic coagulation cascade. This pathway intersects with the intrinsic cascade through the interactions with Factor X resulting in thrombin generation, which promote platelet activation and, in turn, a further thrombin production by the release of procoagulant molecules. Moreover, impaired fibrinolysis contributes to clot stability resulting in denser fibrin networks with thinner fibres, increased clot opacity, and delayed clot breakdown (28). So, sepsis is characterized by a hypercoagulable state, with microvascular thrombi, NET formation, and endothelial injury. This state can cause DIC, an important complication associated with bleeding, due to the consumption of clotting factors, anticoagulant proteins and platelets (26). In fact, the level of antithrombin, activated protein C, and endothelial glycosaminoglycans, like heparan sulphate, is reduced, thus leading to the suppression of endogenous anticoagulant pathways. This is further exacerbated by the inhibition of thrombomodulin and endothelial protein C receptors, which leads to a decrease in protein C activation (5). Interestingly, coagulation factors and anticoagulant proteins also activate specific signalling pathways by binding cell receptors like Protease-activated receptors (PARs). PARs 1,3 and 4 are activated by thrombin and PAR-2 by TF-factor VIIa complex, factor Xa, and trypsin. PAR-1 also bind TF-factor VIIa complex and factor Xa. These pathways are associated with coagulation and inflammation responses with both systems reciprocally influencing the shape of the clinical course of sepsis disease (Figure 2) (26). Thrombocytopenia, defined as platelet count <150.000/ μL, is common feature in patients with sepsis and the incidence reaches up to 70% in Intensive Care Unit (ICU) (28). During infections, when bacteria gain access to the circulatory system, they activate platelets causing platelet consumption through specific pathways clearly detailed in the review by Dermot Cox

(29). After a primary increase in platelet production, the body’s capacity to generate platelets is outpaced by their rate of consumption. Once this imbalance occurs, septic patients begin to develop thrombocytopenia which is also negatively correlated with the outcome (29). For this reason, platelet count is included in the SOFA score. A retrospective observational study (30), analysing 1024 septic patients, revealed that thrombocytopenia, categorized as severe, moderate, or relative, was present in 33%, 40%, and 9% of cases, respectively. Moreover, mortality increased from 17.4% in non-thrombocytopenic patients to 40.6%, 21.1% and 27.8% in those with severe, moderate, and relative thrombocytopenia, respectively. So it is well established that the development of thrombocytopenia in sepsis patients doubles the risk of mortality (31). Mean Platelet Volume (MPV) is a platelet index which quantifies the average size of circulating platelets, with a reference range of 7.2 - 11.7 Femtoliters (fL). An elevated MPV reflects both compensatory thrombopoiesis by the bone marrow and platelet activation. A prospective observational study (32) showed that MPV, as well as its ratio with platelet count, is predictive of clinical worsening and increased mortality in septic patients. Additionally, MPV levels were found to be significantly higher in neonates with sepsis compared to non-septic patients. Within the first 24 hours after birth, an increase in MPV was associated with elevated C-Reactive Protein (CRP) levels and a higher risk of neonatal mortality (33). An emerging and under investigation biomarker of platelet activation is Platelet Distribution Width (PDW). PDW reflects and quantifies the heterogeneity of platelet morphology and increases upon activation. Currently it is not validated for diagnostic purposes, and its use is limited to clinical research. PDW may be a useful parameter when interpreted alongside other biomarkers, but results regarding its accuracy are discordant and there is no established cut-off point (34, 35). High PDW values have been associated with worsening clinical condition and an increased 90-day mortality among internal medicine patients (36). Among the emerging platelet parameters, the Immature Platelet Fraction (IPF) is an indicator of reactive thrombopoiesis and platelet consumption in sepsis. However, available literature studies report moderate diagnostic accuracy and substantial inter-instrument variability, leading to uncertain diagnostic variability. Therefore, IPF should not be used alone as biomarker of sepsis but as a complementary additional parameter that may improve diagnostic performance when combined with conventional proven biomarkers (37-40). In conclusion, platelets are essential effector cells in both haemostasis and inflammation. They are critically involved in the pathogenesis of sepsis contributing to clinical complications. Platelet indices are rapidly and cost-effective markers that, reflecting both dysfunctional coagulation and the systemic inflammatory response in sepsis, could serve as part of an integrated approach to facilitate the early diagnosis and improve the management of septic patients (41).

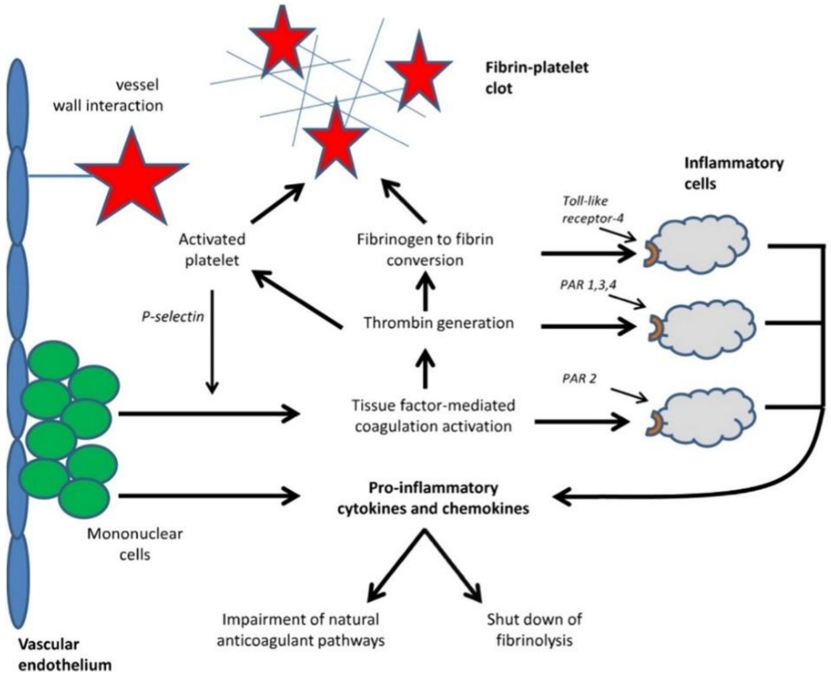

Figure 2 "Interaction between inflammation and coagulation in sepsis": the expression of TF by mononuclear cells leads to thrombin generation and fibrin formation. In the meanwhile, the activation of platelets contributes to clot formation and platelet-derived P-selectin increases the expression of TF. The binding between PARs with TF, thrombin and other activated coagulation proteases, as well as the interaction between fibrin with TLR4 on inflammatory cells, trigger the release of pro-inflammatory cytokines and chemokines; these mediators modulate coagulation and fibrinolysis (26)

Extracellular Histones: definition, origin, and pathological effects

Histones are intra-nuclear proteins highly conserved across species, which play an essential role in the organization and regulation of DNA (42). The positive charge of histones interacts with the negative one of DNA forming nucleosome, the fundamental unit of chromatin. Moreover, histones are categorized as core or linker histones. The core histones are H2A, H2B, H3, and H4, while the linker histones are H1 and H5. Another classification is based on aminoacid composition: lysine-rich (H1, H2A, and H2B) and arginine-rich (H3 and H4). A nucleosome consists of a 147-base-pair segment of DNA that is rolled up into an octameric complex, which is formed by one H3/H4 tetramer and two H2A/H2B dimers. Each nucleosome is separated by 10-60 base pairs of DNA, resulting in a 10 nm diameter chromatin fibre. This arrangement is folded into a 30 nm diameter condensed fibre by the binding of a linker histone to each nucleosome core (43). Both histones and DNA can undergo covalent modifications that alter the chromatin organization promoting the folding or unfolding of specific chromatin regions, thereby changing the gene expression (44). Specifically, histones are subjected to covalent post-translational modifications (PTMs) including acetylation, methylation, phosphorylation, ubiquitylation, and citrullination, enhancing the epigenetic control of gene expression. In particular, the citrullination of histones leads to the conversion of arginine residues to citrulline through the peptidyl arginine deaminase (PAD). Among the PAD family, PAD4 is particularly known for its role in the formation of NET. PAD4-mediated histone citrullination is considered a crucial step in NETosis because of the promotion of chromatin decondensation, which allows nuclear material to be released into the extracellular space (45, 46). The release of histones occurs following the rupture of the nuclear and plasma membranes under conditions of cellular stress or a “hostile” microenvironment, through three main mechanisms: (1) as free histones, or (2) in form of DNA-bound nucleosomes, typically from dying cells during necrosis, or (3) as component of ET, actively released by immune cells through ETosis (Figure 3) (47). As anticipated, ETs are composed of DNA, histones, and granular proteins such as myeloperoxidase, neutrophil elastase, and matrix metalloproteinases. They exert an important role in antimicrobial host responses, entrapping microorganisms through their three-dimensional web-like structures (48). However, excessive or dysregulated ET formation has been associated with pathogenic conditions, particularly in inflammatory progression, autoimmune diseases and thrombosis (49). ETosis has been reported in neutrophils (NETs), macrophages (METs), eosinophils (EETs), basophils (BETs), mast cells (MCETs), plasmacytoid (pETs), and monocytes (48). Among these, NETosis is the best described mechanism. Well known triggers of NETosis are Phorbol 12-Myristate 13-Acetate (PMA), the stimulus that led to the discovery of NETosis, infections, platelet activation, cytokines like IL-8 and TNF-α, auto-antibodies, hydrogen peroxide, ionophores, etc (50). Then, the expression of PAD4 is fundamental for the initiation of NETosis, thanks to the conversion of the positive charge of arginine with the neutral one of citrulline, reducing the electrostatic interaction with the negative charge of DNA. The resulting unfolded DNA, with the dissolution of the nuclear membrane, is exposed to the cytoplasmic granules and forms a fibrous structure, NET, then excretes because of the rupture of the cell membrane (48). NETs include anti-microbial substances useful to kill microorganisms, inactivate the bacterial virulence factors, and improve phagocytosis by recruiting leucocytes in the trap site. On the other side, this is a double-edge sword because excessive NETs formation or incorrect removal causes several pathological effects and consequences (51). NETs exert significant effects on vascular system. They are toxic to endothelial cells, causing endothelial dysfunction that amplifies NETs formation, leading to a vicious cycle of injury (52). They can also be a scaffold for the adhesion of platelets and erythrocytes, promoting their aggregation. So, NETs cover a pivotal role in thrombosis, also acting through histone-mediated procoagulant effects (50). This is also confirmed by literature data demonstrating NETs degradation through DNase I or PAD4 inhibition, as well as neutrophil elastase reduces the formation of thrombus in vivo (53). Once released, extracellular histones damage host cells and induce an inflammatory response, acting through various mechanisms, that can be summarized as a pathogenic triad of cytotoxicity, immune stimulation, and coagulation (Figure 4) (54). In particular, histones exert their harmful effects (1) acting as chemokines or inducing chemokine release, (2) stimulating the production of cytokines, (3) causing the apoptosis of adjacent cells and leukocytes, (4) showing direct cytotoxicity, (5) inducing platelet activation, aggregation, and thrombin generation, (6) activating Peripheral Blood Mononuclear Cells (PBMCs) which, in turns, release cytokines, and (7) stimulate the release of Reactive Oxygen Species (ROS) by neutrophils (55) (56). These effects are carried out by histones, well-known DAMPs, via TLRs (2, 4, and 9), MyD88, NF-kB and the NRLP3 inflammasome. Literature data show that, when histones are injected in renal artery of rats, they induce the release of IL-6, TNF-α, and iNOS, all effects that are limited in TLR2 and TLR4 knock-out mice. Moreover, a dose of 10 μg/mL of histones induces in ARPE-19 cells the increase of IL-6 and IL-8, leading to the phosphorylation of ERKs, p38 MAPK and JNK. The inhibition of these kinases blocks the cytokine production. Noteworthy, the effect of a higher concentration of histones (50 μg/mL) is significantly different, causing cell death not prevented by kinase inhibitors (55). Moreover, H3 and H4 fractions increase the expression of E-selectin, ICAM-1 and VCAM-1 promoting leukocyte adhesion, rolling and transmigration via TLR9 (57).

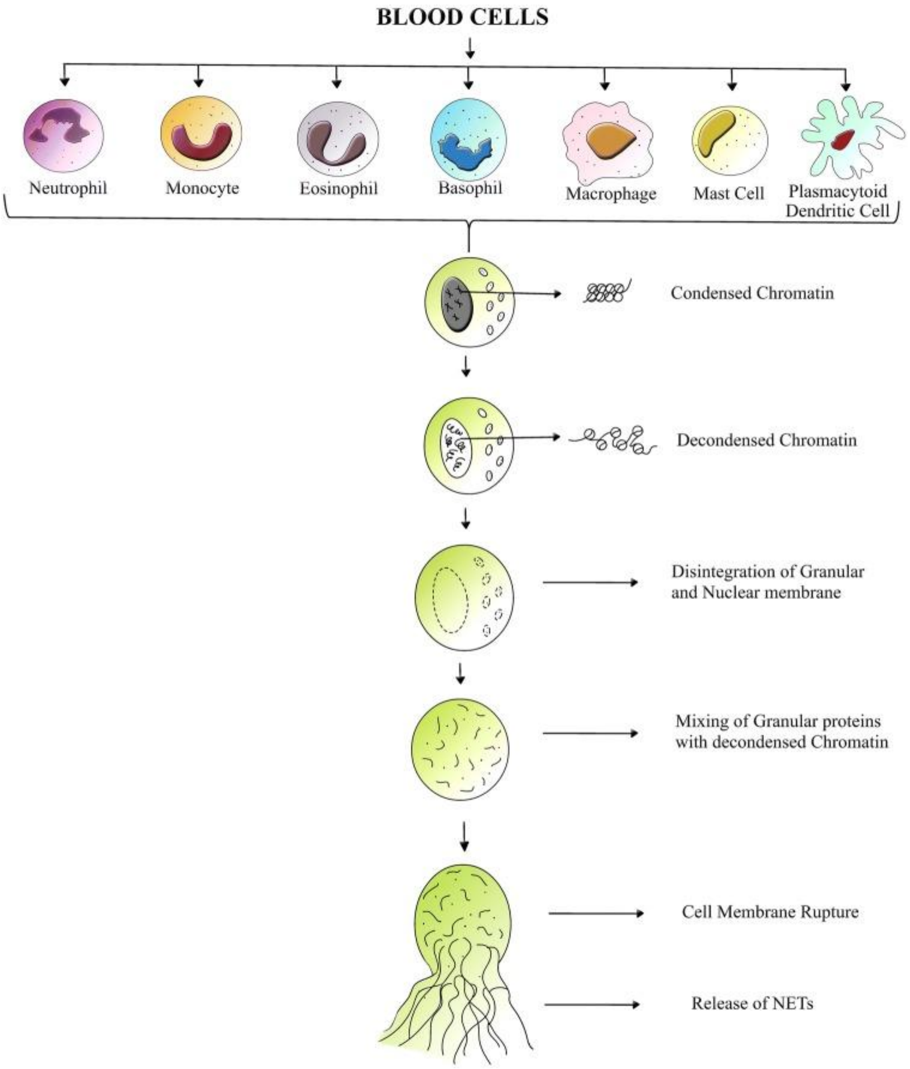

Figure 3: Mechanisms of ET formation in blood cells like neutrophils, monocytes, eosinophils, basophils, mast cells, macrophages and plasmacytoid. The main steps consist of: decondensed chromatin, disintegration of granular and nuclear membrane, mixing of granular proteins with decondensed chromatin, cell membrane rupture and release of NETs (48).

The role of Histones in Sepsis

In sepsis condition, circulating histones are released in high concentration and play a crucial role as DAMPs, driving inflammation, cytotoxicity, and multi-organ failures (58). The physiological serum level of histones is ~0.06 ng/mL, and values exceeding 3 ng/mL are associated with endothelial damage, inflammation, and coagulopathy (59). Core histones H3 and H4 are most frequently reported in plasma from sepsis patients (55), and have been associated with pro-inflammatory and pro-aggregating effects (58). Eichhorn et al. characterized a panel of 44 inflammatory mediators released in critical sepsis, including DAMPs. Results showed that extracellular histones were significantly higher in non-survivors (30-fold) compared to survivors (60). Ekaney et al. demonstrated that H4 plasma concentration was markedly higher in septic patients compared to ICU controls, contributing to disease progression through TLR4-mediated inflammation and promoting poor outcome (61). Lu et al. proved that H4 level is an independent predictor of mortality, and ROC analysis confirmed its prognostic value. Moreover, H4 levels positively correlated with SOFA scores and plasma cardiac troponin I (62). Wildhagen et al. found that H3 levels were associated with increased mortality of sepsis patients in ICU and inversely correlated with antithrombin levels and platelet count, also highlighting its potential use as biomarker of sepsis disease (63). During sepsis, PAMPs and DAMPs activate immune cells (*e.g.* neutrophils and macrophages) and endothelial cells through PRR receptors (TLRs, C-type lectin receptors, and NOD-like receptors). This peculiar activation induces inflammatory cell death programs such as NETosis, necrosis, and pyroptosis, which exacerbate in turn the release of histones (58). Circulating histones, with the mechanisms described above, lead to the production of cytokines (IL-1, IL-6, IL-18, TNF-α), promote NETs release and upregulate TF expression in monocytes, linking inflammatory processes to coagulation cascade. At the vascular level, histones induce membrane permeabilization, calcium influx, and barrier dysfunction that result in altered blood flow and leukocyte recruitment, combined by cytokine release, increased TF, prostacyclin, and superoxide, and reduced thromboxane A_2 and nitric oxide. All these changes induce DIC via TF-driven activation of prothrombin and via histone-prothrombin-FXa complex formation (26, 58). Moreover, histones trigger platelet aggregation and thrombin generation via TLRs (64). The combination of uncontrolled inflammation, endothelial barrier dysfunction, coagulation activation, and microvascular thrombosis, flows out in Multiple Organ Dysfunction Syndrome (MODS), characterized by circulatory failure, acute respiratory distress syndrome (ARDS), renal injury, and liver failure, leading to death in severe sepsis (47, 58). Histones are not the only components of NETs, and also the cell-free DNA (CFDNA) exert damaging effects to the host: for example, by triggering blood coagulation *via* the intrinsic pathway or inhibiting fibrinolysis by impairing plasmin-mediated fibrin degradation. CFDNA is elevated in septic patients, being useful as prognostic indicator, but its contribution to the pathogenesis of sepsis remains unknown or at least elusive (65). It is noteworthy to understand the different role of CFDNA, histones, and nucleosome *in vivo*. Medeiros et al. investigated these effects injecting them in healthy and septic mice (65). Their results suggested that DNA mask the effects of histone and the administration of histones contributes to the harmful effects found in septic conditions, while this does not occur with the administration of nucleosome or DNA (65). Due to the pivotal role of extracellularly circulating histones in sepsis (but also in other disease settings including cancer, neurodegenerative diseases, acute infections etc.), to prevent NET formation or directly to block histones activity may represent a feasible intervention to reduce cellular damage (54). NET formation is counteracted by several approaches, such as the inhibition of NADPH oxidase, PAD2 and PAD4. Also, the inhibition of the receptors of histones, including TLRs and C-type lectin receptors, may represent an interesting approach to prevent the cytotoxic effects of extracellular extranuclear histones (54). Furthermore, direct methods to neutralize histone effects are represented by complexation or fragmentation procedures. Molecules with negative electrostatic charge, such as albumin, polysialic acid, heparins/heparinoids etc., were demonstrated to be able to complex the positive charges of histones. The most promising strategy consists in the use of glycosaminoglycan compounds (66) like unfractionated heparin (UFH), and low-molecular-weight heparin (LMWH) which already have been used in the treatments of sepsis complications (54). Preclinical studies in animal models of sepsis demonstrated the reduced organ damage and mortality induced by heparins (67). Sharma et al. compared the effectiveness of UFH, LMWH, Vasoflux, and Fondaparinux in counteracting the effects of histones (68), demonstrating that not all heparins and derivatives exert the same protective effects against histone-mediated cytotoxicity, as their activities depend on different molecular sizes and compositions (68). For example, Fondaparinux is a synthetic analogue of the heparin pentasaccharide (1.7 kDa) that binds antithrombin with high affinity, inhibiting factor Xa but not thrombin. Due to its small molecular size, Fondaparinux shows limited affinity for histones, and fails to reduce tissue factor expression, indicating that histone-neutralizing activity requires heparin fragments larger than 1.7 kDa (68). In the frame of the studies of our research group on heparin and histones, we analysed the effects of Danaparoid sodium, a well-known glycosaminoglycan mixture of heparan sulphate, dermatan sulphate, and chondroitin sulphate (5-5.5 kDa), exerting both anticoagulant effects through inhibition of factor Xa and IIa and anti-inflammatory actions by downregulating NF-kB dependent cytokines such as IL-6, TNF-α, and HMGB1. In murine models of LPS-induced systemic inflammation, danaparoid administration reduced pulmonary injury and systemic cytokine release, while increased levels of IL-

10 and macrophage activation, improving survival. These findings highlighted that the ability to counteract histone-mediated cytotoxicity and procoagulant activity is dependent on the molecule size, and additional anti-inflammatory properties may broaden the therapeutic potentiality in sepsis conditions. Our papers discussing the role of histones in relation to thrombocytopenia are presented in the following chapters: 3, 4, 5, and 6.

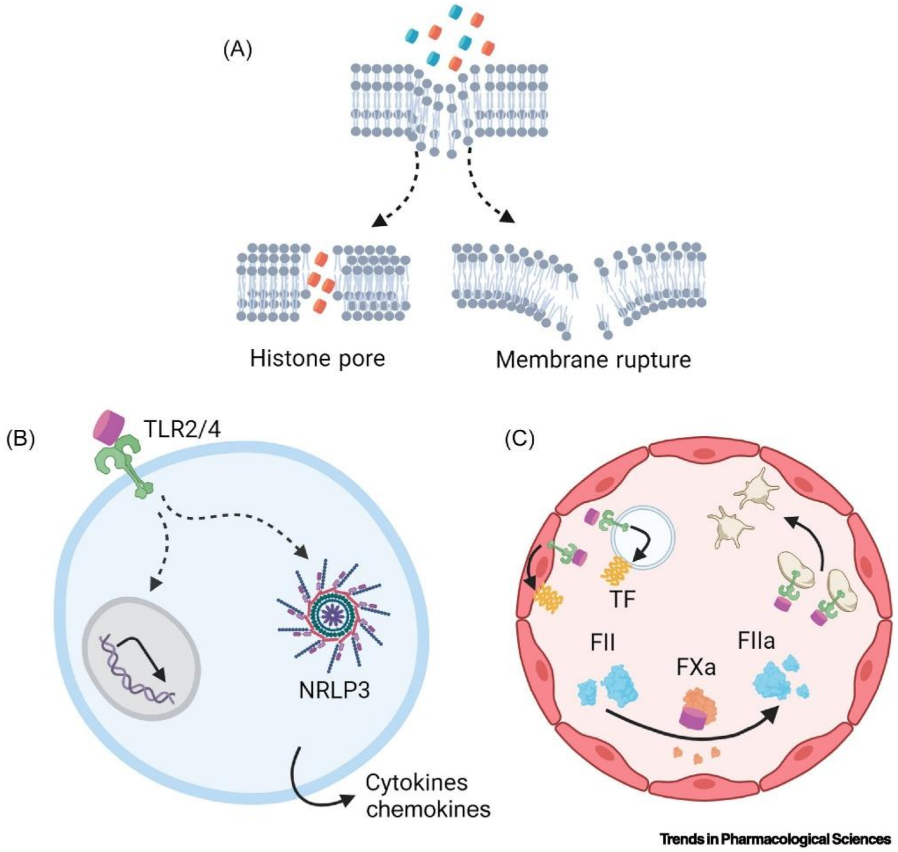

Figure 4: Pathogenic triad of cytotoxicity, immune stimulation, and coagulation induced by histones. (A) H3 and H4 are cytotoxic, causing membrane bending which induces membrane rupture or membrane pore formation. (B) Histones promote the release of pro-inflammatory mediators through the activation of TLR2/TLR4 and NLRP3 inflammasome (C). (D) Histones promote TF expression in myeloid and endothelial cells through TLRs and bind FXa leading to thrombin generation. Histones activate platelets also by TLR interactions (54).

Biomarkers of Sepsis

Biomarkers of sepsis are crucial for early diagnosis, therapeutic decisions, and prognosis. A total of 4801 studies about sepsis-related biomarkers have been published between October 2019 and October 2024, due to their importance in clinical practice (69). Blood culture represents the Gold Standard for bloodstream infection detection, but it has limits for screening sepsis in the Emergency Department (ED). Firstly, the identification of a pathogen is not necessarily associated with the diagnosis of sepsis, which is not sustained only by bacteria, the reason why half of sepsis cases are culture negative. Moreover, blood culture is a time-consuming test that takes 12 hours or more to be completed, limiting its usefulness in the ED (70). Among the most widely studied and used diagnostic biomarkers of sepsis we can list: C-reactive protein (CRP), procalcitonin (PCT), lactate, White Blood Cell (WBC) count, Presepsin (sCD14), and pro-Adrenomedullin (Pro-ADM) (4, 70). It must be specified that no single biomarker is 100% sensitive and specific in discriminating sepsis from other inflammatory diseases. The application of a multi-biomarker strategy, added to symptoms recognition, and laboratory markers of organ dysfunction, is essential to provide a more comprehensive evaluation of sepsis (70, 71). Biomarkers need to be interpreted in combination with the clinical contexts of SOFA, due to the variability of their expression in different patients, and their changes should be monitored over time to provides more accurate prognostic information (72, 73). Researchers shed light also on novel biomarkers, highlighting the molecular complexity of septic patients. The new biomarkers can be categorized into 3 groups: (1) the transcriptomic multi-analyte panels, which use RNA transcripts of multiple genetic markers combined with algorithms and data from patients, to create a test; (2) tests based on physical cell properties, using for example flow cytometry to measure changes in WBC morphology. These tests are rapid, due to their inclusion in the haematology analyzer; (3) Tests based on the artificial intelligence, which use preexisting data from clinical databases, to predict sepsis (70). SeptiCyte (Immunexpress, Inc) is a test from the first group; it uses RT-qPCR to measure immune-related mRNA markers and helps to discriminate sepsis from non-infectious inflammation. Results are provided in one hour with a risk score, from 1 (low) to 4 (high). This test is approved for use in ICU settings (70). Monocyte Distribution Width (MDW) (Beckman Coulter, Inc.) and IntelliSep (Cytovale, Inc.) are markers of the second group: they detect physical changes in leucocytes during the host response. Both are used in ED. MDW is an early biomarker, obtainable as part of the Complete Blood Count (CBC) (70), which is discussed in depth in the next chapter dedicated to. IntelliSep provides results about immune cell morphology changes in 10 minutes, but it is a standalone test which requires a specific request (70).

Finally, MeMed BV (MeMed, Ltd.) is a test of the third group which through the combination of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), interferon gamma-induced protein 10 (IP-10) and CRP, distinguish bacterial infections from viral infections, in 15 minutes. This test is confirmed for use in children (70). Here is a brief overview of the most used biomarkers of sepsis in clinical practice:

CRP is a protein produced and released only by hepatocytes in response to an infection or a status of inflammation (4). It is an acute-phase protein, its levels rise 4-6 hours after the inflammatory trigger, with a half-life of 19 hours (73). CRP is commonly used as a marker of sepsis; elevated plasma levels of CRP correlated with the severity of the infection, while its rapid decrease is associated with the remission of inflammation (74). CRP can't be used to discriminate against bacterial and non-bacterial infections, due to its low sensitivity (68-92%) and specificity (40-67%) (75).

PCT is a 116-aminoacid precursor of calcitonin, a hormone produced by C-cells present in the thyroid gland. PCT concentrations significantly rise in response to bacterial toxins, from a basal level of 0.05 ng/mL to 0,5 ng/mL, which may indicate the risk of sepsis (76). Generally, the increase starts in 2-4 hours from sepsis onset and peaks in 24-48 hours. Moreover, PCT may be useful to discriminate between infectious and non-infectious causes (77). Its sensitivity and specificity range for sepsis diagnosis is respectively 77-85% and 75-83% (73). PCT can also guide antimicrobial therapy but can increase also in other conditions like cardiogenic shock, pancreatitis, etc. (4).

Cytokines (e.g. IL-6) are additional biomarkers to improve PCT and CRP results. They rise early in response to systemic inflammation, with low specificity, but correlating with worse outcomes (4).

Lactate is a marker of tissue hypoperfusion or impaired oxidative metabolism (4). In sepsis conditions there is a significant correlation between lactate levels (< 2 mmol/L) and disease severity and mortality (78), but it is elevated also in other metabolic conditions . Early lactate clearance is associated with improved clinical outcomes (79).

Presepsin (sCD14). CD14 is a glycoprotein expressed on monocytes and macrophages surface that works as receptors for Lipopolysaccharide (LPS). In inflammatory conditions the N-terminal of CD14 is cleaved and secreted as soluble CD14 subtype (sCD14). sCD14 normal serum levels range from 2 to 6 mg/mL, while increase after microorganism infection, faster than procalcitonin or IL-6 (74, 80). Literature data emphasize the significant different levels of sCD14 in survivors and non-survivors, recognizing its valuable role as sepsis biomarker (80).

Pro-ADM is a stable precursor of ADM studied for its role in sepsis diagnosis. Pro-ADM is found in the plasma of sepsis patients due to the degradation of ADM (4). ADM is a 52-aminoacid peptide mainly secreted by endothelial and vascular smooth cells, where it acts as vasodilator during physiological stress (80). Pro-ADM may detect sepsis-related organ dysfunction up to 24 hours before clinical onset, being a promising tool to guide ICU admission, optimize treatment strategies, and improve risk stratification (81).

WBC count (4-11 x10^3/µL) is one of the most frequently performed laboratory tests in patients with suspected infection. WBC are composed of subpopulations: granulocytes (neutrophils, eosinophils, and basophils), lymphocytes, and monocytes. They are part of CBC which is a test provided by haematology analyser routinely executed to evaluate individual health status. An elevated WBC count usually reflects inflammatory of infectious processes, but it may also increase in non-infectious conditions like haematological or autoimmune disorders. On the other hand, WBC can remain in the normal range or decrease in septic patients. For these reasons WBC has a low specificity in sepsis diagnosis, limiting its role as a standalone biomarker, requiring to be integrated in a multi-biomarker panel (82).

Of all sepsis biomarkers studied, parameters related to CBC are valuable tools due to several advantages, including their universal availability, low cost, rapid turnaround time, ease of execution, and routine use across all clinical settings, from ED to the ICU. In addition to WBC, CBC parameters can be categorised into Red Blood Cells (RBC) and Platelets (PLT). The CBC of a septic patients usually presents lymphocytopenia, neutrophilia, eosinopenia, thrombocytopenia, increased RDW, and increased Neutrophi-to-lymphocyte ratio (NLR). The inclusion of PLT in the SOFA score highlights the importance of thrombocytopenia. Also, the anaemia, reflected by the reduction of haemoglobin and haematocrit, is common feature during sepsis. As mentioned before, MDW is a new biomarker of sepsis included in the CBC as part of Cell Population Data (CPD). CPD parameters are studied as early potential sepsis biomarkers, they are generated by the new generation analysers and they provide quantitative information about volume, granularity, and complexity for each WBC (82). Details about all CPD are described in the third Chapter.

Monocyte Distribution Width (MDW)

Definition

Monocyte Distribution Width (MDW) is a new cell population data parameter obtained with the most advanced generation of DxH hematology analyzers by Beckman Coulter. MDW quantifies monocyte heterogeneity, reflecting their morpho-functional changes associated with a dysregulated immune response. In 2019, the USA Food and Drug Administration (FDA) approved its use in the ED as early sepsis biomarkers (83, 84). The importance of an immediate and appropriate diagnosis of sepsis has been well discussed in the first chapter, here we will explore the role played by MDW in this context. MDW is a parameter included in CBC, a first-line test, which makes it easy to perform and inexpensive, it has a low turn-around time, and it is rapidly available (84). Monocytes are key players in both innate and acquired immunity and MDW measures monocyte anisocytosis through Volume, Conductivity and Scattering (VCS) technology (85). VCS system enables white blood cells characterization using three key measurements: (1) single cell volume, (2) high-frequency conductivity, and (3) five laser light scattering. By combining these informations is possible to acquire detailed information on physical and functional blood cell properties (86). In particular, MDW is calculated using a mathematical formula based on the standard deviation of monocyte volume values, representing their peculiar variability (87). All information acquired using VCS is reported in Two- and Three- dimensional Dataplots and Surface Plots (86). The FDA guidelines recommend MDW as early sepsis indicator for adult patients presenting to Emergency department. It is obtained from venous whole blood collected in K2EDTA tubes and analysed within two hours. An MDW value above 20.0 (cut-off), when interpreted with other laboratory parameters and clinical evaluation, supports the identification of patients with sepsis or at increased risk of developing sepsis during the 12 hours after hospital admission (88). García-Álvarez et al. studied the reference value of MDW in whole blood samples from 281 healthy donors collected in K2EDTA, age-range 18-66 years, demonstrating that males and females presented MDW values of 17.48 (SD 1.85) and 17.95 (SD. 1.8), respectively, suggesting that the reference interval is the same for both genders (89). MDW can be obtained also in K3EDTA whole blood samples, with a cut-off of 21.5 to identify the risk of sepsis (90). The cut-off in K3EDTA samples is higher than in K2EDTA due to the different baseline reference range in healthy subjects. Agnello et al. evaluated the reference interval of MDW in K3EDTA whole blood from 486 selected subjects, age-range 18-70 years. Their results showed that male and females displayed similar MDW values, with values of 19.29 and 19.49, respectively (91). Importantly, MDW levels are not influenced by sex and age (83).

Research evidence on MDW in early sepsis recognition

The early identification of sepsis remains an important clinical challenge and several recent studies have focused their attention on the role of MDW, which in combination with other biomarkers can facilitate timely diagnosis and more accurate intervention. Upon encountering PAMP, monocytes become activated through recognition of PPRs, triggering a cascade of immune responses, including cytokine release and morphological changes. All these functional and structural changes are mirrored by MDW increase (Figure 5).

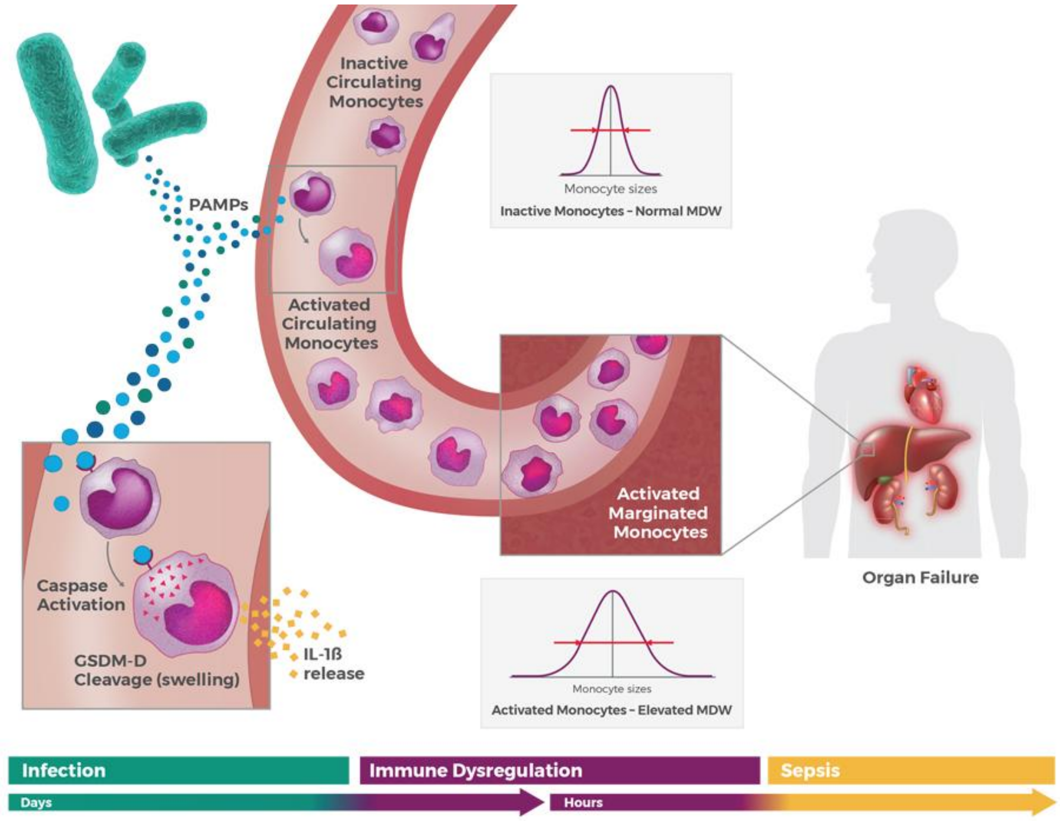

Figure 5: MDW quantifies changes in the volume of monocytes after an infection, reflecting monocyte activation in response to pro-inflammatory mediators release by PAMPs (92).

Several research studies investigated the role of MDW as diagnostic and prognostic sepsis biomarker, also comparing its accuracy with other conventional markers. Here a revision of the main recent and interesting results.

An observational study by Agnello et al. considered 62 septic patients (M:F 60:40%; median age 70 years), diagnosed based on Sepsis-3 criteria, and 131 controls (M:F 63:37%; median age 70 years); samples were collected in K3EDTA and analysed through UniCel DxH 900 hematology analyzer Beckman Coulter. Their results showed that septic patients had significantly higher MDW than control patients, median values of 28.4 and 20.1, respectively. CRP, PCT and lactate were also higher in septic patients than controls. Noteworthy is that the Area Under the Curve (AUC) of MDW was significantly higher than AUCs of CRP, PCT and lactate, demonstrating that MDW has got a higher accuracy in sepsis detection compared to conventional biomarkers. Moreover, they assessed the prognostic role of baseline levels and of the kinetics during the first five days of ICU stay of each biomarker. Results emphasized that a marked reduction in MDW values correlates with improved survival, whereas an increase in MDW values were linked to higher mortality rate. Therefore, monitoring MDW during the early days of hospitalization offers valuable prognostic insights in septic patients. These findings indicate that MDW is a reliable biomarker of sepsis but also provides better prediction of short-term outcomes compared to traditional sepsis biomarkers (93).

Liu et al. evaluated MDW in K2EDTA whole blood samples from 252 septic patients (162 males and 90 females). The Authors demonstrated that MDW levels were not significantly different between survivors and non-survivors on the first day after hospital admission while on the third day of hospitalization they showed a significant prognostic association with 28-day mortality. MDW levels above 26.20 have been linked to a fivefold increase in the risk of death compared to patients with lower levels. Moreover, the AUC of MDW on the third day after admission reached 0.731, higher than SOFA scores, WBC, NLR, CRP and PCT (85).

Levin et al. in their study developed the CBC Sepsis Index (CBC-SI) incorporating MDW measurement to the CBC with differential leucocyte formula (CBC-diff) for sepsis diagnosis. A retrospective cohort of adult patients (age ≥18) admitted in ED with CBC-diff, including MDW, NLR and WBC values were enrolled. Among 51407 accesses in

ED, 1683 met the Sepsis-3 criteria within 24 hours, of which 79.8% had obvious signs while 20.2% were without clear signs. CBC-SI index ranges from 0 to 5, for most of ED visits was 0 (63-2%), followed by those with 1 point (19.2), 2 points (9.4%), 3 points 4.7%, 4 points (2.4%, and five points (1.1%). The probability of sepsis increases with the score, from 3.2% at 1 point to 38.1% at 5 points. The AUC of CBC-SI is 0.83, higher than the other leucocyte indices alone (NLR:0.81; MDW:0.76; WBC:0.72). Interestingly, removing MDW from CBC-SI resulted in the strongest drop in AUC, compared to the remotion of NLR or WBC, emphasizing the fundamental contribution of MDW in CBC-SI index. Moreover, CBC-SI performed even better in patients without obvious sign of sepsis: an index ≥1 had a sensitivity of 81.1% and specificity of 69.1% for the group without obvious signs compared with a sensitivity of 83.5% and specificity of 39.4% in the group with obvious signs of sepsis. Finally, CBC-SI outperformed qSOFA, especially for detecting occult sepsis and a score of zero showed a stronger negative predictive value for excluding sepsis compared with qSOFA (84).

Theodoridis et al. conducted a prospective study including 136 adult patients (mean age 73.5, 54.4% female), equally divided into septic and non-septic controls based on Sepsis-3 criteria. Septic patients were also distinguished into survivors (n=34) and non-survivors (n=34). They evaluated MDW, CRP, PCT, IL-6, lactate, ferritin, TNF-α and coagulation indices in whole blood samples collected in K2EDTA. Results showed that 50% of septic patients died. Traditional markers such as CRP and PCT lacked prognostic value, while lactate, IL-6 and TNF-α levels were found higher in non-survivors. MDW emerged as a strong prognostic parameter, with levels significantly elevated in non-survivors, and with strong predictive performance on days 3 and 5 (AUC>0.9). The Authors concluded that MDW is an independent predictor of outcome in patients with sepsis admitted in ICU (86).

Agnello et al. explored the accuracy of MDW and presepsin through an observational prospective study which enrolled 104 patients (mean age 70 years, M:F 56%:44%) admitted in ICU. Among the patients, 29% were defined as septic patients following Sepsis-3 criteria. They evaluated MDW, PCT, CRP and presepsin at admission, after 24h, 48h, 72h, after 5 days and at discharge. Results demonstrated that, at each time considered, patients diagnosed with sepsis showed elevated concentrations of PCT and presepsin compared with non-septic individuals. Conversely, CRP showed significant differences only at T0, while MDW had statistical relevance at T0 and at

T24h. In particular, presepsin emerged as an independent predictor of ICU mortality, at 48h and 72h after hospital admission. Moreover, presepsin kinetics demonstrated prognostic value, because of increasing levels at 48h were associated with reduced median survival. They concluded that MDW and presepsin should be integrated into clinical practice, with application in detecting and monitoring sepsis, respectively. Since MDW is included in CBC, it may contribute to the identification of sepsis, even in cases where clinical symptoms are occulted. On the other hand, presepsin offers useful information in monitoring disease progression and in recognizing patients who may require intensive therapeutic management (94).

Finally, a systematic review and Meta-analysis by Malinovska et al. evaluated the diagnostic performance of MDW as a biomarker for infectious disease, focusing on sepsis and COVID-19. To explore and consolidate this evidence, authors systematically searched PubMed, Embase, Scopus, and Web of Science for studies published between 2010 and early 2022. Out of 215 identified publications, only 29 studies met the inclusion criteria. Most studies evaluated sepsis (19) and COVID-19 (6). About sepsis they demonstrated that MDW has got good diagnostic accuracy for sepsis, even though results depend on the reference definition, Sepsis-2 or Sepsis-3, and on the type of anticoagulant used, K2EDTA or K3EDTA. Taking these variations into account, the AUC ranged from 0.74 to 0.94, with mean sensitivity of 0.69 to 0.79 and mean specificity of 0.57 to 0.89. For COVID-19 the AUC of MDW was 0.79, mean sensitivity 0.79 and mean specificity 0.59. This meta-analysis emphasized that integrating MDW into routine CBC testing enables the early detection of sepsis, even in patients not initially suspected of infection. Compared to biomarkers that require targeted ordering, MDW offers a broad range of applicability. Authors suggest that MDW should be integrated with other CBC parameters to improve specificity (95).

MDW and infecting pathogen

Monocytes are crucial effectors of the immune system and mediate the response to invading bacteria through phagocytosis and the production of NO, ROS, and TNF. Monocytes may also produce and release cytokines such as IL-12, IFN, and chemokines, like CXCL9 which triggers the activity of other effectors for bacterial clearance. About viral infections, monocytes are the first cells to encounter the virus (83). This induces monocyte alterations, such as cell polarization, motility, and cytokine expression, that allows the virus to replicate in them. Finally, the immune response to fungi is also orchestrated by monocytes. In particular, PAMPs induce a cascade of signals that lead to phagocytosis and to the elimination of fungi (83). Due to their substantial involvement during immune response, monocytes undergo major morphological and functional changes mirrored by MDW. MDW is a sensitive indicator of monocyte activation that increase in response to bacterial, viral and fungal infection, but the evidence that MDW reliably and specifically identifies and discriminates the type and species of infecting microorganisms is very limited with a paucity of literature data (96). Cusinato et al. revealed that MDW values at hospital admissions were comparable for bacterial infection and viral infection (COVID-19) (97). Jo et al. performed a prospective observational study based on the potential relation between MDW levels and blood culture results. They suggested that MDW was significantly higher in positive blood cultures than in negative ones, but they didn’t evaluate whether the bacteria were mainly characterized as Gram positive or Gram negative. However, they showed that MDW increase also in patients with sepsis caused by viruses like in influenza, hepatitis A and C, and in COVID-19 condition (98). Piva et al. performed a prospective observational study on 506 patients (age range 18-89 years, 346 males and 169 females) admitted in ICU evaluating MDW, in whole blood samples collected in K2EDTA, and conventional biomarkers (PCT and CRP). Following Sepsis-3 criteria and SOFA score, 394 patients were classified as no sepsis, 108 as sepsis, and 4 as septic shock. In particular, infective microorganisms were defined as: 22.3% gram-negative (*E. coli*, *K.pneumoniae*, etc.), 25% gram-positive (*Enterococcus faecalis*, *S.aureus*, etc.), 25% viruses (*SARS-CoV-2*, influenza, etc.), 3.6% fungi (*Candida spp*., *Aspergillus fumigatus*, etc), and 17.9% culture-negative. The Authors reported that MDW values were significantly higher in patients with sepsis (IQR 23.48-29.83) or septic shock (IQR 21.27-37.21) than in those without sepsis (IQR 19.86-24.36). The highest median values were observed in septic shock (28.97). ROC curve analysis yielded and AUC of 0.785, with an optimal cut-off of 24.63, corresponding to a sensitivity of 66.9% and specificity of 77.8%. The diagnostic accuracy of MDW was comparable to that of PCT and superior to both CRP and WBC, confirming its important role as biomarker for sepsis detection in ICU. Relevant too is the increase of MDW values in relation of the causative infecting pathogen: MDW did not differ significantly across bacterial, viral or fungal sepsis. On the contrary, PCT showed the highest concentration in gram-negative infections, while viral and fungal sepsis were associated with lower levels, particularly in SARS-CoV-2 conditions. CRP usually increased in all groups compared to non-septic patients, with the highest values in fungal infections. The study of Piva et al. concluded that MDW values were elevated across sepsis of different aetiology and demonstrated a prognostic value. Survivors had a significant decrease in MDW while non-survivors maintained high values (87). Based on these fragmented pieces of literature and paucity of scientific data, MDW is actually defined not a pathogenspecific/pathogen-related biomarker, but further studies are needed to better define and understand this field of research. For this reason, a frame of our research focused on this topic, and in chapter 2 is reported the first papers which analyse MDW alterations in *E. coli* sepsis, in particular comparing in vivo and ex vivo models.

Cell Population Data (CPD)

Definition

Sepsis is a global medical emergency, and a rapid intervention is essential to avoid the worse outcome. Researchers and clinicians have focused their efforts on finding rapid, inexpensive and accurate biomarkers. Assuming that the crucial characteristic in patients at risk for sepsis is a dysregulated immune response to an infection, biomarkers that quantify morphological and functional alterations in immune cells are promising candidates. Neutrophils and monocytes are principal actors in the defence against infecting microorganisms; they react promptly through phagocytosis and releasing inflammatory cytokines. The most advanced haematology analysers enable the evaluation of new parameters alongside CBC, providing additional information on the morphology and function of leucocytes. Cell Population Data (CPD) are research-use-only (RUO) parameters which describe neutrophils, monocytes and lymphocytes providing quantitative information about their morphological and functional characteristics. Due to their several advantages CPD are potential sepsis biomarkers. They are automatically included in the CBC without the need for an additional sample or elaboration. They are always available and are the first test requested by clinicians when patients are at risk of sepsis. Therefore, CPD are fast, useful and inexpensive biomarkers to be evaluated in a panel together with other conventional biomarkers (99).

Beckman Coulter haematology analysers (Beckman Coulter Inc., Miami, FL, USA) employ VCS technology, which assess leucocyte morphology using three independent measurement modalities. The V component, through direct current impedance, quantifies cell volume, providing the degree of size variability. The C component, through radio frequency conductivity, evaluates the internal composition of cells, mirroring their structural and biochemical properties. The S component, through laser light scatter, quantify cytoplasmic granularity and nuclear morphology (99, 100). The underlying “Coulter principle” monitors changes in electrical resistance while cells, suspended in a conductive diluent, pass through a small opening. Electrodes positioned on either side of this opening detect transient increased in resistance caused by each cell, generating electrical pulses. From these pulses is determined the white blood cell count (99). In the latest Coulter model, the UniCel DxH 800 (Beckman

Coulter Inc., Miami, FL, USA), the advanced flow cell design enables the collection of multi-angle light scatter measurements, thus obtaining a CPD panel including mean (MN) and standard deviation (SD) of volume, conductivity, and five-angle light-scattering parameters, for neutrophils, monocytes, lymphocytes, and eosinophils (101). In particular, the five-angles are: median angle light scatter (MALS), lower MALS (LMALS), and upper MALS (UMALS), which provide information on granularity and membrane surface characteristics, axial light loss (AL2) which reflect cellular transparency), and the low-angle light scatter (LALS) which is a complexity index (Figure 6) (99).

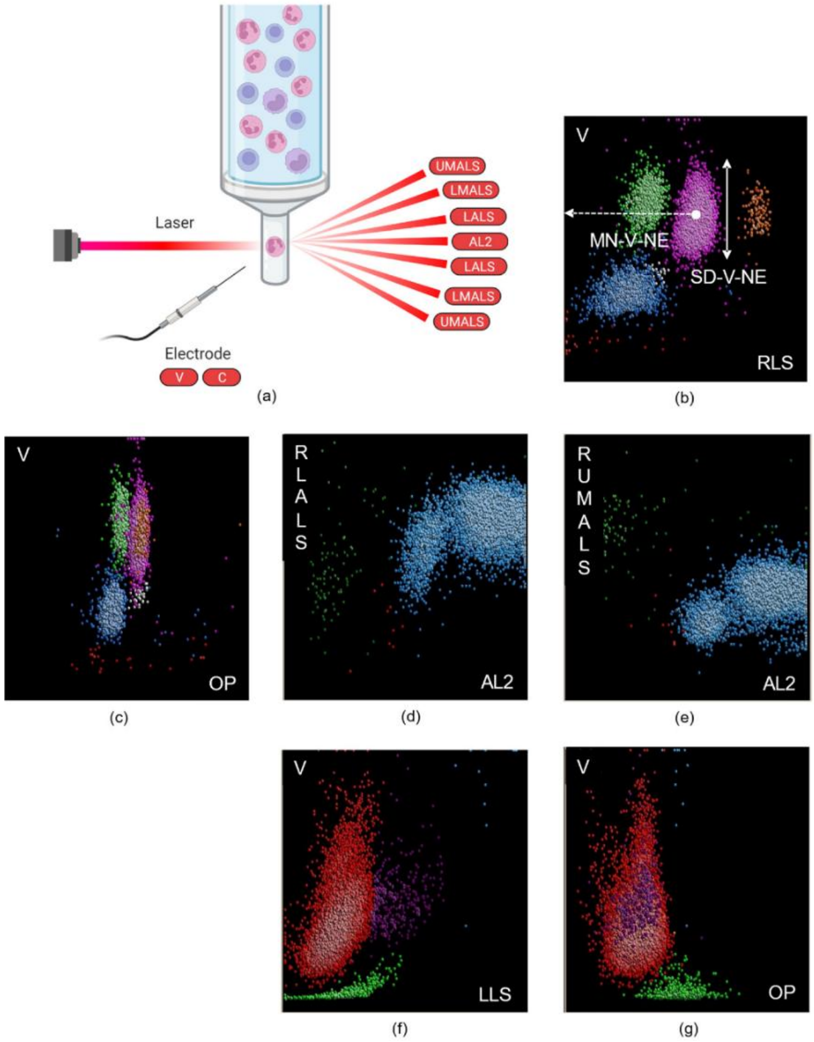

Figure 6: Coulter technology. (a) The light scattering is measured at different angles: UMALS, LMALS, LALS, AL2. (b) Scatterplot of V versus Rotated Light Scatter (RLS) identifies the five leucocyte subpopulations. (c) Scatterplot of V versus opacity (OP) differentiating leucocytes. (d) Nucleated red blood cell (NRBC) plot: scatterplot of rotated low-angle light scatter (RLALS) versus AL2. (e) NRBC plot: scatterplot of reflected upper-median-angle light scatter (RUMALS) versus AL2. (f) Scatterplot of V versus linear light scattering (LLS) showing in green platelets and debris, in purple reticulocytes and in red mature RBCs. (g) Scatterplot of V versus OP (102).

On the other hand, Sysmex analysers (Sysmex Corporation, Kobe, Japan) use fluorescence flow cytometry to measure CPD. This technique employs fluorescent dyes, staining nucleic acids and proteins, blood-cell membrane surfactant reagents, to generate information about cell-shape and scatter-light to measure granularity and size of cells (103). Combined with automated gating, this technology enables real-time and reproducible evaluation of neutrophils and monocyte activation. In the XN analysers, leucocyte populations (neutrophils, monocytes and lymphocytes) are visualized in 3D scattergram, where side scatter (X-axis) reflects internal complexity, fluorescence (Y-axis) indicates nucleic acid content, and forward scatter (Z-axis) corresponds to cell size. Means, standard deviations, and distribution widths are recorded for each leucocyte subset to evaluate the population heterogeneity (99, 104).

Role of CPD in sepsis diagnosis

Several research studies proved the clinical utility of CPD in detecting sepsis, representing promising parameters to be included in the management of sepsis (99). Here an overview of principal results is reported. Roccaforte et al. demonstrated that neutrophils complexity (NE-SSC), neutrophils fluorescence intensity (NE-SFL), width of dispersion of neutrophils fluorescence (NE-WY), monocytes complexity (MO-X), monocytes fluorescence intensity (MO-Y) are significantly different between septic and non-septic patients. Moreover, Neutrophils size (NE-FSC), NE-Y, width of dispersion of neutrophil size (NE-WZ), and MO-X are able to discriminate sepsis and septic-shock. This study was conducted using Sysmex XN 3000 hematology analyzer and they concluded that NE-SSC, NE-SFL, width of dispersion of neutrophils complexity (NE-WX), MO-X, MO-Y, PCT and CRP have the best diagnostic value for sepsis (104). Mishra et al. in their study, using Sysmex XN1000 analyser, found that LY-X, LY-Z, MO-X, MO-Y, NE-WZ, LY-WX, LY-WY, and LY-WZ were significantly high in septic patients. Particularly, MO-X, MO-WY, LY-X, and LY-Z are best discriminators. MO-X had 95.83% sensitivity and 73.61% specificity (AUC 0.876), MO-WY had 84.72% sensitivity and 45.83% specificity (AUC 0.597), and LY-X had 93.06% sensitivity and 48.61% specificity (AUC 0.685). Moreover, MO-X, LY-X, and LY-Z correlates with SOFA score, and MO-X and High Fluorescence Lymphocyte Count (HFLC) correlates with procalcitonin. They emphasized the potential role of CPD as quick markers of sepsis, highlighting that further studies are needed to optimize the clinical application of CPD (105). Kaneda et al. using Sysmex XN-2000 demonstrated that NE-WY and NE-SFL changed in a time-dependent manner in patients with bacterial infections. In particular, NE-WY levels rose earlier after the onset of sepsis compared with NE-SFL. The strong correlation between NE-WY and circulating bacteria suggests that this CPD may serve as indicator of sepsis. Furthermore, the rapid decline of NE-WY in patients who improved their condition of sepsis, supports its role as marker of therapeutic response (106). Unlu et al. evaluated the diagnostic performance of CPD generated by DxH 900 Beckman Coulter for their application in the detection of neonatal sepsis. They demonstrated that mean neutrophil volume (MN_V_NE), the standard deviation of neutrophil volume (SD_V_NE), the standard deviation of monocyte volume (SD_V_MO), and MDW were significantly higher in culture-confirmed sepsis group compared with both the clinical sepsis and control group. This study proved that CPD, particularly MN_V_NE, MDW and their combination had a diagnostic relevance in neonatal sepsis (100). Çevlik et al in their prospective study assessed the diagnostic performance of CPD in sepsis, comparing them with cases of non-systemic infection and both acute and chronic non-infectious inflammatory conditions. They used the UniCel DxH 800 Beckman Coulter. MN_V_NE was significantly higher in sepsis group compared to others, SD_V_NE was also elevated in sepsis, distinguishing sepsis from other groups. Combinations of CPD such us SD-V-NE + SD-V-LY + SD-V-MO, SD-V-NE + SD-V-MO, and MN-V-NE + SD-V-NE + SD-C-LY + SD-V-MO achieved higher AUC than procalcitonin. They concluded that CPD appear to be promising tools for distinguishing sepsis from non-sepsis conditions (107). Park et al. evaluated the diagnostic utility of CPD in the screening of sepsis and fungemia through DxH 800 haematology analyser Beckman Coulter. They reported that in sepsis samples neutrophil and monocyte volumes were significantly increased, whereas neutrophil light scatter values were markedly reduced. ROC curve demonstrated high diagnostic accuracy for lymphocyte SD (sensitivity 78–89%, specificity 78–87%), monocyte volume (sensitivity 88.2%, specificity 87.3%), and monocyte volume SD (sensitivity 93.1%, specificity 91.0%). About fungemia, only the lower-angle light scatter of lymphocyte SD showed a discriminatory performance between fungemia and bacteraemia (sensitivity 74.1%, specificity 72.4%). Authors suggested that incorporating CPD into management of sepsis could enhance the screening and early identification of sepsis in clinical practice (101). He et al. proposed a retrospective study concluding that the following leucocyte parameters, obtained through Sysmex XN9000, HFLC#, Immature granulocyte absolute concentration, NE-WX, LY-WX, LY-

WY, and MO-WX, cover an auxiliary role in the diagnosis of sepsis. Moreover, PCT showed a lower predictive ability of clinical outcome compared to the combination of WBC and LY-X, at discharge (108).

CPD are studied not only to discriminate bacterial sepsis conditions, but also in viral states such as those associated with COVD-19. Vasse et al. studied the role of CPD, provided by DxH 800 analyser Beckman Coulter, in the identification of SARS-CoV-2 infection. They developed an algorithm based of the combination of 4 monocyte CPD (SD-V-MO, MN-C-MO, SD-LALS-MO, and SD-UMALS-MO) to discriminate patients with SARS-CoV-2 infection from patients without infection. Among 222 patients, 60.5% were successfully identified through the algorithm. False negatives were mainly patients with a low inflammatory condition while false positive were principally sepsis patients. They concluded that CPD should contribute to the early diagnosis of COVID-19 (109).

Örmen et al. studied the morphological and functional changes of leucocytes in patients with COVID-19, using DxH 800 Beckman Coulter. They found that neutrophil count increased while lymphocyte count decreased in patients with severe infection compared to mild infection. A statistically significant increase in both the percentage of neutrophils and the neutrophil-to-lymphocyte ratio was observed in patients with severe disease, compared to those with moderate or mild infections. Moreover, the alterations in neutrophil volume and conductivity varied significantly according to disease severity. In severe cases, lymphocyte volume, lymphocyte volume standard deviation, and lymphocyte conductivity, as well as monocyte volume and its standard deviation, were markedly increased compared to patients with mild disease. The increase of lymphocyte and monocyte volumes in the severe group is also significantly different from that in the moderate group. In conclusion, COVID-19 disease induces alterations in leukocyte populations and CPD are related to disease severity (110).

In summary, CPD are being studied for their ease of use, speed of acquisition, cost-effectiveness, and, most importantly, their usefulness in diagnosing bacterial and viral sepsis in combination with conventional biomarkers. Further studies and clinical trials are needed to obtain results on larger populations and to establish standard of use, as currently they remain parameters for research use only.

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Chapter 2

Comparative analysis of Monocyte Distribution Width alterations in Escherichia coli sepsis: insights from in vivo and ex vivo models

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Comparative analysis of monocyte distribution width alterations in *Escherichia coli* sepsis: insights from *in vivo* and ex vivo models

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Abstract

Objectives: Monocyte distribution width (MDW) is an early sepsis indicator measuring monocyte heterogeneity during massive infection. We compared MDW changes in *Escherichia coli* sepsis patients with the effects of living *E. coli* and lipopolysaccharide in an *ex vivo* sepsis model. We also investigated the dynamics of monocyte morpho-functional and inflammatory responses in the sepsis model.

Methods: Whole blood from healthy participants was *in vitro* stimulated with live *E. coli* (10^6–10^{10} CFU/mL) and LPS (0.1–10 µg/mL). Complete blood counts, including MDW, were evaluated at different time-points using DxH 690T Hematology Analyzer (Beckman Coulter). MDW values were compared with those retrospectively obtained from sepsis patients (n=23). May-Grunwald-Giemsa-stained blood smears were analyzed by digital cell morphology (CellaVision DM software). A panel of 27 inflammatory mediators was quantified in plasma (Bio-Plex 200).

Results: MDW values were early and significantly increased in a dose- and time-dependent manner by live *E. coli* and LPS treatments ($p<0.01$). MDW values were significantly higher in sepsis patients compared to controls and overlapped those observed in the *ex vivo* model. IL-1β, TNF-α, IL-8, MIP1-α, MIP-1β, Eotaxin, G-CSF, and PDGF-bb were significantly modulated after treatments.

Conclusions: Our findings confirm the clinical utility of MDW in sepsis diagnosis and sustain the reliability of the whole blood assay as *ex vivo* sepsis model. *E. coli* and LPS directly promote early monocyte morpho-functional modifications, mirrored by high MDW values and pro-inflammatory mediators. These results improve the knowledge on the biological basis of sepsis, providing novel evidence on the usefulness of MDW in septic conditions.

Keywords: biomarker; digital morphology analysis; *E. coli*; inflammation; monocyte distribution width; sepsis

Introduction

Sepsis is a life-threatening organ dysfunction due to a dysregulated host response to infections (Sepsis-3) [1]; this definition emphasizes the crucial role of the individual immune response to a critical infective pathogen, which may be significantly amplified by endotoxins and/or endogenous factors.

Bacterial infections represent the primary cause of sepsis, and *Escherichia coli (E. coli)* is among the most frequently isolated gram-negative species in sepsis patients [2]. Lipopolysaccharide (LPS) from the Gram-negative bacterial membrane is the most studied endotoxin, due to its

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well-known role as trigger of pro-inflammatory and immune responses [3].

Once activated, immune cells mount a series of defensive responses, characterized by hemodynamical changes and blood cell morpho-functional modifications. Moreover, the release of inflammatory mediators and reactive oxygen species from activated immune cells can directly damage endothelial cells and fuel the activation of other immune cells, finally leading to endothelial dysfunctions, micro/macro-circulatory alterations, and multi-organ failure [4]. The kaleidoscope of the immune responses found in sepsis shares these complex cascades and activation mechanisms, with a wide individual variability of the responses [5], being some patients moving from excessive immune activation toward extensive immune suppression, up to a coexistence of simultaneous hyperinflammation and sepsis-induced immune suppression [6].

Several studies have explored the effects of both LPS and living or heat-inactivated *E. coli* bacteria in a whole blood model to simulate *in vitro* a sepsis condition and provide insights on inflammatory and immune pathways activated by endotoxins and pathogens [7–13].

Monocyte distribution width (MDW) has been CE-marked as an early sepsis indicator in the emergency department, making it a potential marker for the early diagnosis of sepsis [14]. MDW is an IVD parameter available on Beckman Coulter's DxH690T and DxH900 hematology analysers that quantify the heterogeneity and anisocytosis of the monocyte population circulating in blood. It can be obtained quickly and simultaneously with the complete blood count (CBC) and the differential leukocyte formula, being automatically calculated from the standard deviation of the monocyte volume, through a patented mathematical formula, exploiting the VCS (volume, conductivity, and scatter) technology, using three independent and simultaneous energy sources (direct current impedance to measure cell volume of all cell types; radio frequency opacity to characterize conductivity for the internal composition of each cell; a laser beam to measure light scatter for cytoplasmic granularity and nuclear structure).

Increasing evidence underlines that higher values of MDW are found in sepsis patients, and these values significantly increase with the worsening of the sepsis conditions [15–19]. MDW has been shown to be increased in sepsis of bacterial, viral, and fungal origin, without apparently different levels among pathogens, despite that these microorganisms exploit characteristic and diverse pathways to promote immune activation [20]. Moreover, we recently demonstrated that extracellular histones, acting as DAMP proteins, promote monocyte morpho-functional alterations and a MDW increase [21–23] in an *ex vivo* whole blood model.

Here we aimed to compare the MDW alterations measured in patients with sepsis from *E. coli* with the effects of LPS and living *E. coli ex vivo* stimulation to investigate both basic mechanisms and pathways established during active infections, with particular emphasis on the ability of LPS and living *E. coli* to induce morphological alterations in blood cells, MDW modifications, and the ability to trigger a peculiar pattern of cytokine production by blood cells.

Materials and methods

Sample collection, hematological analysis, and digital morphology

Peripheral venous blood was collected in EDTA-K_2 tubes (BD Vacutainer®, 6 mL) from healthy adult subjects (n=24 for *E. coli* studies and n=10 for LPS curve studies) recruited as anonymous volunteers at the Blood Transfusion Centre of the Hospital Santa Maria della Misericordia of Urbino during their periodic blood donation session. Healthy donors fulfill all the requirements needed for blood donation. In detail, the inclusion criteria were donors of all genders, aged 18–65 years, ≥50 kg, BP≤180/100; HR=50/100 bpm; Hb=13.5/12.5 g/dL (M/F), no known hematological, oncological, and infective diseases. Exclusion criteria included: positive tests for HbsAg, anti-HCV antibodies, anti-HIV 1–2 antibodies and HIV 1–2 antigens, anti-Treponema Pallidum (TP) antibodies. All donors signed an informed consent form. Samples were processed within 4 h from blood collection at the Clinical Biochemistry Laboratories of the University of Urbino.

Routine complete blood cell counts and MDW were analysed on a UniCell DxH 690T Hematology Analyzer (Beckman Coulter, Inc., Brea, USA). The instrument performance has been daily validated by using three different commercially available internal quality controls: COULTER 6C Plus Cell Control (three concentration levels) to monitor the DxH 690T system performance for CBC, DIFF and NRBC parameters, COULTER Retic-X Cell Control (three concentration levels) for monitoring system performance of the reticulocyte parameters, and COULTER LATRON CP-X Control, a suspension of latex particles of standardized size used to monitor VCSn technology (volume, conductivity, and light scatter measurements). Each sample has been assayed once, except for the samples treated with the highest *E. coli* concentrations, which required multiple measurements due to the appearance of instrumental flags.

Manually prepared air-dried smears were stained through the automated slide preparation system (Sysmex SP-50) with May-Grunwald-Giemsa-stain for digital cell morphology analyses with the CellaVision DM software (DI-60).

This observational non-interventional *ex vivo* study was approved by the local Ethical Committee (on the basis of an official document of accordance with the Blood Transfusion Center of "S. Maria della Misericordia" Hospital in Urbino (PU), Italy) and all investigations have been conducted according to the Declaration of Helsinki principles as revised in 2013.

Bacterial strain, preparation of bacterial suspensions, and growth condition

The bacterial strain used in this study was *E. coli* ATCC 25922. Bacteria were routinely grown at 37 °C on Muller–Hinton (MH) agar.

For each experiment, one colony obtained from a fresh culture was transferred in 20 mL of MH broth and incubated at 37 °C for 14–18 h (usually overnight) with the cap slightly loosened.

The next day, the overnight culture was harvested, washed, resuspended twice in saline solution (NaCl 0.9 %), and then adjusted by spectrophotometry at optical densities at 600 nm (OD_{600}), corresponding to a concentration of 10^{10} colony forming unit (CFU)/mL. Then, from this bacterial suspension, 10-fold serial dilutions in saline solution (NaCl 0.9 %), from 10^{-1} to 10^{-4} were prepared in 10 mL final volumes. Aliquots (1 mL) of this dilution were placed into a microcentrifuge tube and centrifuged for 15 min at 6000×*g*, to form a pellet, and the supernatant was carefully removed with a pipette to ensure the pellet was not dislodged; blood was immediately added to the pellet. Amounts equivalent to a final concentration of 10^6, 10^8, and 10^{10} CFU/mL were used as stimuli for the human whole blood model.

Ten microliters of each dilution of *E. coli* suspension were plated in triplicate on tryptone soya agar (TSA) plates and incubated at 37 °C for 18–24 h for the final enumeration of CFU/mL.

All culture materials were purchased from ThermoFisher Scientific (Waltham, MA USA).

Whole blood *in vitro* stimulation with LPS or living *E. coli*

Aliquots of 1 mL of EDTA-K_2 whole-blood from each volunteer were immediately incubated with 0.1, 0.5, 1.0, 5.0, and 10 μg/mL of lipopolysaccharide (LPS from *E. coli* O127:B8, Sigma-Aldrich cod. L3129) or living *E. coli* suspension to a final concentration of 10^6, 10^8, and 10^{10} CFU/mL at RT (about 25 °C).

CBC results, including MDW, were measured at 0, 30, 60, and 180 min after careful mixing to avoid blood cell sedimentation. After 3 h, all blood samples were centrifuged (2000×*g*, 15 min) to obtain plasma for further analyses. All tubes, tips and solutions were endotoxin free to avoid artefactual introduction of endotoxin.

Determination of the total protein concentration

Plasma samples were assayed by Bradford Protein Assay (Bio-Rad, USA) to determine the total protein concentration through Onda V-30 Scan Spectrophotometer (Giorgio Bormac, Italy).

MDW measurement in sepsis patients

MDW values and clinical data of sepsis patients (n=23; age range: 44–87 years; age mean: 67 years) diagnosed with *E. coli* bloodstream infection were retrospectively extracted by data archives of the Laboratory Medicine Department, Laboratori Clínic Metropolitana Nord (LCMN), University Hospital Germans Trias i Pujol, Badalona, Spain. At the same time, MDW values and clinical data from unaffected controls (n=135; age range: 19–80 years; age mean: 54 years) were collected to overcome the possible confounding factor due to the different anticoagulants used (EDTA-K_3) compared with the *ex vivo* study.Data from septic patients and control subjects were collected from two different studies carried out in the Emergency Department and Intensive Care Unit of Germans Trias i Pujol University Hospital. Both studies were carried out according to the basic ethical principles of the Helsinki Declaration (Fortaleza, October 2013). Both *in vivo* studies were reviewed and approved by the Clinical Research Ethics Committee of the Hospital (PI-18-249 and PI-18-140). All patients included in these studies have signed an informed consent previously approved by the CEIC (Ethics Committee for Clinical Investigation).

Cytokine determination by multiplex immunomagnetic assay

After 3 h of treatments, all plasma samples obtained were assayed to evaluate a panel of 27 inflammatory biomarkers through the Pro™ Human Cytokine 27-plex assay (including: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin/CCL11, bFGF, G-CSF, GM-CSF, IFN-γ, IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, PDGFbb, RANTES, TNF-α, and VEGF) according to the manufacturer's instructions (BioPlex, Bio-Rad Labs) as previously reported [22]. The cytokine concentrations (pg/mL) were calculated through a 8-point standard curve performed in duplicate.

Statistical analysis

Regarding CBC parameters, differences among groups and times were determined using a mixed-effects model followed by Tukey's multiple comparison test. The Mann-Whitney test was selected for comparing MDW differences between two groups and Kruskal–Wallis with Dunn's Multiple comparison test was chosen for comparing differences among multiple groups. Concerning inflammatory mediators, differences among groups were determined using a mixed-effects model followed by Dunnet's multiple comparison test. Values are expressed as mean±standard deviation (SD) unless otherwise specified, and p-values <0.05 were considered significant. All statistical tests were performed using GraphPad Prism 9.0.

Results

Digital morphology analyses

The treatment of whole blood aliquots with increasing concentrations of *E. coli* and LPS was associated with the early appearance of monocyte anisocytosis. Monocytes lose their round shape with a reniform nucleus (Figure 1A) in favour of an increased cell volume with intracellular vacuolization and nuclear alterations (Figure 1B–G) and clear evidence of phagocytosis of live *E. coli* bacteria (Figure 1B–E).

Complete blood count and MDW determination in LPS-stimulated blood samples

CBCs, including MDW, were anonymously determined in 10 healthy donors (6 M, 4 F; age range: 31–65; mean age: 46.1 years) before and after stimulation with increasing LPS doses. MDW values in controls reached a mean ± SD value of 17.4 ± 1.6 at T0, 17.8 ± 1.3 at T30, 17.7 ± 1.9 at T60, and 18.4 ± 1.3 at T180, without significant differences among all times considered.

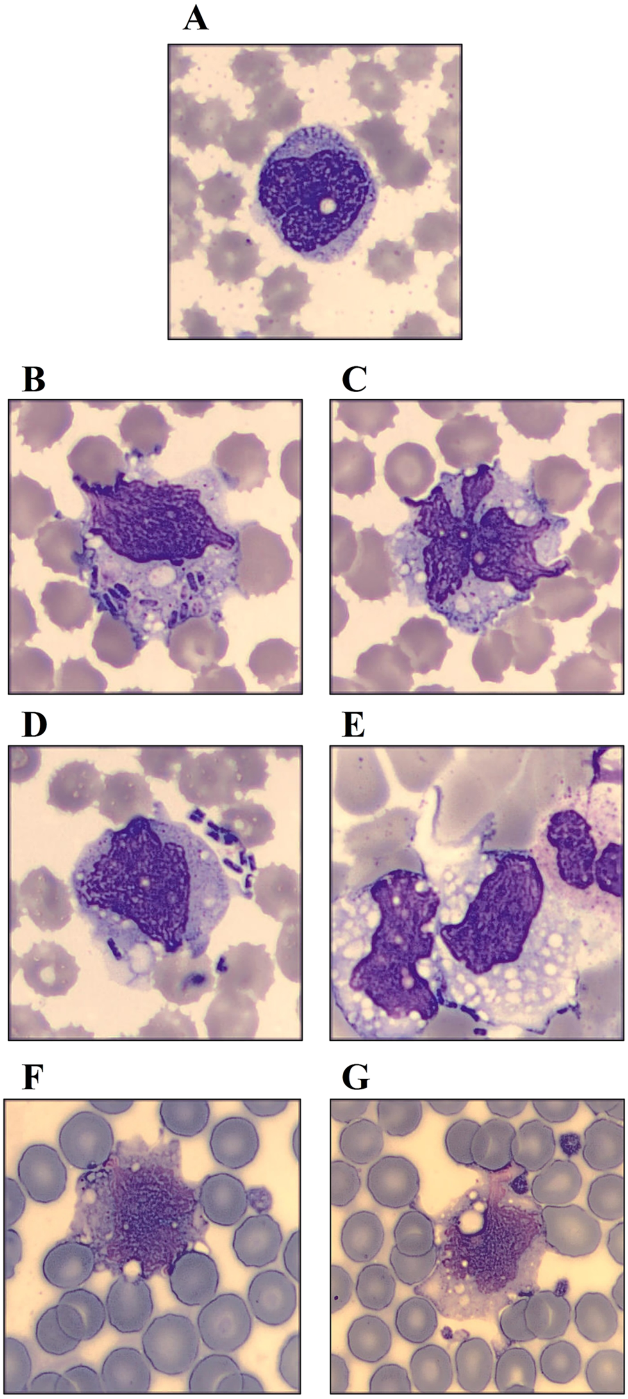


Figure 1: Digital light microscopy images of peripheral blood monocytes representative of untreated whole blood controls (A), and after 3 h of treatments with *E. coli* 10^6 CFU/mL (B, C), *E. coli* 10^8 CFU/mL (D, E) and LPS 1 µg/mL (F, G). (May-Grunwald-Giemsa Staining, ×100).

The *in vitro* stimulation of whole blood with increasing concentrations of LPS from *E. coli* induced a significant time- and dose-dependent MDW increase without affecting the monocyte count (Figure 2A–C). In detail, MDW reached values of 20.4 ± 1.6, 21.4 ± 1.4, 23.7 ± 1.7, 27.5 ± 4.1, and 26.9 ± 4.1 (mean ± SD) after 3 h of treatment with 0.1, 0.5, 1.0, 5.0, and 10.0 μg/mL of bacterial LPS. The two highest concentrations of LPS showed overlapping MDW modifications at each time point explored, highlighting a possible plateau effect (Figure 2A). A representative time- and dose-dependent two-dimensional data plots of all WBC subpopulations separated according to volume and RLSs values is reported in Figure 2D, where the green cloud represents the monocyte population; a focus on the monocyte population is also reported as surface plots (Figure 2E).

Complete blood count and MDW determination in *E. coli*-stimulated blood samples

CBCs and MDW were evaluated in 24 healthy donors (18 M, 6 F; age range: 18–65; mean age: 42.5 years) before and after stimulation with increasing concentrations of live *E. coli*. MDW values in controls reached a mean ± SD value of 17.2 ± 1.4 at T0 and T30, 17.8 ± 1.6 at T60, and 18.8 ± 1.4 at T180, without significant differences among all times considered. The treatment of whole blood with living *E. coli* determined a time- and dose-dependent MDW increase (Figure 3A). In detail, *E. coli* 10^6 CFU/mL induced a significant time-dependent increase of MDW at 30, 60, and 180 min (mean±SD) vs. respective controls (19.2 ± 2.1, p=0.0078; 20.5 ± 2.2, p<0.0001; 21.7 ± 3.7, p=0.0005, respectively) (Figure 3A).

MDW increases to 21.2 ± 2.6 (p<0.0001) after 30 min, 23.5 ± 3.4 (p<0.0001) after 60 min, and 26.1 ± 5.0 (p<0.0001) at 180 min were observed by treating whole blood with *E. coli* 10^8 CFU/mL.

The treatment with *E. coli* 10^{10} CFU/mL promoted a MDW increase to 23.0 ± 2.1 (p<0.0001) after 30 min, 26.3 ± 2.8 (p<0.0001) after 60 min, and 29.0 ± 4.9 (p<0.0001) at 180 min (Figure 3A).

These Gram-negative related MDW modifications were not accompanied by a change in the monocyte population count, neither in the total count (Figure 3B) nor in percentage values (Figure 3C), whose mean values were distributed within the physiological ranges (0,2-1,2 × 1000/μL and 2–12 %, respectively). A representative time- and dose-dependent two-dimensional data plots of all WBC subpopulations separated according to volume and RLSs values is reported in Figure 3D, where the green cloud represents the monocyte population; a focus on the monocyte population is also reported as surface plots (Figure 3E).

MDW determination in sepsis patients

The retrospective analyses of MDW values in sepsis patients affected by *E. coli* infection, compared with unaffected controls recruited at the same Hospital, revealed that MDW values in sepsis patients were significantly higher compared to control subjects (MDW in sepsis – min-max: 22.4–49.3; median: 28.2; MDW in controls-min-max: 15.2–28.3; median: 20.0; p<0.0001) (Figure 4A).

Of note, these samples have been collected in EDTA-K_3, thus, to ensure a correct comparison between *in vivo* and *ex vivo* results, we normalized MDW from patients and treated samples (at 3h) for the median MDW value of the control cohort, and for the corresponding untreated controls in the *ex vivo* dataset, respectively. The percentage fold-of-change calculated after normalization confirmed the differences emerged in the two models between sepsis patients and controls, as well as between *E. coli* treated samples and controls, revealing also the absence of significant differences in MDW levels between sepsis patients and whole blood samples *ex vivo* triggered with *E. coli* for 3h (Figure 4B).

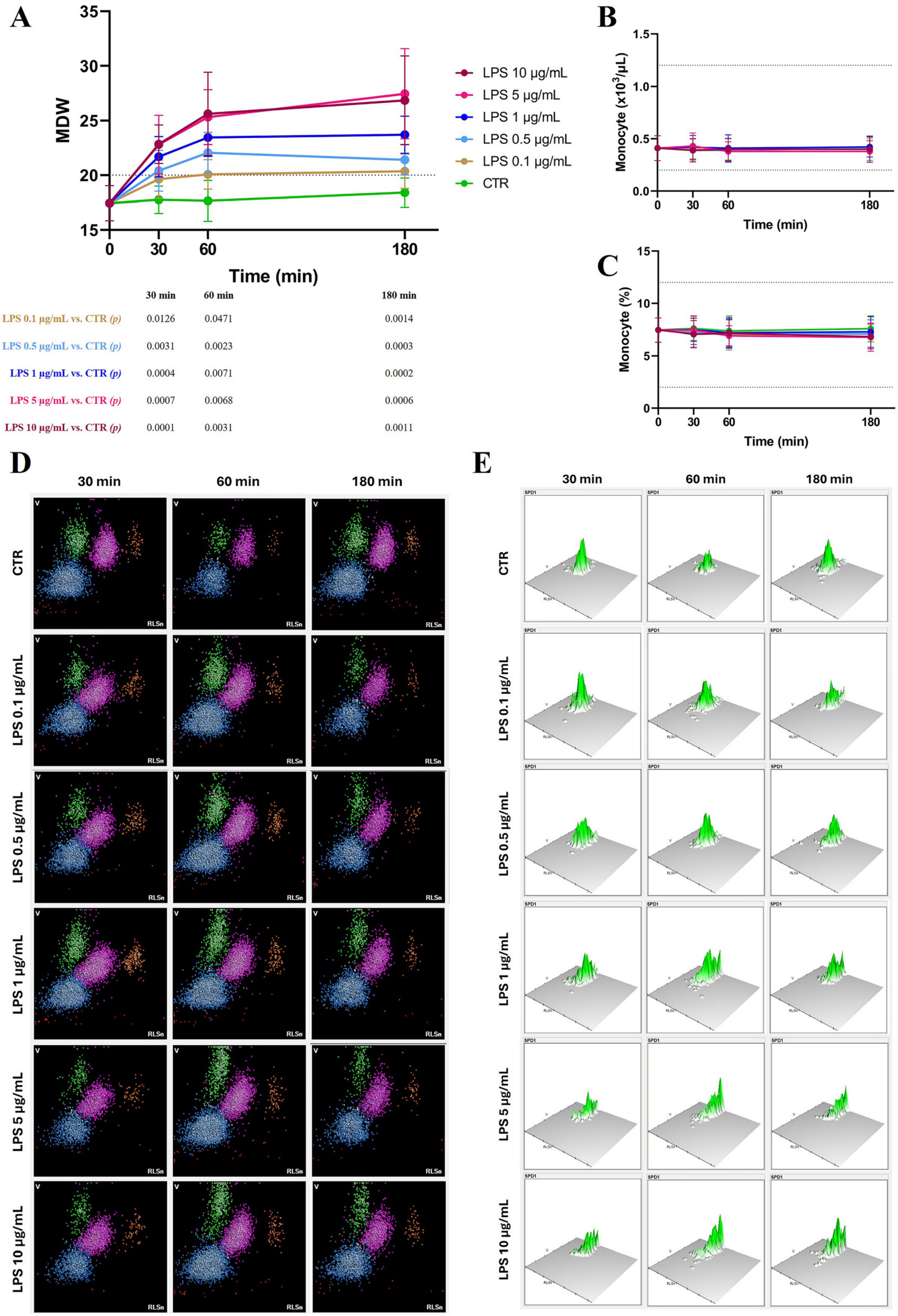
Cytokine quantification by multiplex Immunomagnetic assay

The total protein content was not significantly different between plasma from controls and *E. coli*/LPS-stimulated blood cells (mean mg/mL±SD; CTR: 45.8 ± 5.3; EC10^6 CFU/mL: 45.6 ± 3.9; EC10^8 CFU/mL: 46.2 ± 4.9; EC10^{10} CFU/mL: 46.6 ± 3.3; LPS: 45.6 ± 9.1).

After 3 h of treatment with *E. coli* 10^6 and 10^8 CFU/mL (due to multiple CBC readings of *E. coli* 10^{10}-treated whole blood, the remaining sample volume was not sufficient to quantify the cytokine panel), we observed a dose-dependent increase of peculiar cytokines, as shown in Figure 5.

In detail, we highlighted that the levels of MIP-1α/CCL3 were significantly increased by all triggers (*E. coli* 10^6, 10^8 CFU/mL and LPS) compared with untreated controls, showing the strongest responses (fold of change vs. controls: *E. coli* 10^6 CFU/mL: 23.4-fold, p<0.05; *E. coli* 10^8 CFU/mL: 21.0-fold, p=0.001–0.01; LPS: 48.2-fold, p<0.05) (Figure 5).

The levels of IL-8/CXCL8 also showed a significant increase of about 9.1-fold (p<0.05), 17.0-fold (p=0.001–0.01), and 10.2-fold (p=0.001–0.01) after treatments with *E. coli* 10^6 CFU/mL, *E. coli* 10^8 CFU/mL, and LPS, respectively. Levels of TNF-α were significantly up-regulated from 5- to 10-fold vs. untreated controls, after living *E. coli* and LPS treatments (Figure 5).



	30 min	60 min	180 min
LPS 0.1 µg/mL vs. CTR *(p)*	0.0126	0.0471	0.0014
LPS 0.5 µg/mL vs. CTR *(p)*	0.0031	0.0023	0.0003
LPS 1 µg/mL vs. CTR *(p)*	0.0004	0.0071	0.0002
LPS 5 µg/mL vs. CTR *(p)*	0.0007	0.0068	0.0006
LPS 10 µg/mL vs. CTR *(p)*	0.0001	0.0031	0.0011

Figure 2: Time- and dose-dependent MDW variations (A) and monocyte absolute (B) and percentage (C) counts in whole blood stimulated with increasing concentrations of LPS from *E. coli* (0.1, 0.5, 1.0, 5.0, 10 µg/mL). Values are expressed as mean±SD. The table under Figure A reports the significance values resulting from the statistical analysis (the comparisons were calculated among treatments and respective controls at each time). Representative time- and dose-dependent two-dimensional data plots (D) and monocyte surface plots (E) resulting from whole blood stimulated with increasing concentrations of LPS from *E. coli* (0.1, 0.5, 1.0, 5.0, 10 µg/mL). In (D) all WBC subpopulations were separated according to volume and RLSs values; in (E) volume and RLS of monocytes are plotted (RLS, Rotated Light Scatter); in (D) and (E) the monocyte population is represented in green.

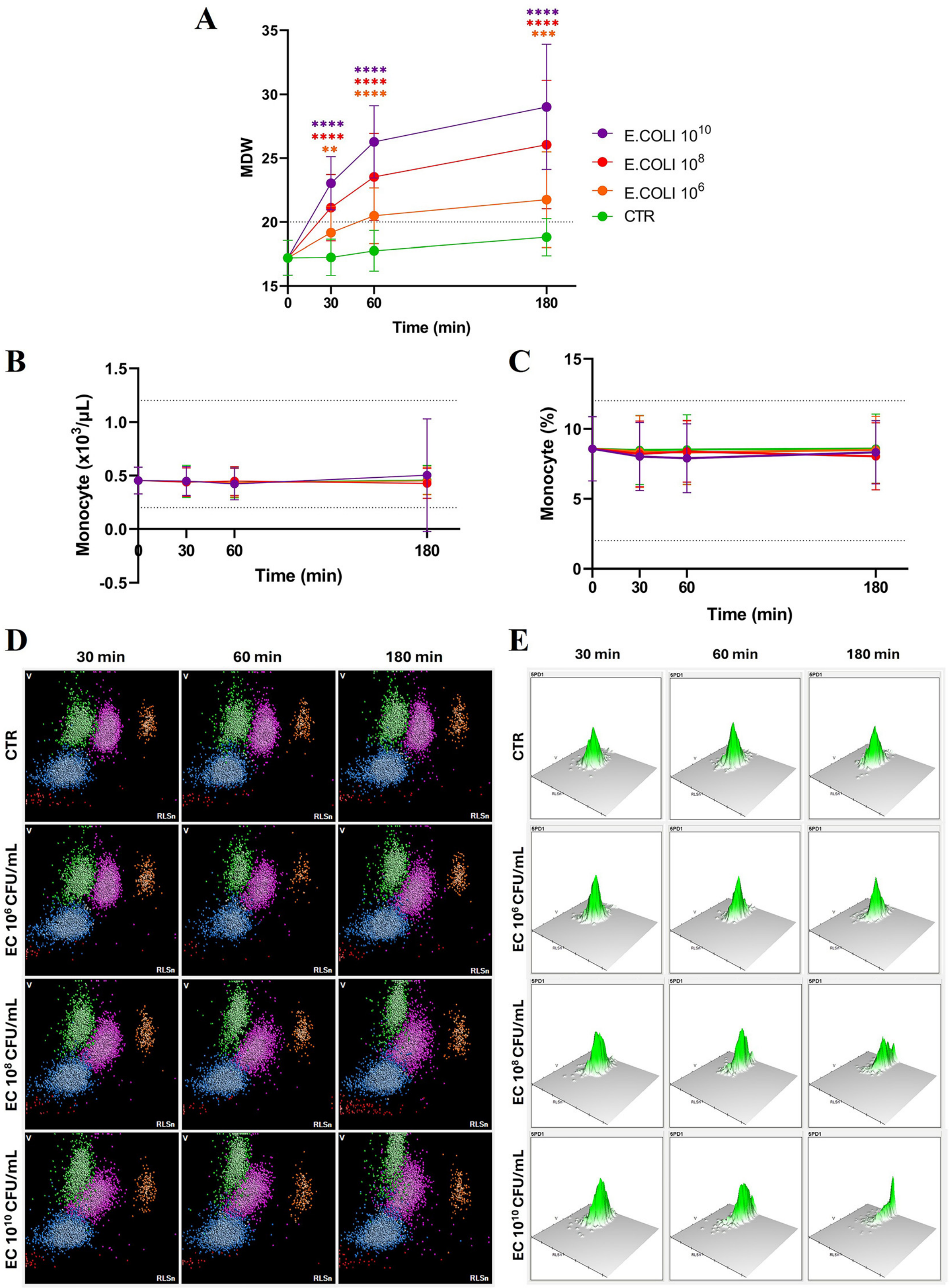


Figure 3: Time- and dose-dependent MDW variations (A) and monocyte absolute (B) and percentage (C) count in whole blood stimulated with increasing concentrations of living *E. coli* (10^6, 10^8, 10^{10} CFU/mL). Values are expressed as mean±SD. The dotted line indicates normal values. The comparisons were calculated among treatments and respective controls at each time. (**p=0.001–0.01; ***p=0.0001–0.001; ****p<0.0001). Representative time- and dose-dependent two-dimensional data plots (D) and monocyte surface plots (E) resulting from whole blood stimulated with increasing concentrations of live *E. coli* (10^6, 10^8, 10^{10} CFU/mL). In (D) all WBC subpopulations were separated according to volume and RLSs values; in (E) volume and RLS of monocytes are plotted (RLS, Rotated Light Scatter); in (D) and (E) the monocyte population is represented in green.

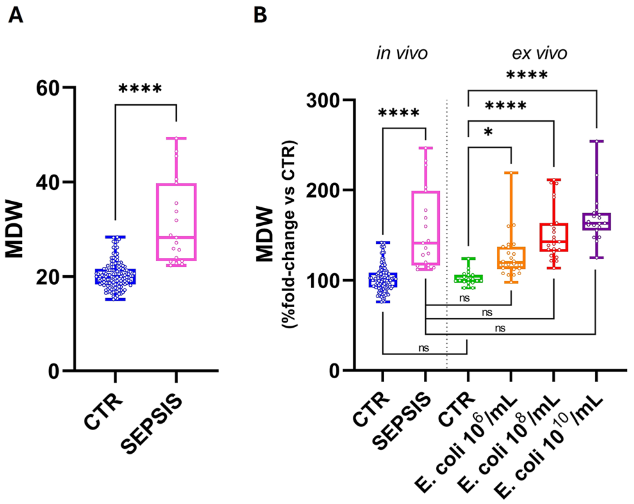


Figure 4: MDW variations in sepsis patients affected by *E. coli* infection and unaffected controls. In (A) MDW values are expressed as absolute values, as measured by DxH900 in EDTA-K$_3$ samples; in (B) MDW values are reported as percentage fold-of-change to of the values measured *in vivo* and, in the *ex vivo* model (further details in the main text). Values are expressed as box and whiskers, showing min to max values, median and 25th to 75th percentiles. Statistical tests: Mann–Whitney (A) and Kruskal–Wallis with Dunn's Multiple comparison test (B); *p=0.01–0.05; ****p<0.0001.

G-CSF levels were significantly increased by about 2.9-fold (p<0.05), 3.9-fold (p=0.001–0.01), and 5.5-fold (p=0.001–0.01) after treatments with *E. coli* 10^6 and 10^8 CFU/mL, and LPS, respectively.

Plasma levels of IL-1β showed a significant increase of about 1.1-fold, 1.6-fold (p<0.05), and 1.8-fold after treatments with *E. coli* 10^6 CFU/mL, *E. coli* 10^8 CFU/mL, and LPS, respectively.

A minor significant up-regulation was observed for MIP-1β/CCL4 of 1.2-fold, 1.2-fold (p<0.05), and 1.7-fold after treatments with *E. coli* 10^6 CFU/mL, *E. coli* 10^8 CFU/mL, and LPS, respectively.

Finally, a significant decrease of Eotaxin/CCL11 was observed after *E. coli* 10^6 and 10^8 CFU/mL treatments (p=0.01–0.05); a significant down-regulation of PDGF-bb was also observed after *E. coli* 10^8 CFU/mL stimulation (p=0.01–0.05) (Figure 5). According to the 8-points standard curves and the Limit of Detection (LOD) of the assay IL-2, IL-15, and VEGF were removed from the analyses due to the limited number of samples with measurable levels of cytokines.

A complete overview of cytokine behavior, according also to age and gender differences is provided in Figure 6. The heat map shows the mean value (pg/mL) of each cytokine measured in untreated controls, *E. coli* 10^6 and 10^8 CFU/mL, and LPS treated samples, obtained both in the whole cohort of donors, and classified according to gender and age (cut-off 45 years).

These findings suggest preliminary indications on potential age- and sex-related differences in cytokine-mediated immune responses associated with bacterial infection. Indeed, certain cytokines appeared to be differently modulated by the same trigger in subjects of different sexes (e.g., IL-8) and age ranges (e.g., MIP-1α, IL-8, and PDGFbb). Further experiments in a larger cohort of subjects are required to confirm this observation.

Discussion

The present study investigated the ability of live *E. coli* and its endotoxin LPS to promote monocyte morpho-functional alterations and the dynamic of various cytokines and chemokines produced by blood cells, and to evaluate how these could be reflected and quantified by MDW.

Firstly, a wide variety of anticoagulants have been described in literature to collect whole blood for exploring the effect of live or heat-killed bacteria on selected targets (e.g., to avoid the complement system inhibition [10] and to inhibit thrombin [24]). Importantly, according to the current guidelines for the measurement of MDW and multiplexing of cytokines, we collected whole blood samples with EDTA-K$_2$ as anticoagulant [14, 25].

Noteworthy, EDTA by chelating the divalent cations Ca^{2+} and Mg^{2+} limits but not completely excludes any further activation of the complement system, which can still be achieved [26].

The bacterial load found in bloodstream infections ranges from 1 to 10 CFU/mL to 1×10^3 and 1×10^4 CFU/mL [27]. According to literature data, the *E. coli* doses used in our study were similar to those reported in other studies performed in whole blood models [7] with similar incubation times, reaching bacteria concentrations of up to 10^8 CFU/mL [7, 8, 12].

There are several investigations focused on the biochemical mechanisms adopted by Gram-negative bacteria during sepsis; however, to the best of our knowledge, none of them was oriented in determining the impact of live *E. coli* on MDW.

Only two studies screened MDW levels according to the pathogen involved in sepsis [18, 28], and a recent review [20] suggested that an increase in MDW values is not associated with specific type of microorganisms.

Herein, we highlighted that monocyte morpho-functional modifications induced by live *E. coli* could be measured and quantified by MDW and are directly associated with the bacterial load and incubation time, thus confirming the ability of MDW to early detect bacteria-activated monocytes [16–18, 28].

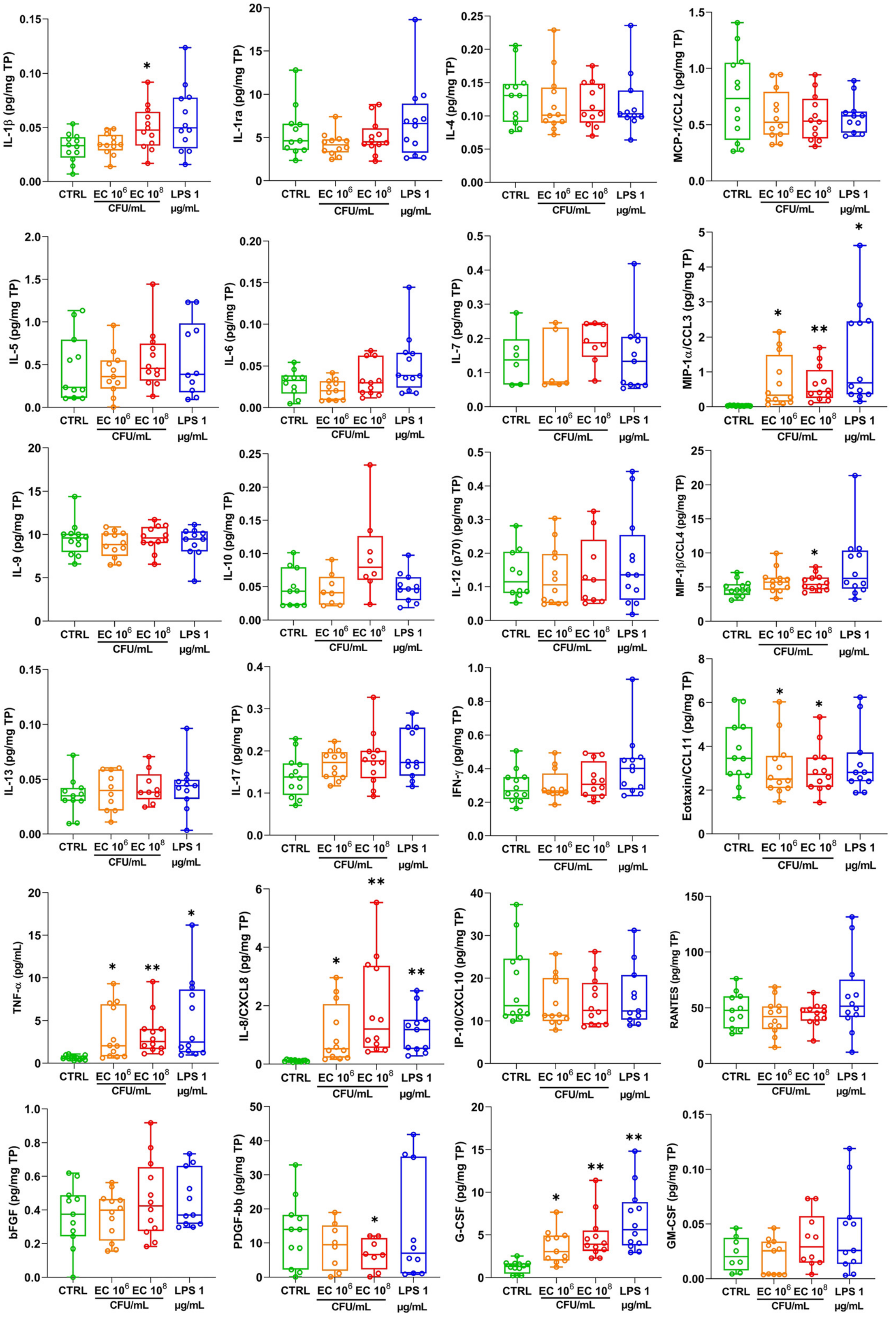


Figure 5: Cytokine values in plasma samples (n=12) from whole blood after 3h of stimulation with increasing concentrations of living *E. coli* (10^6 and 10^8 CFU/mL) and LPS from *E. coli* (1 µg/mL). (Mixed-effects model followed by Dunnet's multiple comparison test: *p=0.01–0.05; **p=0.001–0.01; comparisons were calculated among treatments and respective controls). Values are expressed as box and whiskers, showing min to max values, median and 25th to 75th percentiles of the pg/mg total proteins.

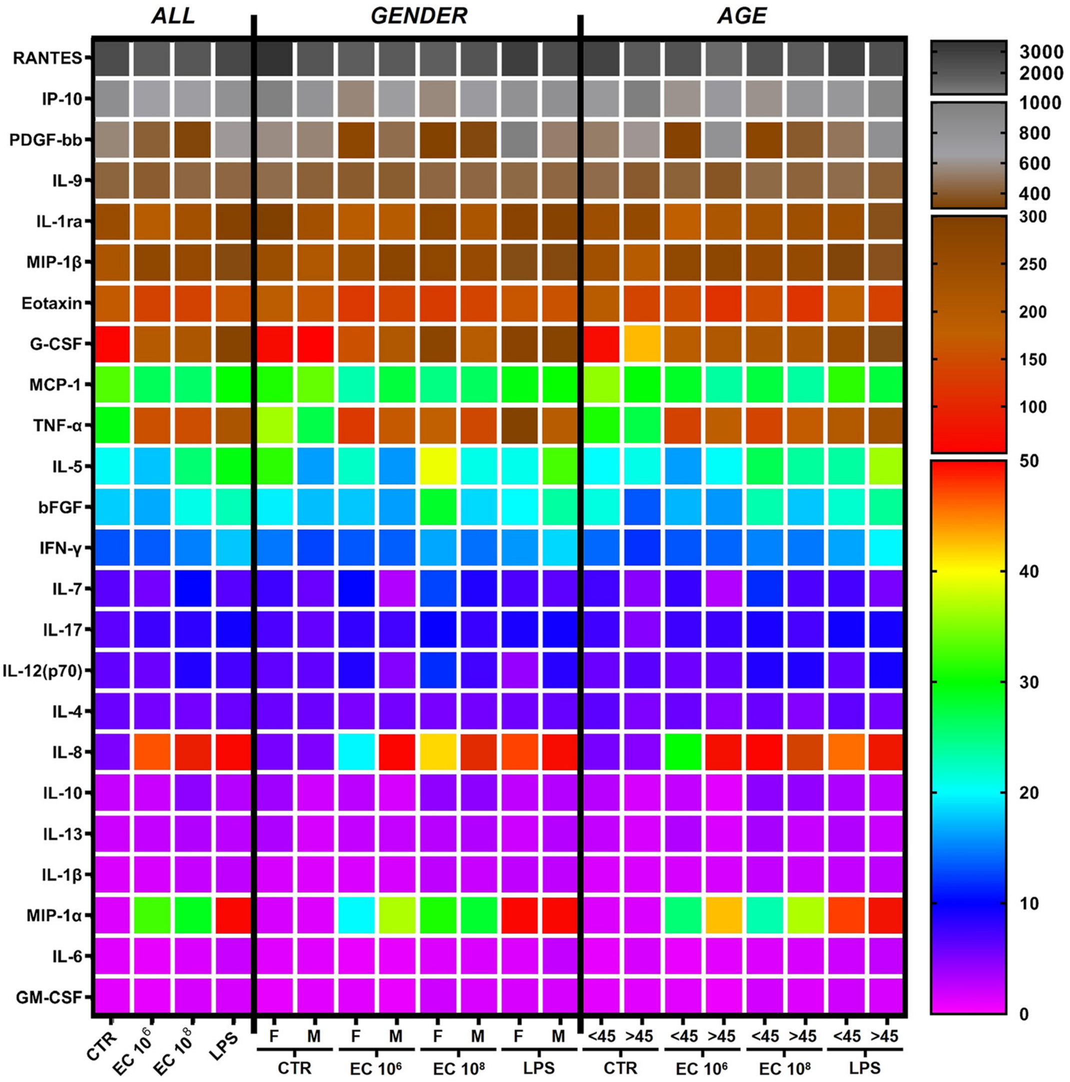


Figure 6: The heat map represents the variations of cytokines, chemokines, growth factors and colony stimulating factors according to gender and age differences. On the left are indicated all the inflammatory parameters. The first group of columns (all) highlights the mean cytokine values obtained from the analyses of the entire population (n=12). The other groups of columns (gender and age) show the mean cytokine values obtained from the analyses of the population divided according to the gender (F, n=3; M, n=9) and the age (<45 years, n=7; >45 years, n=5). Cytokine concentrations (pg/mL) are depicted as colours ranging from magenta, blue, green, yellow, orange, red, brown, and grey, indicating increasing levels of concentration.

Our findings confirmed that MDW values are significantly higher in sepsis patients, compared to unaffected controls [14, 16, 17, 20, 29–31] and that MDW measurements obtained in our *ex vivo* model significantly overlap those measured in sepsis patients, thus sustaining the reliability of the *ex vivo* model.

In full agreement with our previous findings [21] and other published literature [32], we also underlined that *in vitro* treatment of whole blood with increasing concentrations of LPS and live *E. coli* induced MDW enhancement in a dose- and time-dependent manner, reaching overlapping levels.

In fact, LPS, the major component of the outer leaflet of the membrane of Gram-negative bacteria, is widely recognized as a prototypal signal for the activation of monocyte/macrophage inflammatory responses by inducing the upregulation and release of several inflammatory mediators and activating TLR-mediated signaling [8, 32–35].

E. coli- and LPS-induced MDW changes were accompanied by morpho-functional alterations of monocytes, as confirmed by digital microscopy of MGG-stained blood smears. These findings are in agreement with the monocyte morphological alterations described *in vivo*, in both bacterial [36] and viral [37, 38] sepsis, as well as in *ex vivo* models of human whole blood stimulated with LPS and/or histones [21, 22, 32]. Monocyte morphological abnormalities, including increased cell size, vacuolization, intracellular hypergranulation, nuclear deformations with chromatin decondensation, and bacteria phagocytosis (only with live *E. coli* stimulation) are features representative of monocyte activation, despite that different pathways were evoked by LPS and live *E. coli*. The recognition of microbial PAMP can trigger phagocytosis, but opsonization of pathogens provides the best strategy to optimize microbial engulfment [39]. Monocyte vacuolization in LPS-triggered blood samples can be the result of a "priming" mechanism leading to monocyte morpho-functional activation toward a pro-inflammatory phenotype, without a "true phagocytosis" activation, due to the absence of microorganisms. In fact, previous studies established that LPS is able to promote the formation of vacuolar structures, but these structures are not of lysosomal or phagosomal origin [40].

On the other hand, we unexpectedly observed different inflammatory responses, in terms of both cytokine mediators and intensity of the inflammatory responses. Previous studies reported that *in vitro* stimulation of whole blood with *E. coli* was associated with a dose-dependent up-regulation of peculiar cytokines (e.g., TNF-α, IL-1β, IL-2, IFN-γ, IL-6, IL-8, MIP-1α, IL-1ra, G-CSF and MIP-1β), whose expression was dependent on the activation of CD-14 and complement pathways, and on incubation temperature [7, 8, 12, 13, 41].

Herein, we observed a significant increase in several proinflammatory cytokines, including MIP-1α/CCL3, IL-8/CXCL8, TNF-α, IL-1β and a minor significant up-regulation was observed for MIP-1β/CCL4. Interestingly, we also observed an increased level of anti-inflammatory cytokine IL-10 but only after treatment with the highest concentration of *E. coli*. This observation is in agreement with an earlier publication [42] on detailed analysis of gene expression in patient monocytes during sepsis and after recovery, which demonstrated plasticity of monocytes in the course of disease. Significant up-regulation of pro-inflammatory cytokines (IL-1β, IL-6) and chemokines (MIP-1α/CCL3 and RANTES/CCL5) in sepsis monocytes compared with monocytes after recovery has been observed, but at the same time, anti-inflammatory cytokine IL-10 was found to be up-regulated in sepsis monocytes. Together with the previous publication [42], our *in vitro* results support the possible functional heterogeneity of monocytes in sepsis, which can result in morphological variability, detected by elevated MDW. Preliminary observations also suggest potential age- and gender-related immune responses, underlining that peculiar cytokines (e.g., MIP-1α, IL-8, and PDGFbb) could be sensitive to different ages and genders. Despite highlighted in a limited number of subjects, these findings provide possible hypotheses on how biological sex- and age-related factors could sustain different susceptibility to infective agents, immune responses, disease outcome and response to therapeutic strategies, providing novel evidence for a gender-based medicine [43].

In vitro treatment of whole blood with live bacteria better reflects the cellular and inflammatory responses elicited *in vivo*, since *in vitro* stimulation with LPS does not predict the degree and pattern of cytokine production as it occurs *in vivo* due to the cooperation with microenvironmental tissue-derived inflammatory mediators [44].

Previous studies have also described that cytokine release is achieved upon *in vitro* stimulation with heat-killed or antibiotic-killed bacteria, due to the liberation of LPS and other bacterial components [45], which stimulate the secretion of pro-inflammatory cytokines mainly by mononuclear cells [46].

Further differences in the levels and timing of cytokine release may rely on (I) the rapid live pathogen internalization by phagocytes vs. the long-lasting LPS effect; (II) the type of Lipid A moiety within the LPS structure and the length of the acyl chains [33]; (III) the relative position of the Lipid A moiety in LPS molecule when it is in its soluble or membrane-bound form [47].

Despite our results obtained in an *ex vivo* whole blood model cannot fully represent the complexity of multicellular and humoral responses attainable *in vivo*, our present findings further support and confirm the clinical utility of MDW in early sepsis diagnosis and the reliability of the whole blood assay as *ex vivo* sepsis model. Moreover, we reveal that monocyte morphological alterations, as assessed by digital microscopy and quantified by MDW variations, could be induced not only by live bacteria through the activation of phagocytic mechanisms, but also by pro-inflammatory pathways associated with the release of bacterial endotoxins. Ongoing investigations are focused on the possible species-specific bacteria modifications of MDW according to the microorganisms and strains and also with the associated PAMPs.

MDW is a low cost and rapid-to-obtain biomarker available as part of the routine CBC. It has a high negative predictive value [19], and a high accuracy in excluding sepsis diagnosis when MDW values are below the decisional cut-off [48].

Finally, our results support and strengthen the use of MDW as an early screening tool for the recognition of sepsis conditions of bacterial origin in a timely manner, which can represent the greatest and crucial challenge in critical sepsis clinical and laboratory managements.

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Research ethics: The non-interventional *ex vivo* study was approved by the local Ethical Committee based on an official document of accordance between the University of Urbino Carlo Bo and the Blood Transfusion Center of “S. Maria della Misericordia” Hospital of Urbino – Italy. The retrospective study included data obtained from previous *in vivo* studies reviewed and approved by the Clinical Research Ethics Committee of the University Hospital Germans Trias i Pujol, Badalona, Spain (PI-18-249 and PI-18-140). All patients included in these studies signed an informed consent previously approved by the CEIC (Ethics Committee for Clinical Investigation).

Informed consent: Informed consent was obtained from all participants involved in the studies.

Author contributions: Conceptualization: Ligi D, Della Franca C, and Mannello F. Methodology: Ligi D, Della Franca C, Pelloso M, Martinez-Iribarren A, Leis A, Fabbri E, Salvatori F, Schiavano GF. Investigation: Ligi D, Della Franca C, Pelloso M, Martinez-Iribarren A, Leis A, Fabbri E, Salvatori F, Schiavano GF, Brandi G. Visualization: Ligi D, Della Franca C, and Mannello F. Funding acquisition: Mannello F. Project administration: Ligi D, and Mannello F. Supervision: Ligi D, Sukhacheva EA, and Mannello F. Writing – original draft: Ligi D, Schiavano GF, Brandi G, and Mannello F. Writing – review & editing: Ligi D, Della Franca C, Sukhacheva EA and Mannello F. All authors have accepted responsibility for the entire content of this manuscript and approved its final submission.

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Data availability: The dataset used in this paper is not publicly available since it is still under elaboration for publication by the Authors but is available from the corresponding author Daniela Ligi (daniela.ligi@uniurb.it) upon reasonable request.

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Chapter 3

Deciphering conundrums on heparins and circulating histones: hype or hope?

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JTH COMMENTARY

Deciphering conundrums on heparins and circulating histones: hype or hope?

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The release and accumulation of histones in the extracellular microenvironment represents one of the pieces of the complex "extracellular traps" jigsaw, a process involved in both homeostasis and disease [1], including host defense, immune regulation, immunothrombosis, sepsis, and neoplastic diseases. This process involves a coordinated activation of several cells (eg, neutrophils, monocytes, and platelets) and a plethora of biomolecular components (eg, enzymes, proteinases, complement and coagulation factors, and crucial damage- and pathogen-associated molecular patterns, including circulating histones) [2,3].

Histones are highly basic and conserved chromatin proteins involved in binding DNA and regulating gene expression. At the extracellular level, histones act as bioactive and proinflammatory molecules [4], able to induce a plethora of biological events summarized as "pathogenic inflammatory triad of cytotoxicity, immune stimulation and coagulation" [5,6].

After microbial/viral infections and the release of endogenous danger signals [2,3], neutrophils and monocytes (but also basophils and eosinophils) are mainly activated to form extracellular traps [7] through an intricate and finely regulated mechanism involving chromatin decondensation and enzymatic and proteolytic activities (eg, neutrophil/monocyte elastase and myeloperoxidase) [1]. This ultimately results in the release of cell-free DNA and proteins, approximately 70% of which are represented by free histones [1,8].

Excessive release and accumulation of free histones in extracellular compartments, particularly but not limited to the histone H3 and H4 subtypes, has been identified and demonstrated to be a contributing cause of significant local and systemic detrimental effects. This includes cell membrane disruption, increase of intracellular calcium, stimulation of Toll-like receptors, activation of complement and inflammasomes, thrombin generation, and inflammothrombosis and immune dysregulation, common hallmarks found in autoimmunity, sepsis, neoplasia, and cardiovascular events besides the recently described involvement in viral processes [1,3,5].

In this context, a huge number of studies have been focused on the biocompounds active in reducing the entity of cell, tissue, and organ damage and/or the risk of adverse clinical events and death related to the release of circulating histones in blood and their accumulation in human organs [6–8]. The main mechanisms explored were (i) the neutralization by complexation of circulating histones through direct interaction between positive charge residues of histones and the negative charge of glycosaminoglycan-based molecules, polyanionic agents, natural plasma proteins (including albumin, C-reactive protein, osteopontin, and fibrinogen); and (ii) the proteolytic cleavage/degradation of circulating free histones, mainly catalyzed by elastases, serine proteases, cathepsins, and activated protein C, especially those secreted by granulocytes, monocytes and mast cells [9].

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The histone complexation process obtained with glycosaminoglycans (including heparins, heparinoids, nonheparin molecules, and nonanticoagulant heparins) has shown a different ability and degree of complexation and diverse degree of efficacy (ie, from null activity to significant capability to prevent histone-mediated cytotoxicity and improving survival rate), also depending on the experimental models, as reviewed in [6,10–13].

Although the proteolytic cleavage of circulating histones was first evaluated as an epigenetic mechanism priming macrophage differentiation [14], only recently has the possibility of using proteolytic cleavage to neutralize the cell-destructive potential and harmful proinflammatory mechanisms of circulating histones been evaluated [15].

In their recent insightful study published in *Journal of Thrombosis and Haemostasis*, Huckriede et al. [16] aimed to shed light on the effects of unfractionated heparin (a mixture of physiologically derived high- and low-molecular-weight porcine heparins) on the proteolytic neutralization of extracellular histone isoforms by neutrophil elastase, through *in vitro* and *ex vivo* approaches analyzing cytotoxicity, Toll-like receptor activation, and inflammatory pathways.

They found that activated neutrophil elastase can fully cleave all classes of core histones into several fragments overlapping those found in citrated plasma of patients admitted to the intensive care unit. This demonstrates the complex regulation of their release and physiological counteractions during sepsis and thromboinflammatory processes. Interestingly, the *in vitro* addition of unfractionated heparin (ie, isolated heparin from porcine, composed of both long and short tails of glycosaminoglycans and the pentasaccharide chain) significantly increased the elastase-dependent cleavage rate of all classes of histones (average increased rate 34.5%), suggesting a further role of these glycosaminoglycan compounds other than as anticoagulant, profibrinolytic, and inflammatory agents.

In contrast to data in the literature demonstrating glycosaminoglycans as anti-elastase and anti-inflammatory drugs [17], surprisingly, Huckriede et al. [16] described that histone H3 is cleaved by activated neutrophil elastase in the presence of unfractionated heparin.

Moreover, it is well known that histones (in particular, histone H3) are triggers able to dose-dependently induce Toll-like receptor activation, explaining in part their crucial role as significant thromboinflammatory stimuli [4,11]. Notably, Huckriede et al. [16] reported that the cleavage of H3 did not reduce the activation of Toll-like receptor, suggesting that neither unfractionated heparin complexation nor elastase proteolysis of histones limit the stimulation of Toll-like receptor pathway and ultimately inflammasome signaling and inflammatory cascade.

Interestingly, the *in vitro* incubation of histone H3 with activated neutrophil elastase resulted in an increase of cell viability of approximately 30%, up to almost 100% of recovery with elastase plus unfractionated heparin, compared to the well-documented 80% of cytotoxicity induced by histone H3 treatment [16]. This finding highlights the potential usefulness of either unfractionated heparin complexation of circulating histones and/or enhancement of neutrophil elastase cleavage of histones as important mechanisms in counteracting the well-documented histone-mediated cytotoxicity.

Collectively, the study of Huckriede et al. [16] underscores the role of proteolytic neutralization of the cytotoxic effects of extracellular histones by neutrophil elastase and highlights the potential of unfractionated heparin as proteolysis-enhancing scaffold that does not affect Toll-like receptor proinflammatory/thrombotic pathway activation. In this respect, this evidence raises more questions than answers, and further analyses are warranted to elucidate *some still opened conundrums:* (1) Are there specific histone regions involved in Toll-like receptor signaling and ultimately inflammasome cascade? (2) Are cleaved histones still able to induce monocyte/neutrophil morphologic alterations (as observed during sepsis)? (3) Are proteolytically fragmented histones still involved in platelet aggregation and thrombocytopenic events? (4) Besides unfractionated heparin, are there any other classes of glycosaminoglycan-based drugs able to revert platelet aggregation? (5) Are there any other glycosaminoglycan-based drugs able to induce similar effects as unfractionated heparin, acting as proteolytic scaffolds and anti-inflammatory compounds?

Among the plethora of studies investigating the use of glycosaminoglycan-based compounds in sepsis and thromboinflammatory disease (both *in vitro* and in emergency units) [12,13], contrasting results on their effects on histones create potential confusion [6,10]. On the other hand, critical aspects of clinical usefulness have emerged including their heterogeneity (from unfractionated to highly purified fractions and synthetic pentasaccharide), complex structural properties (broad distribution in relative molecular weights and charge density due to sulfation degree), selective interaction with multiple proteins (including histones), and "old and novel" pharmacologic functions and clinical applications, not limited to the coagulation cascade, open new clinical frontiers and medical perspectives [18]. Currently, there are unexpected and contrasting results regarding the diverse classes of "heparins" (more appropriately defined as "glycosaminoglycan-based drug") against circulating histones, ranging from no effect (eg, for synthetic heparins) to significant complexing/inhibiting action (eg, noncoagulant heparins and drug-based mixtures of glycosaminoglycans) [6,10,13].

Sepsis and thromboinflammatory diseases are among the world's leading causes of death and remain poorly understood at the molecular level (eg, the exact involvement of neutrophil extracellular traps and histones), and their control is facing new and extensive challenges.

Due to the pivotal role of extracellular histones in promoting platelet aggregation [19], mainly in sepsis condition in which they are massively released, future studies should prioritize elucidating the specific histone-platelet interaction mechanisms and assessing how different glycosaminoglycan compounds can modulate these pathways. Furthermore, given the well-established role of Toll-like receptors (primarily Toll-like receptors 2 and 4) in mediating histone-induced platelet activation, these receptors represent promising therapeutic targets that could potentially break the vicious cycle between inflammation and coagulation. Novel mechanistic biomarkers and better understanding of the pathophysiology of sepsis

[3,8,20], and novel/improved heparin-based therapeutic treatment [11,13,18] may fundamentally improve sepsis care, even though those advances have not yet translated into sustained population-level gains or trial endpoints.

The "hype" of the scientific progress in circulating histone immunobiology during NETosis [1,7] and the phenotypic responses of sepsis patients [20] may represent the "hope" to fill in the major persisting knowledge gaps about histone-targeting strategies, aligning neutrophil extracellular trap driver biomarkers (including circulating histones) with novel therapeutics of sepsis (including glycosaminoglycan-based drugs) to promote better identification and management of patients affected by histone-related and neutrophil extracellular trap-related diseases [1,8]. Opening new avenues in the integration of antihistone strategies in sepsis treatment [12] could improve patient outcomes by managing both inflammation and thrombosis[6]. This dual action highlights the therapeutic relevance of glycosaminoglycan-based drugs for septic patients, with applications that extend beyond conventional anticoagulation.

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AUTHOR CONTRIBUTIONS

D.L., C.D.F., and F.M. drafted, finalized, and approved the final version of this commentary.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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Chapter 4

Platelet Distribution Width (PDW) as a significant correlate of COVID-19 infection severity and mortality

Daniela Ligi, Chiara Della Franca, Kin Israel Notarte, Nathaniel Goldrich, David Kavteladze, Brandon Michael Henry and Ferdinando Mannello.

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Review

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Platelet distribution width (PDW) as a significant correlate of COVID-19 infection severity and mortality

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Abstract: SARS-CoV-2 infection may cause a wide spectrum of symptoms, from asymptomatic, to mild respiratory symptoms and life-threatening sepsis. Among the clinical laboratory biomarkers analyzed during COVID-19 pandemic, platelet indices have raised great interest, due to the critical involvement of platelets in COVID-19-related thromboinflammation. Through an electronic literature search on MEDLINE, CINAHL, PubMed, EMBASE, Web of Science, and preprint servers we performed and updated a systematic review aimed at providing a detailed analysis of studies addressing the potential clinical utility of platelet distribution width, platelet distribution width (PDW), in laboratory medicine, exploring the possible association between increased PDW levels, disease severity, and mortality in COVID-19. Our systematic review revealed a wide heterogeneity of COVID-19 cohorts examined and a lack of homogenous expression of platelet indices. We found that 75 % of studies reported significantly elevated PDW values in COVID-19 infected cohorts compared to healthy/non-COVID-19 controls, and 40 % of studies reported that patients with severe COVID-19 showed increased PDW values than those with less-than-severe illness. Interestingly, 71.4 % of studies demonstrated significant increased PDW values in non survivors vs. survivors. Overall, these results suggest that platelets are critically involved as major players in the process of immunothrombosis in COVID-19, and platelet reactivity and morphofunctional alterations are mirrored by PDW, as indicator of platelet heterogeneity. Our results confirm that the use of PDW as prognostic biomarkers of COVID-19 sepsis still remains debated due to the limited number of studies to draw a conclusion, but new opportunities to investigate the crucial role of platelets in thrombo-inflammation are warranted.

Keywords: COVID-19; platelet; platelet distribution width; PDW; SARS-CoV-2; systematic review

Introduction

The novel coronavirus disease 2019 (COVID-19) is a pandemic infectious disease sustained by a member of the Coronaviridae family, finally called acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The range of clinical pictures is quite heterogeneous, with most patients experiencing only mild respiratory symptoms or being asymptomatic, especially among young children, with a relevant role in spreading the disease [1]. The proportion of patients with COVID-19 who progress towards severe or even critical illness, requiring sub-intensive or intensive care varies but is decreasing over time, in line with the increased population immunity, improved early diagnostic procedures, and advanced therapeutic strategies. Accordingly, the death rate is highly variable worldwide depending on genetic, epigenetic, and environmental factors [2].

Among the plethora of clinical laboratory biomarkers and potential hematological parameters analyzed during COVID-19 pandemic [3, 4], particular attention has been

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focused on platelet functions and activities, as well as platelet-linked laboratory indices, due to a great deal of information available in COVID-19 patients suggesting the hyper-activation of coagulative cascade, finally leading to thrombosis and thrombocytopenia [5–8]. Interestingly, these studies shed light also on the missing pieces of the intricate puzzle of COVID-19.

According to the widely accepted association between platelet parameters and COVID-19, several recent studies on the diagnostic and prognostic value of routine hemocytometry markers underlined the clinical usefulness of platelet distribution width (PDW) in COVID-19, emphasizing the role of this measure for distinguishing and stratifying the risk of developing critical illness and/or dying. To this end, the hematological parameter PDW, linked to heterogeneity of platelet volume, has recently emerged as a predictive factor of multiorgan dysfunction, enhanced micro-thrombotic processes and increased risk of death in several physio-pathological conditions [9–12].

According to standard hematological procedures, PDW is generated alongside other platelet volume indices (mean platelet volume, MPV; plateletcrit, PCT) and represents a parameter mathematically based on the measure of platelet volume and standard deviation of volume distribution within the platelet population. In fact, PDW is an indicator of heterogeneity in platelet size, reflecting morphologic changes in reactive/activated/giant platelet cells [13, 14].

In a general perspective, higher PDW values are associated with a wider range of platelet size, which could result from platelet activation processes, platelet destruction mechanisms, or platelet consumption [13, 15–17].

Thrombotic events and hypercoagulability are also associated with increased PDW values due to the high number of platelets being destroyed and consumed (thrombocytopenia), and the activation of thrombopoiesis, which stimulates the release of younger and larger platelets from the bone marrow in the blood circulation [5, 6].

On these bases, this parameter enhances the attainment of crucial details through classic optical microscopic evaluation of peripheral blood smears, providing further crucial information on heterogeneity and volume modifications of platelets upon massive inflammo-thrombotic processes.

We describe here the results of an updated systematic review aimed to provide a detailed analysis of studies that have addressed the potential clinical utility of PDW in routine laboratory medicine, exploring the possible association between increased PDW levels, disease severity, and mortality in COVID-19 patients.

Methods

This systematic review adheres to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement of 2020. As this manuscript is a systematic review, no Ethical Committee approval was required.

Search strategy

Electronic literature searches were conducted by two different authors on the following databases: MEDLINE, CINAHL, PubMed, EMBASE, and Web of Science databases, as well as on preprint servers medRxiv and bioRxiv, for studies published between 2020 and present time (i.e., latest search date: December 2, 2022). Database search strategies were conducted with the assistance of an experienced health science librarian. We also screened the reference list of identified papers for capturing black literature. Searches were limited to human studies and English language citations by using the following combinations of terms: “COVID-19” OR “SARS-CoV-2”, “platelet distribution width”, “severity”, “mortality”, “pregnancy”, “pulmonary embolism”, “acute respiratory distress syndrome”. The search strategy combined these terms using Boolean operators for the main databases is detailed in Table 1.

Selection criteria

This review included observational cohort, cross-sectional, and case-control studies. A series of comparisons were made: infected vs. uninfected healthy controls; severe vs. non-severe disease; non-survivors vs. survivors. Severe disease was clinically defined as patients needing intensive care unit (ICU) admission, mechanical (forced) ventilation, COVID-19 related hospitalization, pneumonia, or onset of critical symptoms and/or shock and/or presence of organ failure. Due to lack of comparable data between multiple studies, pediatric populations were excluded from analysis and only adult populations were considered. All studies fulfilling these criteria were then included in a systematic literature review.

Two authors reviewed the title and abstract of those publications identified in databases. Duplicates were then removed. The title and abstract were screened for eligibility and posterior full-read text. The reference list of the documents included in our analysis was also scrutinized with forward and backward citation tracking to detect other potentially eligible studies (Figure 1).

Table 1: Database formula during literature search.

PubMed, MEDLINE/CINAHL (via EBSCO), WOS (EMBASE)/Web of Science search formula
((“COVID-19)” OR “SARS-CoV-2” AND (“platelet distribution width”)) AND ((“severity”) OR (“pregnancy”) OR (“pulmonary embolism”) OR (“acute respiratory distress syndrome”))

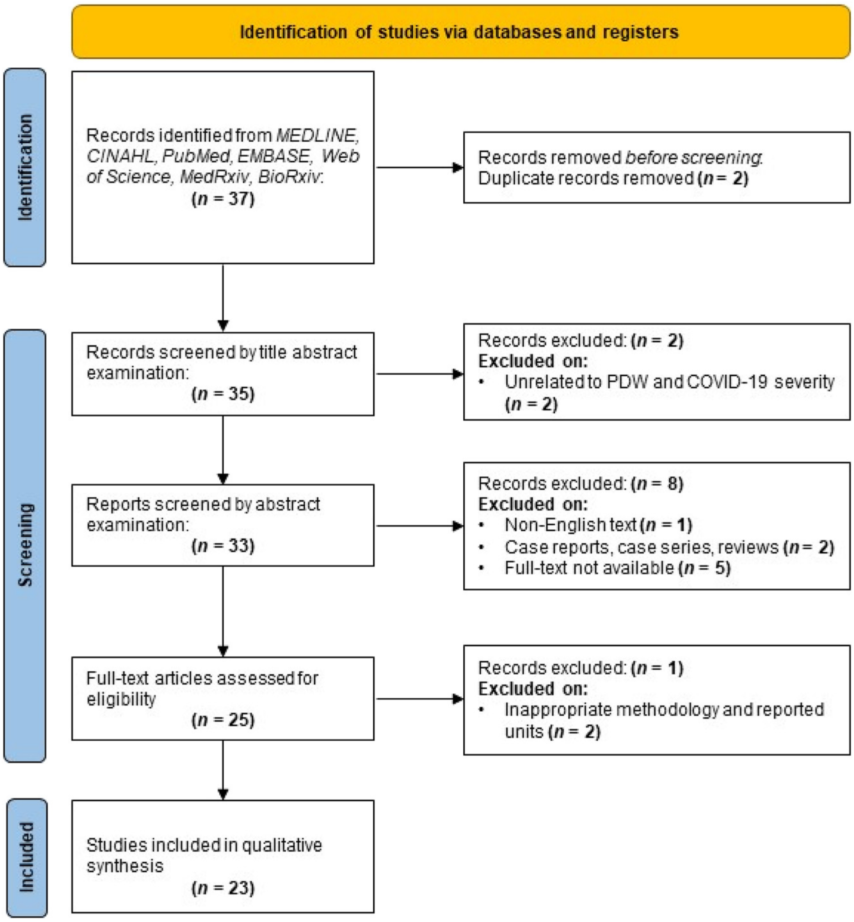

Figure 1: Preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram.

Data collection

Data including authors, years, country, sample size, PDW measurements (in % and femtoliters, fL) and their associated p-values were extracted from each study. Disagreements between authors with respect to study eligibility were resolved by discussion and consensus among authors, and discrepancies between reviewers at any stage of the screening process were resolved by asking a third author when necessary.

Quality assessment

The NIH Quality Assessment Tool was used to evaluate all studies used for analysis. Two authors grouped the studies into two categories: case-control studies (Table 2) and observational cohort/cross-sectional studies (Table 3). The same two authors assessed each study using the provided checklist to characterize the quality of the papers. Case control studies were evaluated by research question, study population, target population and case representation, sample size justification, groups recruited from the same population, inclusion and exclusion criteria prespecific and applied uniformly, case and control definitions, random selection of study participants, concurrent controls, exposure accessed prior to outcome measurement, exposure measures and assessment, blinding of exposure assessors, and statistical analysis parameters, where applicable. Observational cohort/cross-sectional studies were evaluated by research question, study population, groups recruited from the same population, sample size justification, exposure accessed prior to outcome measurement, sufficient timeframe to see an effect, different levels of the exposure of interest, exposure measures and assessment, repeated exposure assessment, outcome measures,

Table 2: NIH Quality Assessment Tool for evaluating case-control studies.

Case-control study	Overall quality rating	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12
Al-Buthabhak [18]	Good	Y	Y	Y	Y	Y	Y	N/A	N	Y	Y	N/A	N
Alnor [19]	Good	Y	Y	N	Y	Y	Y	N/A	Y	Y	Y	N/A	N/A
AydinyiImaz [20]	Good	Y	Y	Y	Y	Y	Y	N/A	N	Y	Y	N/A	Y
Nori [21]	Good	Y	Y	N	Y	Y	Y	N/A	Y	Y	Y	N/A	Y
Shankaralingappa [22]	Good	Y	Y	N	Y	Y	Y	N/A	Y	Y	Y	N/A	N/A
Yovchevska [23]	Good	Y	Y	N	Y	Y	N	N/A	Y	Y	Y	N/A	Y

Y, yes; N, not; N/A, not applicable.

Table 3: NIH Quality Assessment Tool for evaluating observational cohort and cross-sectional studies.

Observational cohort/ cross-sectional study	Overall quality rating	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Q13	Q14
Asrie [24]	Fair	Y	Y	Y	Y	N	Y	Y	N	N	N	Y	N/A	N/A	Y
Bommenahalli Gowda [25]	Fair	Y	Y	N/A	Y	N	Y	Y	N/A	Y	N	Y	N/A	N/A	N
Suarez Castillejo [26]	Good	Y	Y	Y	Y	Y	Y	Y	N/A	Y	Y	Y	N/A	Y	N
Çelikkol [27]	Fair	Y	Y	N/A	N/A	N	Y	Y	Y	Y	N	Y	N/A	N/A	N
Covali [28]	Fair	Y	Y	Y	Y	N	Y	Y	N/A	Y	N	Y	N/A	N/A	N
Güçlü [12]	Good	Y	Y	N/A	Y	N	Y	Y	Y	Y	Y	Y	N/A	Y	N
Hajian [29]	Good	Y	Y	Y	Y	N	Y	Y	Y	Y	N	Y	N/A	N/A	N
Khalid [30]	Good	Y	Y	Y	Y	N	Y	Y	Y	Y	N	Y	N/A	N/A	Y
László [31]	Fair	Y	Y	N/A	Y	N	Y	Y	Y	Y	N	Y	N/A	N/A	N
Ouyang [32]	Fair	Y	Y	N/A	N	N	Y	Y	Y	Y	Y	Y	N/A	N/A	N
Ozcelik [33]	Fair	Y	Y	N/A	Y	N	Y	Y	N	Y	N	Y	N/A	N/A	N
Pujani [34]	Fair	N	Y	N/A	Y	N	Y	Y	Y	Y	N	Y	N/A	N/A	N
Ravindra [35]	Fair	N	Y	N/A	N/A	N	N	Y	Y	Y	N	Y	N/A	N/A	Y
Suliman [36]	Fair	Y	Y	Y	Y	N	Y	Y	N/A	N/A	N	Y	N/A	N/A	N
Wang [10]	Fair	N	Y	N/A	Y	N	Y	Y	N	N/A	N	Y	N/A	N/A	N
Ye [37]	Good	N	Y	N/A	Y	N	Y	Y	Y	Y	N	Y	N/A	N/A	Y
Zhong [38]	Good	N	Y	Y	Y	Y	Y	Y	N	N/A	Y	Y	N/A	Y	Y

Y, yes; N, not; N/A, not applicable.

blinding of outcome assessors, follow up rate, statistical analysis parameters, where applicable. Studies that achieved 75 % of the criteria or above received a score of "good"; 50 % of the criteria or above received a score of "fair"; less than 50 % of the criteria received a score of "poor".

Results

Study selection

Twenty-three studies with a total population of n=12,767 participants were selected in this systematic review to quantify platelet distribution width (PDW) in patients with COVID-19. The studies were conducted in the following countries: Iraq [18, 21], Turkey [12, 20, 27, 33], Spain [26], Romania [28], India [22, 25, 34, 35], Iran [29], Hungary [31], China [10, 32, 37, 38], Saudi Arabia [30, 36], Denmark [19], Ethiopia [24], and Bulgaria [23]. The patients were further divided into study-specific groups such as non-severe (n=778, including mild and moderate), and severe (n=510) COVID-19 cohorts; survivor (n=1,270) and non-survivor (n=360) cohorts; and presence of comorbidities including acute respiratory distress syndrome, influenza, pulmonary embolism, multisystem inflammatory system, and pneumonia. It is also worth noting that articles under these categories were nonexclusive, as many studies included more than one factor in their analysis.

Meta-analysis was not deemed appropriate due to the high heterogeneity between studies. In accordance with the guidelines outlined in the Cochrane Handbook and best practices in evidence synthesis, we did not to perform a meta-analysis due to the presence of methodological and clinical heterogeneity that cannot be adequately addressed by statistical methods alone.

By acknowledging the limitations imposed by high heterogeneity, we aimed to ensure the robustness and reliability of our findings, and to avoid combining studies with such significant differences that could introduce bias or distort the overall conclusions of the analysis.

Accordingly, we conducted a synthesis of the data reported by addressing population, patient level differences, limitations with different assays, sensitivities, and reported units, and methodological quality.

Study assessment

All studies used for analysis received scores of either good or fair. No studies received a score of poor. The studies that were deemed good include [12, 18–23, 26, 29, 30, 37, 38].

The studies that were classified as fair include [10, 24, 25, 27, 28, 31–36]. These characterizations are provided in Tables 2 and 3.

PDW trends

As reported in Tables 4 and 5, six out of eight (75 % of studies) reported significantly elevated PDW (expressed as fL or %) values in COVID-19 infected cohorts as compared to healthy

Table 4: PDW (%) in COVID-19 patients.

Author **Country of origin** **Design**	**Sample size**	**PDW measurement**	**PDW significance (p-value)**
Çelikkol et al. [27] Turkey Retrospective cohort	Confirmed COVID-19 patients n=56 Unconfirmed COVID-19 patients n=46 Healthy patients n=30 Mild COVID-19 cohort n=31 Severe COVID-19 cohort n=25	PDW (%) Confirmed COVID-19 patients: 16.02 ± 2.96 % Unconfirmed COVID-19 patients: 15.04 ± 3.29 % Healthy patients: 14.5 ± 1.9 % Mild COVID-19 patients: 16.05 ± 3.24 % Severe COVID-19 patients: 15.98 ± 2.63 %	Confirmed vs. healthy: 0.017 Severity: 0.921
Ozcelik et al. [33] Turkey Retrospective	COVID-19 cohort n=54 Influenza cohort n=43	PDW (%), median (IQR) COVID-19 patients: 16.2 (15.9–16.6) % Influenza patients: 12.2 (10.6–14.2) %	Diagnostic (COVID-19 vs. influenza pneumonia): <0.001
Pujani et al. [34] India Prospective cross-sectional	COVID-19 cohort n=506 Non-COVID-19 cohort n=200 Mild-moderate COVID-19 cohort n=337 Severe COVID-19 cohort n=118 Very severe COVID-19 cohort n=51 Survival subclassification COVID-19 survivor cohort n=473 COVID-19 non-survivor cohort n=33	PDW (%) Total COVID-19 patients: 16.12 ± 3.6 % Non-COVID-19 patients: 15.69 ± 2.30 % Very severe COVID-19 patients: 17.43 ± 3.78 % Severe COVID-19 patients: 16.24 ± 3.51 % Moderate COVID-19 patients: 15.99 ± 3.6 % Survivors: 16.08 ± 3.64 % Non-survivors: 17.37 ± 3.00 %	Case vs. Control (COVID-19 vs. Non-COVID-19): 0.03 Severity: 0.012 Survival: 0.047
Shankaralingappa et al. [22] India Retrospective case-control	COVID-19 cohort n=199 Non-COVID-19 cohort n=198 High PDW (>25 %) subclassification COVID-19 cohort n=1 Non-COVID-19 cohort n=5	PDW (%) *Normal reference range: 0–25 %* COVID-19 patients: 15.83 ± 5.92 % Non-COVID-19 patients: 14.44 ± 2.83 %	Case vs. Control (COVID-19 vs. Non-COVID-19): 0.003 PDW>25 %: 0.101
Covali et al. [28] Romania Prospective cohort	Positive COVID-19 cohort n=46 Negative COVID-19 cohort n=411	PDW (%), mean values (and SD) on the upper line Pregnant COVID-19 positive patients at term: 16.98 ± 2.70 % Pregnant COVID-19 negative patients at term: 16.97 ± 3.17 % PDW (%), median values (quartile 1, quartile 2) on the lower line Pregnant COVID-19 positive patients at term: 16.59 (15.10, 18.41) % Pregnant COVID-19 negative patients at term: 16.63 (14.79, 18.77) %	Case vs. Control (COVID-19 vs. COVID-19 negative in pregnant women): 0.804
Aydınyılmaz et al. [20] Turkey Retrospective case-control	All patients n=5,412 Intensive care cohort n=871 Hospital ward cohort n=4,541 ASA use (+) cohort n=118 ASA use (−) cohort n=255	PDW (%), median (IQR) Total COVID-19 positive patients: 12.0 (10.8–13.5) % Intensive care: 12.8 (11.5–14.5) % Ward: 11.9 (10.7–13.3) % COVID-19 positive patients with MPV>10.45 fl and D-dimer >500.2 ng/dL in ICU: ASA use (+): 14.40 (13.2–15.08) % ASA use (−): 14.42 (12.53–15.44) %	Hospitalization for all patients: <0.001 ASA use for MPV>10.45 fl and D-dimer >500.2 ng/dL patients in ICU: 0.415

Table 4: (continued)

Author Country of origin Design	Sample size	PDW measurement	PDW significance (p-value)
Güçlü et al. [12] Turkey Retrospective cohort	Moderate COVID-19 Measurement 1: n=80 Measurement 2: n=70 Difference: n=69 Severe COVID-19 Measurement 1: n=13 Measurement 2: n=124 Difference: n=123 COVID-19 survivors Measurement 1: n=158 Measurement 2: n=147 Difference: n=146 COVID-19 non-survivors Measurement 1: n=54 Measurement 2: n=47 Difference: n=46	PDW (%), mean ± SD PDW measurement 1 Moderate COVID-19 patients: 17.37 ± 2.32 % Severe COVID-19 patients: 17.72 ± 2.52 % Survivors: 17.44 ± 2.35 % Non-survivors: 18.02 ± 2.69 % PDW measurement 2 Moderate COVID-19 patients: 17.96 ± 1.43 % Severe COVID-19 patients: 18.13 ± 1.66 % Survivors: 17.89 ± 1.55 % Non-survivors: 18.63 ± 1.56 % PDW difference (%), mean ± SD Moderate COVID-19 patients: 0.61 ± 2.34 % Severe COVID-19 patients: 0.55 ± 2.45 % Survivors: 0.46 ± 2.35 % Non-survivors: 0.93 ± 2.57 %	PDW measurement 1 Severity: 0.142 Survival: 0.040 PDW measurement 2 Severity: 0.144 Survival: 0.006 PDW difference Severity: 0.913 Survival: 0.389
Hajian et al. [29] Iran Cross-sectional	All patients n=59 Severe COVID-19 cohort n=21 Critically ill COVID-19 cohort n=38	PDW (%), median serum (IQR) All patients: 12.0 (11.0–14.0) % Severe patients: 11.70 (10.90–13.45) % Critically ill: 12.00 (11.12–14.20) %	Severity: 0.745
Ravindra et al. [35] India Retrospective single-center	Mild COVID-19 cohort n=51 Severe COVID-19 cohort n=49 COVID-19 survivor cohort n=88 COVID-19 non-survivor cohort n=12	PDW (%), mean (SD) Mild COVID-19 patients: 17.11 (7.3) % Severe COVID-19 patients: 16.47 (2.16) % COVID-19 survivors: 15.4 (2.13) % COVID-19 non-survivors: 16.5 (3.12) %	Severity: 0.064 Survival: 0.078
Bommenahalli Gowda et al. [25] India Retrospective cross-sectional	COVID-19 survivor cohort n=75 COVID-19 non-survivor cohort: n=25	PDW (%) *Normal reference range: 15–17; mean ± SD: 16.7 ± 2.7; median: 17.4; Minimum: 1.2; Maximum: 21.5* Non-survivors: 17.58 ± 2.84 % Survivors: 16.37 ± 2.59 % Testing PDW=17 % as cut off value for influence on survival ≤17.0: Non-survivors (n=21), survivors (n=42) >17.0: Non-survivors (n=4), survivors (n=33) Odds ratio (Confidence Interval) Mortality occurrence between PDW≤17.0 % and >17.0 % indices: 4.1(1.3–13.2)	Survival: 0.05 Differentiating survival with PDW=17 % cutoff: 0.012
László et al. [31] Hungary Retrospective descriptive analysis of prospectively collected data	ICU cohort n=95 Non-ICU cohort n=111 COVID-19 survivor cohort n=130 COVID-19 non-survivors n=76 COVID-19 ICU survivor cohort n=60 COVID-19 ICU non-survivors n=35	PDW (%), Median (25–75 % confidence interval) COVID-19 survivors:14.4 (11.6–45.1)% COVID-19 non-survivors: 21.4 (14.9–57.5)% ICU patients, survivors: 16.7 (12.3–57.8)% ICU patients, non-survivors: 51.5 (15.2–57.6)%	ICU stay: <0.001 ICU survival: 0.09

Table 4: (continued)

Author **Country of origin** **Design**	**Sample size**	**PDW measurement**	**PDW significance (p-value)**
Ouyang et al. [32] China Retrospective	COVID-19 survivor cohort n=82 COVID-19 non-survivor cohort n=25	PDW (%) *Normal reference range: 15–17* First laboratory tests COVID-19 survivors: 16.18 % COVID-19 non-survivors: 16.63 % Last laboratory tests COVID-19 survivors: 16.14 % COVID-19 non-survivors: 16.74 %	First laboratory tests Survival: <0.001 Last laboratory tests Survival: <0.001
Al-Buthabhak et al. [18] Iraq Retrospective case-control	Mild pneumonia (no hospital admission) n=64 Moderate-severe pneumonia ICU admission n=24 Mechanical ventilation n=22 In-hospital death n=9 Complete recovery (no persistent symptoms) n=54 Post-recovery shortness of breath (O_2 dependent) n=23 Post-recovery fatigue n=19	PDW (%) COVID positive patients: 12.6 ± 2 % Odds ratio (Confidence Interval) High PDW: 0.3 (0.4–1.9)	Length of ICU stay: <0.00 Length of hospital stay: <0.00 Degree of lung injury: 0.25 Mechanical ventilation use: 0.12 In-hospital death: 0.13 Complete recovery: 0.08 Post-recovery shortness of breath (O_2 dependent): 0.07 Post-recovery fatigue: 0.05 Length of ICU stay with high PDW: <0.00
Suarez Castillejo et al. [26] Spain Prospective cohort, single-center	All patients n=179 Non-PE COVID-19 cohort n=108 PE COVID-19 cohort n=71	PDW(%) All patients Baseline: 16.3 (15.8–16.8) % Peak: 17.2 (16.9–17.9) % Prior to CTPA: 16.4 (15.9–16.8) % Non-PE COVID-19 patients Baseline: 16.1 (15.7–16.8) % Peak: 17.1 (16.8–17.7) % Prior to CTPA: 16.2 (15.8–16.7) % PE COVID-19 patients Baseline: 16.6 (16.1–17.2) % Peak: 17.3 (16.9–18.2) % Prior to CTPA: 16.6 (16.1–16.9) %	Non-PE COVID-19 patients vs. PE COVID-19 patients Baseline: 0.00 Peak: 0.04 Prior to CTPA: 0.0
Suliman et al. [36] Saudi Arabia Retrospective cohort	Non-COVID-19 cohort n=2,414	PDW (%) Mean Preceding Period (07/2019–04/2020): 13.18 % Mean Lockdown Period (05/2020–09/2020): 12.58 %	Diagnostic (pre-pandemic vs. lock-down): <0.001 (Unpaired t test and F test)
Zhong et al. [38] China Retrospective cohort	MPR≤7.44 cohort n=59 MPR>7.44 group n=26	PDW (%), median (IQR) MPR≤7.44 patients: 12.6 (11.8, 13.9) % MPR>7.44 patients: 14.6 (13.1, 16.8) %	Severity: 0.004

ASA, acetylsalicylic acid; ICU, intensive care unit; PE, pulmonary embolism; MPV, mean platelet volume; MPR, mean platelet volume/platelet count rate; CTPA, computed tomography pulmonary angiography.

unaffected controls or COVID-19 negative cohorts [19, 21, 22, 27, 33, 34].

One study by Covali et al. found no difference in the mean PDW between pregnant COVID-19 patients and pregnant uninfected control [28]. It is possible that this study's lack of significant findings is attributable to comparisons between pregnant cohorts. The study by Khalid et al. indicated a significantly greater median PDW level in healthy controls compared to in the COVID-19 infected cohort [30].

Table 5: PDW (fL) in COVID-19 patients.

Author **Country of origin** **Design**	**Sample size**	**PDW measurement**	**PDW significance (p-Value)**
Alnor et al. [19] Denmark Case-control (nested)	All patients Non-COVID-19 cohort n=228 COVID-19 cohort n=74 Severity subclassification Severe COVID-19 cohort n=16 Non-severe COVID-19 cohort n=58 Patients with CRP<100 mg/L Non-COVID-19 cohort n=54 COVID-19 cohort n=49 Severe COVID-19 cohort n=8 Non-severe COVID-19 cohort n=41	PDW (fL), median (IQR) All patients Non-COVID-19 patients: 11.2 (10.1–12.6) fL COVID-19 patients: 12.2 (10.6–13.4) fL Severe COVID patients: 13.0 (11.6–14.5) fL Non-severe COVID patients: 12.1 (10.5–13.2) fL For patients with CRP<100 mg/L Non-COVID-19 patients: 11.2 (10.2–12.53) fL COVID-19 patients: 12.3 (10.85–13.45) fL Severe COVID patients: 12.90 (11.65–14.48) fL Non-severe COVID patients: 12.20 (10.65–13.30) fL	All patients COVID-19 vs. non-COVID-19 patients: 0.003 Severe COVID-19 vs. non-severe COVID-19 patients: 0.097 Patients with CRP<100 mg/L COVID-19 vs. non-COVID-19 patients: 0.005 Severe COVID-19 vs. non-severe COVID-19 patients: 0.239
Nori et al. [21] Iraq Retrospective case control	COVID-19 cohort n=50 Non-COVID-19 cohort n=50	PDW (fL), mean ± SD/SE COVID-19 positive patients: 14.82 ± 3.18/0.46 fL COVID-19 negative patients: 13.3 ± 2.16/0.39 fL	Case vs. Control (COVID-19 vs. non-COVID-19 in pregnant women): 0.024
Khalid et al. [30] Saudi Arabia Retrospective cross-sectional	COVID-19 cohort n=487 Non-COVID-19 cohort n=300	PDW (fL), median (Min-Max) COVID-19 patients: 12.4 (8.8–23.3) fL Non-COVID-19 patients: 13.2 (10.3–22.1) fL Severity subclassification ICU COVID-19 patients: 12.9 (8.8–23.3) fL ER COVID-19 patients: 12.0 (8.8–22.6) fL Mild COVID-19 patients: 11.4 (9.5–15) fL Non-COVID-19 patients: 13.2 (10.3–22.1) fL	Case vs. Control (COVID vs. Non-COVID): 0.000 Severity: 0.000
Asrie et al. [24] Ethiopia Cross-sectional	All patients n=117 Mild COVID-19 cohort n=45 Moderate COVID-19 cohort n=43 Severe COVID-19 cohort n=29	PDW (fL), median (IQR) All patients: 16.4 (0.75) fL Mild COVID-19 patients: 16.4 (0.65) fL Moderate COVID-19 patients: 16.5 (0.7) fL Severe COVID-19 patients: 17 (1.55) fL	Severity: 0.001
Yovchevska et al. [23] Bulgaria Retrospective analytic case-control, single center	COVID-19 patients with ARDS n=190 COVID-19 patients without ARDS n=303 COVID-19 survivor cohort	PDW (fL) COVID-19 patients with ARDS: 15.10 ± 2.08 fL COVID-19 patients without ARDS: 12.94 ± 2.12 fL	Severity (ARDS): <0.001 Survival: 0.095

Table 5: (continued)

Author Country of origin Design	Sample size	PDW measurement	PDW significance (p-Value)
	n=133 COVID-19 non-survivor cohort n=57	COVID-19 survivors: 14.93 ± 2.16 fL COVID-19 non-survivors: 15.48 ± 1.84 fL	
Ye et al. [37] China Retrospective cross-sectional	Asymptomatic-moderate cohort n=132 Severe or above cohort n=29	PDW (fL) Asymptomatic-moderate patients: 14.98 ± 3.17 fL Severe and above patients: 15.34 ± 2.08 fL	Severity (asymptomatic-moderate/severe and above): 0.559
László et al. [31] Hungary Retrospective descriptive analysis of prospectively collected data	ICU cohort n=95 Non-ICU cohort n=111 COVID-19 survivor cohort n=130 COVID-19 non-survivors n=76 COVID-19 ICU survivor cohort n=60 COVID-19 ICU non-survivors n=35	PDW (fL), median (25%-75 % confidence interval) ICU patients: 19.9 (13.7–57.7) Non-ICU patients: 14.5 (11.6–44.7)	ICU stay: <0.001 ICU survival: 0.09
Wang et al. [10] China Retrospective single center	COVID-19 positive cohort n=40	PDW (fL) Admission group: 11.75 ± 1.227 fL Discharge group: 12.23 ± 1.485 fL	Diagnostic (admission vs. discharge): 0.0186

ARDS, acute respiratory distress syndrome.

Four out of ten (40 % of studies) exhibited significantly increased PDW values in patients with severe COVID-19 vs. in those with less-than-severe illness [20, 23, 24, 34]. All of the remaining studies that tested for a severity-dependent PDW correspondence do not show significance between the two groups [12, 19, 27, 29, 35, 37]. The lack of a significant difference in the study by Güçlü et al. may be a result of the narrower comparison of PDW levels from moderate-to-severe COVID-19 cohorts [12]. Similarly, the report by Hajian et al. compared PDW values in severe vs. critically ill COVID-19 cohorts – such that the differences between groups may be too small to detect a difference in PDW [29].

Five out of seven (71.4 % of studies) demonstrate a significant elevation of PDW values in patients who died from COVID-19 compared with patients who were infected with the virus but survived [12, 25, 31, 32, 34]. The remaining two studies indicated non-significant differences between the groups [23, 35].

Discussion

PDW is a laboratory test that measures volume variability in platelet size, providing an indicator of the heterogeneity in platelet morphology [14]. PDW is widely used as an indicator of platelet function and activation, and it has been reported as a more specific marker of platelet activation, since it does not increase during simple platelet swelling, suggesting higher PDW values on admission to internal medicine wards associated with a more severe clinical profile and increased risk of 90-day mortality [11].

Several reports (n=23) were analyzed by our systematic review, revealing a wide heterogeneity of population cohorts examined and a lack of homogenous expression of platelet indices (e.g., PDW was expressed as fL and percentage).

Overall, the findings of our systematic review revealed that 75 % of studies reported significantly elevated PDW values in COVID-19 infected cohorts compared to healthy/non-COVID-19 controls [19, 21, 22, 27, 33, 34], and that 40 % of studies reported that patients with severe COVID-19 showed increased PDW values than those with less-than-severe illness [20, 23, 24, 34]. However, the lack of a significant difference in some studies may depend on the different selection of COVID-19 cohorts among studies.

In fact, stratifying patients according to their survival, we observed that 71.4 % of studies demonstrated a significant increase of PDW values in patients who died from COVID-19 compared with patients who were infected with the virus but survived [12, 25, 31, 32, 34].

Overall, these results suggest that during Sars-Cov-2 infection platelets are critically involved as major players in the process of immunothrombosis. Platelet reactivity is mirrored by morphofunctional alterations, such as increased MPV and PDW, as indicators of platelet heterogeneity.

Several studies reported that platelets tend to be deformed, to became giant, to form homotypic and heterotypic aggregates, which finally results in a thrombocytopenic condition induced by platelet destruction or consumption, associated with the release of younger and larger platelets from the bone marrow, overall contributing to increased PDW values [5, 6, 13, 15–17].

A similar scenario has also been described to be induced by high levels of circulating histones, which represent critical triggers found at increased concentration in blood samples from COVID-19 and sepsis patients, recognized to be able to induce a thrombocytopenic condition and platelet heterogeneity [39]. Notably, possible heterogeneity in hematological procedures applied for analysis of PDW may also impact the compatibility of PDW data among studies and the conclusions made on present PDW trends. Finally, despite several studies indicating increased PDW values in COVID-19, our results confirm that the use of PDW as possible prognostic biomarkers of COVID-19 sepsis still remains debated due to the limited number of studies to draw a conclusion [40], but new opportunities to investigate the crucial role of platelets in thrombo-inflammation are guaranteed. In this respect, the use of PDW could implement the everyday clinical practice if included in various artificial intelligence (AI) algorithms [41, 42], contributing to the development of innovative diagnostic and prognostic approaches.

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Chapter 5

Thrombocytopenia and hyperinflammation are induced by extracellular histones circulating in blood

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Letter to the Editor

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Thrombocytopenia and hyperinflammation are induced by extracellular histones circulating in blood

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To the Editor,

Circulating histones have been suggested as critical mediators and potential diagnostic/prognostic markers in several hyperinflammatory diseases, including Sars-CoV-2 infection and classical bacterial sepsis, because their levels increase with the worsening of classical and viral sepsis [1–3].

Coagulopathy, thrombocytopenia, platelet aggregation, morphofunctional alterations, immunothrombosis, and thromboinflammation are also critical hallmarks of COVID-19 (Coronavirus Disease-19) and classical sepsis [4].

Several literature data highlighted that histone-platelet interactions could potentially promote thrombocytopenia [5] and platelet activation [6], suggesting that platelets could represent an important target of circulating histones.

Immune cells and platelets are activated to counteract infective stimuli or cell damage, but when they overreact, as during critical sepsis and COVID-19, they could promote a hyperinflammatory response, characterized by an excessive amount of proinflammatory mediators that recruit further immune cells, thus amplifying a dangerous vicious cycle associated with multiorgan failure and death [7].

We recently demonstrated that histones are able to promote morphofunctional alterations in monocytes, thus emerging as critical mediators in monocyte activation during these conditions [8, 9]. Few data are available on the ability of histones to alter platelet indices and associated hyperinflammatory responses. The purpose of this study was to investigate the ability of histones to alter platelet indices (platelet count; Mean Platelet Volume, MPV; Platelet Distribution Width, PDW) and activate hyperinflammatory responses in peripheral blood cells.

Healthy subjects (n=20, age range 24–65) were recruited as volunteers among staff at the Universities of Palermo and Urbino; five samples failed to complete the analytic processes due to insufficient sample volume. Peripheral venous blood, collected in EDTA-K3 tubes, was treated with a mixture of commercially available histones, including H1, H2A, H2B, H3, and H4 (Histone from calf thymus, Sigma, cod. 10223565001) to evaluate the impact of histones at different times (0, 30, 60, 180 min) and concentrations (0, 50, 100, and 200 μg/mL) on platelet indices and on the release of cytokines from blood cells, as previously described [8, 9].

Routine complete blood cell count, including platelet count (PLT), mean platelet volume (MPV) and platelet distribution width (PDW), were analysed on a UniCell DxH900 Hematology Analyzer (Beckman Coulter), according to the manufacturer's instructions.

Automated slide preparation (unit SP-100, DI-60 system workflow, Sysmex) was used to obtain May-Grunwald-Giemsa-stained blood smears, according to the manufacturer's instructions.

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After 3 h all blood samples were centrifuged (2,000×*g*, 15 min), and plasma samples were used to quantify six inflammatory biomarkers through the Pro™ Human Cytokine 27-plex assay (including: MIP-1α/CCL3, PDGFbb, RANTES) and the Pro™ Human TGF-β 3-plex assay (including: TGF-β1; TGF-β2, and TGF-β3) through a multiplex suspension immunomagnetic assays, based on the use of fluorescently dyed magnetic beads covalently conjugated with monoclonal antibodies specific for the target proteins, according to the manufacturer's instructions (Bio-Plex, Bio-Rad Labs, Hercules, CA, USA), as previously described [8, 9]. According to the manufacturer's data, the lowest detection limit was 0.5 pg/mL, while the mean inter-assay and intra-variability was 7.4 and 7.1 %, respectively.

This observational *in vitro* study was approved by the local Ethical Committee and all investigations have been conducted according to the Declaration of Helsinki principles.

The *in vitro* treatment of whole blood samples with increasing concentrations of histones (50, 100, and 200 µg/mL) was associated with a very significant reduction in platelet counts, already starting after 30 min from histone treatment, and remaining stably low at 60 and 180 min (Figure 1A), revealing a dose-dependent mechanism confirmed by significant regression lines (Supplementary Table 1).

MPV was also affected by histone treatments. In fact, besides minimal MPV variations in healthy controls (remaining within the reference range of 7.5–11.7 fL), histone treatments promoted a significant increase in MPV during time-course and at all doses compared to respective controls (Figure 1B).

Moreover, we observed that PDW levels were not significantly changed during time in untreated controls but were significantly increased by treating with all three histone doses, without variations among three time-points (Figure 1C).

The automated digital microscopy of blood smears confirmed morphological changes measured by platelet indices, highlighting platelet alterations related to platelet homotypic and heterotypic aggregations, platelet ballooning, and giant thrombocytes (Figure 1D).

After 3 h of treatment with 50, 100, and 200 µg/mL of histones we observed a significant and dose-dependent increase of MIP-1α, RANTES, PDGFbb, TGF-β1, TGF-β2, and TGF-β3 as shown in Figure 2.

Studies on the interaction between histones and platelets have become more relevant than ever, as both have been found to be critical cellular and molecular mediators contributing to the immunothrombotic manifestations observed in SARS-CoV-2 infection and sepsis.

We highlighted that the *in vitro* stimulation of whole blood from healthy subjects was associated with a rapid dose-dependent decreased platelet count, and an increase of platelet volumetric indices.

In agreement with our results, several studies reported that high levels of circulating histones are associated with a thrombocytopenic condition in classical sepsis and in COVID-19 infection [2, 10], and that histones induce a rapid thrombocytopenia in mice [5], and activate platelets, promoting their pro-coagulant functions [5, 6].

Histones are also able to induce platelet ballooning, generate platelet-derived microvesicles, and promote platelet aggregation by inducing P-selectin expression on platelet membranes, which act as a binding site for leukocytes and endothelial cells, accordingly to our findings on morphological alterations and homo- and heterotypic aggregates induced by histones.

In our *in vitro* conditions we demonstrated that MPV and PDW variations are the result of the variation of dimension as observed for the platelet ballooning and giant thrombocytes. We can assume that PDW and MPV variations, as markers of platelet heterogeneity and increased volume associated to platelet activation, occur mainly due to circulating platelet activation mediated by histones, which alter platelet morphology, activation, and aggregative status, without involving other platelet maturation mechanisms or direct recruitment of precursors.

Furthermore, we observed that MIP-1α, PDGF-bb, RANTES, TGF-β1, TGF-β2 and TGF-β3 were significantly increased by histone treatments in a dose-dependent manner. These findings are in agreement with our previous results [8], and overall reinforce the hypothesis on the critical contribution of histones in the onset of the hyperinflammatory status found in sepsis conditions.

Although the reduced sample size of our study, it is noteworthy that:

(1) the *ex-vivo* whole blood model represents a reliable preclinical model to assess the early modifications of blood cell parameters associated with triggers of bacterial and viral sepsis.
(2) this is the first time that histones have been described as inducers of platelet index alterations in whole blood, highlighting that extracellular histone circulating in blood (i.e., cell-free histones) are critical players in both classical (bacterial) and viral sepsis.
(3) these findings revealed that histones represent a crucial link between inflammation and thrombosis, suggesting their major role for platelet involvement in infections and non-infectious diseases.
(4) the increased concentration of circulating histones in blood affects platelet morphology and activation, inducing thrombocytopenia and thromboinflammation as reported in critical sepsis conditions.

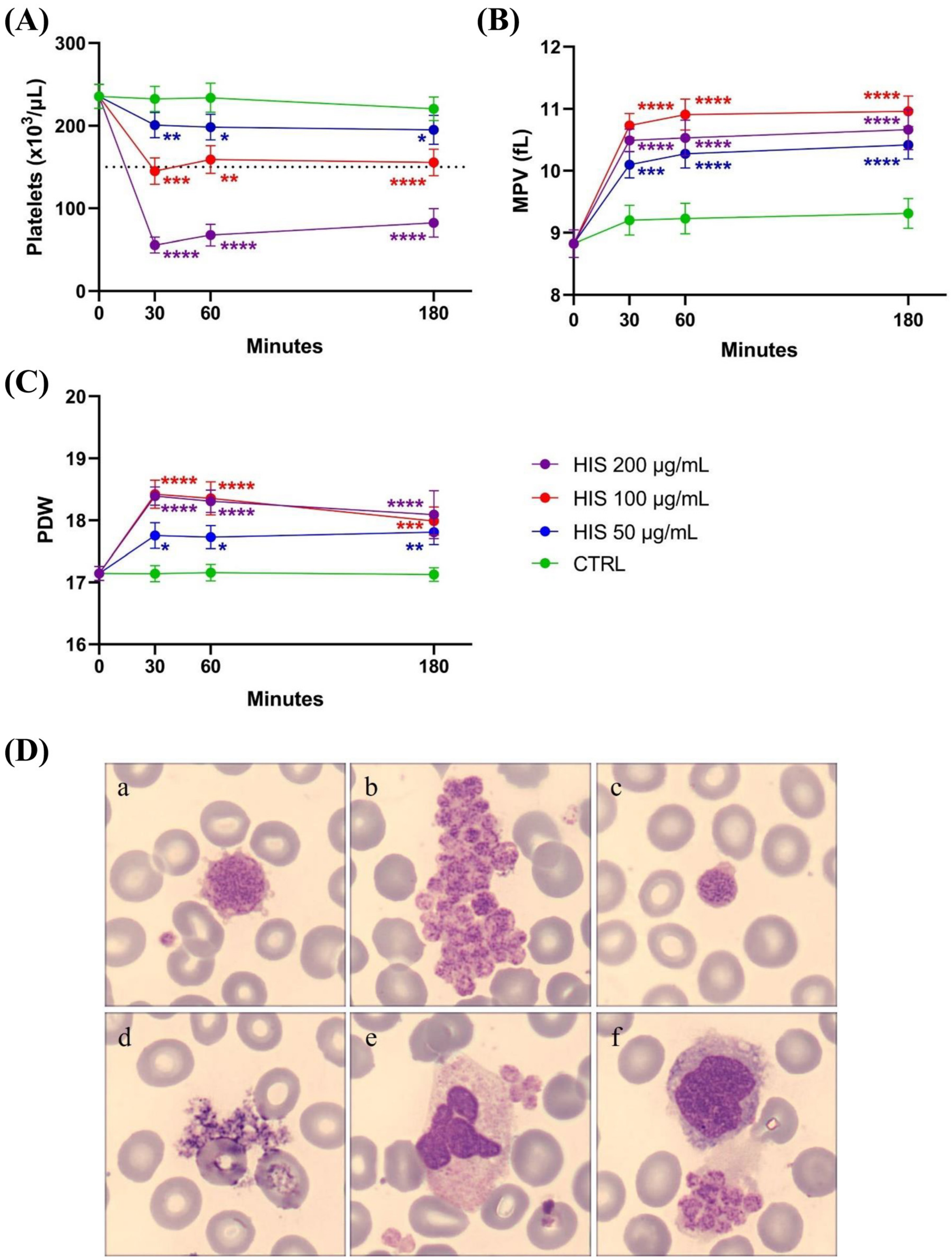

Figure 1: Platelet index and morphology variations after histone treatments. (A) Time- and dose-dependent platelet count variations in histone-treated whole blood samples. (B) Time- and dose-dependent MPV variations in histone-treated whole blood samples. (C) Time- and dose-dependent PDW variations in histone-treated whole blood samples. The graphs show the platelet count, MPV and PDW modification in whole blood samples collected from healthy subjects and treated *in vitro* with 0, 50, 100, and 200 µg/mL of a histone mixture. Values are expressed as mean ± SEM (Two-way ANOVA: *p: 0.01–0.05; **p: 0.01–0.001; ***p: 0.001–0.0001; ****p: <0.0001; the comparisons were calculated among treatments and respective controls at each time). The dotted line in Figure 1A indicates the lower cut-off level, as reported in the literature. (D) Automated light microscopy images of peripheral blood platelets representative of (a) giant thrombocyte; (b) PLT aggregates; (c) Ballooning PLT; (d) PLT-RBC aggregation; (e) PLT-neutrophil interaction; (f) PLT-lymphocyte interaction observed after histone treatments (May-Grunwald-Giemsa, ×100). All statistical tests were performed using GraphPad Prism 9.0. Values are expressed as mean ± standard error mean (SEM) and p-values<0.05 were considered significant. Differences among groups were determined using two-way ANOVA followed by Tukey's multiple comparison test.

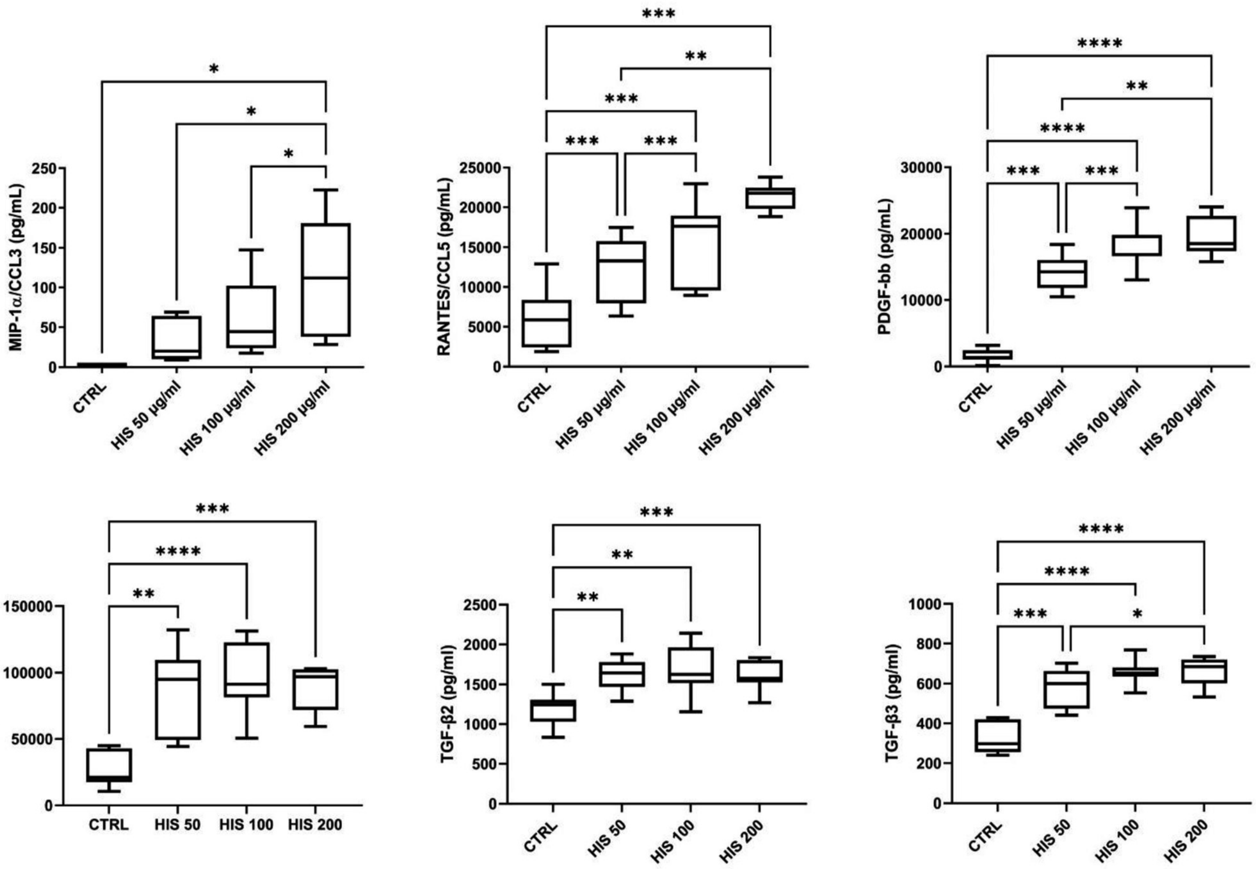

Figure 2: Cytokine values (mean ± SEM) after 3 h of *in vitro* treatment with 0, 50, 100, and 200 µg/mL of a histone mixture (One-way ANOVA: *p: <0.05; **p: 0.001–0.01; ***p: 0.001–0.0001; ****p: <0.0001; comparisons were calculated among n=8 treatments and respective controls and among each treatment). Values are expressed as pg/mL.

Further studies are ongoing to extend the landscape of blood cell alterations induced by crucial bacterial and viral sepsis triggers.

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Competing interests: Authors state no conflict of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: 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. All investigations have been conducted according to the Declaration of Helsinki principles. In line with non-interventional retrospective design of this *in vitro* study, investigations were performed on volunteers without clinical indications and no clinical decisions were made based on measured values.

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Chapter 6

Novel mechanisms of thrombo-inflammation during infection: the harmful impact of circulating histones

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LETTER TO THE EDITOR

Novel mechanisms of thrombo-inflammation during infection: the harmful impact of circulating histones

To the Editor,

We read with great interest the exhaustive review/state of the art by Colicchia et al. [1], in which the new data on thrombo-inflammation mechanisms were shown through the coordinate activation of platelets and neutrophils during bacterial and viral sepsis. We appreciate their careful review, and we wish to comment on some associated issues for integrating and updating the readers about the new biological and biochemical knowledge related to the harmful effects of circulating histones.

Ever increasingly important evidence highlights that histones (intranuclear positively charged proteins, associated with chromatin) show extranuclear/extracellular functions when extruded mainly by activated white blood cells and platelets during bacterial and viral infections [2,3]. In this respect, circulating histones have been demonstrated as inducers of cellular damage through cytotoxic proinflammatory effects, promoting monocyte activation and platelet aggregation, as well as finally acting as damage-associated molecular pattern molecules [2].

A growing number of recent studies underlined the involvement of circulating histones in inflammatory processes and infections (eg, bacterial and COVID-19-associated viral sepsis), shedding light on their roles as both molecular triggers and potential therapeutic targets [3]. Recently, increased levels of circulating histones were also correlated with worsening of several diseases, raising their usefulness to stratify patients at higher risk of morbidity/mortality [2,3].

Other than neutrophils [1], monocytes are well-recognized key players in innate immunity against pathogens [2]; monocyte activation is characterized by peculiar morphologic changes, mirroring cell heterogeneity and anisocytosis, and clinically quantified by the monocyte distribution width (MDW) test, a mathematical parameter based on the measurement of volume (by direct current impedance), conductivity (by radio frequency opacity), and scatter (by laser beam intracellular/intranuclear scatter) of monocyte population [4].

When monocytes overreact to increased levels of circulating histones (as during infection or injury by critical bacterial and viral sepsis) they kick up a cytokine storm [5], a hyperinflammatory reaction inducing a harmful vicious cycle leading patients to a higher risk of severe/critical conditions up-to death [3]. We recently demonstrated that histones trigger MDW changes [4], promoting also hyperinflammatory responses associated with a monocyte anisocytosis [5], mirroring those observed in bacterial and COVID-19-associated viral sepsis [4]. Moreover, we determined the role of circulating histones on the alteration of platelet indices (eg, count, mean platelet volume; platelet distribution width [PDW], and inflammatory cytokines [eg, Platelet-derived growth factor-BB, Regulated on Activation, Normal T cell Expressed and Secreted, transforming growth factor beta-1, beta-2 and beta-3]), revealing that circulating histones may significantly contribute to the thrombocytopenia and cytokine storm observed in COVID-19 and classical sepsis [5].

According to the outstanding focus on neutrophil-mediated platelet activation and the crucial interpretation of the novel mechanisms of thrombo-inflammation during infection by Colicchia et al. [1], as well as considering the novel emerging functions of histones as triggers of both hyperinflammation and monocyte and platelet alterations (determined by MDW and PDW), we would reinforce and integrate their conclusions [1], suggesting that the evaluation of MDW (as an index of activated and heterogenous monocyte populations), PDW (as an index of activated, aggregated and/or ballooning/giant platelets), and histone concentrations may provide a possible future tool to timely predict higher risk of worst outcome in patients with classic bacterial and COVID-19 sepsis.

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AUTHOR CONTRIBUTIONS

D.L. and C.D.F. drafted the manuscript. F.M. participated in the discussion and critical editing of the manuscript. All authors read and approved the final paper.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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Chapter 7

Monocyte Distribution Width alterations and cytokine storm are modulated by circulating histones

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Monocyte distribution width alterations and cytokine storm are modulated by circulating histones

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Abstract

Objectives: Extracellular histone levels are associated with the severity of many human pathologies, including sepsis and COVID-19. This study aimed to investigate the role of extracellular histones on monocyte distribution width (MDW), and their effect on the release of cytokines by blood cells.

Methods: Peripheral venous blood was collected from healthy subjects and treated with different doses of a histone mixture (range 0–200 μg/mL) to analyze MDW modifications up-to 3 h and digital microscopy of blood smears. Plasma obtained after 3 h of histone treatment were assayed to evaluate a panel of 24 inflammatory cytokines.

Results: MDW values significantly increased in a time- and dose-dependent manner. These findings are associated with the histone-induced modifications of cell volume, cytoplasmic granularity, vacuolization, and nuclear structure of monocytes, promoting their heterogeneity without affecting their count. After 3 h of treatment almost all cytokines significantly increased in a dose-dependent manner. The most relevant response was shown by the significantly increased G-CSF levels, and by the increase of IL-1β, IL-6, MIP-1β, and IL-8 at the histone doses of 50, 100, and 200 μg/mL. VEGF, IP-10, GM-CSF, TNF-α, Eotaxin, and IL-2 were also up-regulated, and a lower but significant increase was observed for IL-15, IL-5, IL-17, bFGF, IL-10, IFN-γ, MCP-1, and IL-9.

Conclusions: Circulating histones critically induce functional alterations of monocytes mirrored by MDW, monocyte anisocytosis, and hyperinflammation/cytokine storm in sepsis and COVID-19. MDW and circulating histones may be useful tools to predict higher risks of worst outcomes.

Keywords: COVID-19; cytokine; histones; monocyte; monocyte distribution width; sepsis.

Introduction

Histones are highly conserved, intranuclear, positively charged proteins, which main functions are associated with the maintaining of chromatin stability and the epigenetic regulation of several cellular processes. Increasing evidence shed light on the presence of further extracellular and extranuclear functions for circulating histones. In particular, extranuclear histones can be found in the cytosol and at the cell surface, where they exert anti-microbial effects and promote cell-mediated apoptosis [1]. Moreover, at the extracellular level, histones can be released freely or as a DNA-bound nucleosome passively from dying cells (particularly during necrosis), and also embedded in extracellular traps (ET) during the ETosis process. In this respect, circulating white blood cells extrude ETs in response to a hostile microenvironment, such as during bacterial and viral infections or in sterile conditions due to the activation of immune cells [2]. Extracellular histones induce cellular damages through (I) a direct cytotoxic effect on endothelial cells; (II) the promotion of platelet activation, aggregation, and thrombin generation, resulting in the development of pro-coagulant platelet, endothelial, and monocyte phenotypes; (III) the activation of white blood cells which release cytokines and ROS(2). It is well established that extracellular histones act as damage-

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Daniela Ligi and Bruna Lo Sasso have contributed equally as first authors.

Marcello Ciaccio and Ferdinando Mannello have contributed equally as senior authors.

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associated molecular pattern proteins (DAMPs) and promote pro-inflammatory effects by activating TLR2- and TLR4-mediated signalling [3].

Levels of circulating histones in physiological conditions range from 0.79 to 2.30 µg/mL, but they increase in animals or patients with cancer, inflammation, and infections, suggesting an extracellular role of histones in human diseases [4–6]. In this respect, a growing number of recent studies underlined the involvement of circulating histones in classical bacterial and viral sepsis, emphasizing their potential roles both as triggers and therapeutic targets of diseases. Due to their ability to stimulate inflammatory responses, induce endothelium injuries, and activate coagulation in clinical settings such as bacterial sepsis and COVID-19-associated viral sepsis, histones have raised great attention in the scientific community. In fact, it has been demonstrated that histone levels increase with the worsening of the disease, and thus may be useful to stratify patients at higher risk of mortality [7, 8].

Noteworthy, sepsis infections share common laboratory biomarkers, as well [9, 10]. Currently, great attention is paid to the use of monocyte distribution width (MDW), a measure of monocyte heterogeneity, automatically calculated from the dispersion around the mean of the monocyte population volume in whole blood by last-generation hematology analyzers. MDW is an innovative parameter mathematically based on the measure of specific cell volume index and standard deviation of volume distribution within the monocyte population. MDW measures positional parameters with VCS technology (i.e., volume, conductivity, and scatter), using three independent energy sources simultaneously: direct current impedance to measure cell volume of all cell types; radio frequency opacity, to characterize conductivity for the internal composition of each cell; a laser beam to measure light scatter for cytoplasmic granularity and nuclear structure [11].

MDW has been recently FDA-approved and EC-marked as early sepsis indicator, due to its very early availability during the clinical evaluation when sepsis diagnosis could not be suspected [12] (additional references in Supplementary Material), and recent studies underline its possible prognostic role to stratify COVID-19 patients according to the severity and clinical outcomes [11, 13–19].

Monocytes are pivotal players in the innate immune responses against invading pathogens and their activation is characterized by morphological changes (clinically mirrored by MDW alterations) and the release in biological fluids of inflammatory mediators. When the immune system overreacts to an infection or injury, as during critical conditions of sepsis and COVID-19, it activates a hyperinflammatory reaction, named "cytokine storm". The dysregulated immune response is sustained by a massive release of proinflammatory cytokines, chemokines, and signaling molecules that furtherly attract immune cells, which secrete more cytokines, recruiting even more activated immune cells, thus fueling a dangerous vicious cycle. Despite recruited to block the initial infective stimulus, immune cells and mediators end up attacking the tissues and organs, leading to multiorgan failure and death.

We recently demonstrated that histones trigger MDW changes, mirroring those observed in COVID-19 and sepsis [20], thus emerging as critical contributors of monocyte activation during these conditions. A limited number of data is available instead on the ability of histones to promote hyperinflammatory responses associated with monocyte anisocytosis. The purpose of this study was to investigate the ability of histones to alter MDW index and promote hyperinflammatory responses in peripheral blood cells.

Materials and methods

Sample collection and blood analyses

Healthy subjects (n=8, age range 31–63) were recruited as volunteers among staff at the Department BiND of the University of Palermo and Department DISB of the University of Urbino. Peripheral venous blood was collected in EDTA-K_3 tubes and processed within 4 h from collection. Routine complete blood cell count and MDW were analyzed on an UniCell DxH900 Hematology Analyzer (Beckman Coulter), according to the routine methods and the manufacturer's instructions. Automated slide preparation (unit SP-100, DI-60 system workflow, Sysmex) was used to obtain May-Grunwald-Giemsa-stained blood smears. This observational non-interventional *in vitro* study was approved by the local Ethical Committee and all investigations have been conducted according to the Declaration of Helsinki principles.

Peripheral blood samples and *in vitro* treatments

Aliquots of 1 mL of EDTA-K_3 whole-blood from each volunteer were treated with a mixture of commercially available histones, including H1, H2A, H2B, H3, and H4 (histone from calf thymus, Sigma, cod. 10223565001) to evaluate the impact of histones at different times and concentrations (0, 50, 100, and 200 µg/mL) on the MDW characteristics and on the release of cytokines from blood cells. Whole blood samples were maintained for 30 min at 37 °C for inducing a "priming" effect on circulating blood cells and then maintained at RT to follow and analyze the MDW modifications at 0, 30, 60, and 180 min after careful inversion avoiding blood cell sedimentation. After 3 h all blood samples were centrifuged (2,000×*g*, 15 min) to obtain plasma for further analyses.

Cytokine release

All plasma samples obtained after 3 h of histone treatments were assayed to evaluate a panel of 24 inflammatory biomarkers through the Pro™ Human Cytokine 27-plex assay (including: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, Eotaxin/CCL11, bFGF, G-CSF, GM-CSF, IFN-γ, IP-10/CXCL10, MCP-1/CCL2, MIP-1β/CCL4, TNF-α, VEGF), a multiplex suspension immunomagnetic assays, based on the use of fluorescently dyed magnetic beads covalently conjugated with monoclonal antibodies specific for the target proteins, according to the manufacturer's instructions (BioPlex, Bio-Rad Labs, Hercules, CA, USA).

Levels of all analytes were determined using a Bio-Plex 200 array reader, based on Luminex X-Map Technology (Bio-Rad Labs, Hercules, CA, USA) that detects and quantifies multiple targets in a 96-well plate with a single small fluid volume. Data were collected and analyzed using a Bio-Plex 200 instrument equipped with Bio-Plex Manager analysis software (Bio-Plex Manager Software v. 6.1). The protein concentrations (expressed as pg/mL) were calculated through a standard curve. According to the manufacturer's data, the lower detection limit was 0.6 pg/mL, while the mean inter-assay variability was 7.6%.

Statistical analysis

All statistical tests were performed using GraphPad Prism 9.0. Values are expressed as mean ± standard error mean (SEM), unless otherwise specified, and p values <0.05 were considered significant. Differences among groups were determined using one-way or two-way ANOVA followed by a posthoc test (i.e., Tukey's multiple comparison test). Regression analyses were performed through simple linear regression. Correlations among various biomarkers were tested for significance using the Pearson correlation test.

Results

The treatment of whole blood with increasing doses of histones revealed significantly different profiles of monocyte heterogeneity, ranging from round-shaped and reniform nucleus of normal untreated monocytes (Figure 1A) to a progressively enhanced volume, intracellular vacuolization and granularity, membrane alterations and nuclear structure changes, as observed through blood smears (Figure 1B–D).

The treatment of whole blood with histones results in an MDW increase in a time and dose-dependent fashion. In particular, we observed that MDW values in controls ranged from a minimum of 14.31 and a maximum of 20.70 up-to 3 h, and there were not significant differences among all times considered. The treatment with 50 µg/mL of histones induced a significant time-dependent increase of MDW at 30, 60, and 180 min (mean ± SD) vs. respective controls (20.3 ± 1.2, $p<0.0027$; 21.3 ± 1.4, $p<0.0001$; 22.3 ± 1.6, $p<0.0001$, respectively) (Figure 2A and Supplemental Table 1). This result is also confirmed by the significant regression analysis ($Y=0.02039 \times X + 19.06$; $R^2 = 0.4545$; $p<0.0001$).

The intermediate dose of 100 µg/mL of histones promoted a significant MDW increase of 21.1 ± 1.7 ($p<0.0001$) after 30 min, 22.1 ± 1.4 ($p<0.0001$) after 60 min, and 23.7 ± 2.4 ($p<0.0001$) at 180 min, as also confirmed by the significant regression line ($Y=0.02745 \times X + 19.25$; $R^2=0.5035$, $p<0.0001$) (Figure 2A and Supplemental Table 1).

The highest dose of 200 µg/mL of histones promoted an MDW increase of 21.9 ± 1.4 ($p<0.0001$) after 30 min, 22.8 ± 1.4 ($p<0.0001$) after 60 min, and 24.2 ± 2.6 ($p<0.0001$) at 180 min, as also confirmed by the significant regression line ($Y=0.02983 \times X + 19.62$; $R^2=0.479$, $p<0.0001$) (Figure 2A and Supplemental Table 1).

Interestingly, focusing attention on the effects of increasing doses of histones at each time, we observed that besides the time-dependent increase of MDW for each dose, there was also a significant dose-dependent increase of MDW at each time, as demonstrated by the regression lines reported in Table 1.

These histone-induced MDW modifications were not sustained by a change in the monocyte population count, neither in its total count (Figure 1B), nor in the percentage values (Figure 1C), whose minimal variations were distributed within the physiological ranges (2–12 × 1,000/µL and 0.2–1.2% respectively).

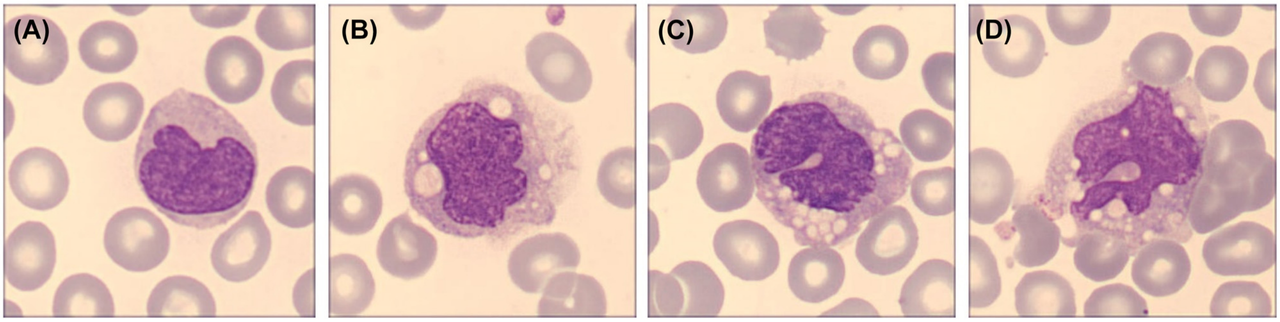

Figure 1: Light microscopy images of peripheral blood monocytes representative of untreated controls (A), HIS 50 µg/mL (B), HIS 100 µg/mL (C), and HIS 200 µg/mL (D) histone-treated whole blood. (May-Grunwald-Giemsa, × 100).

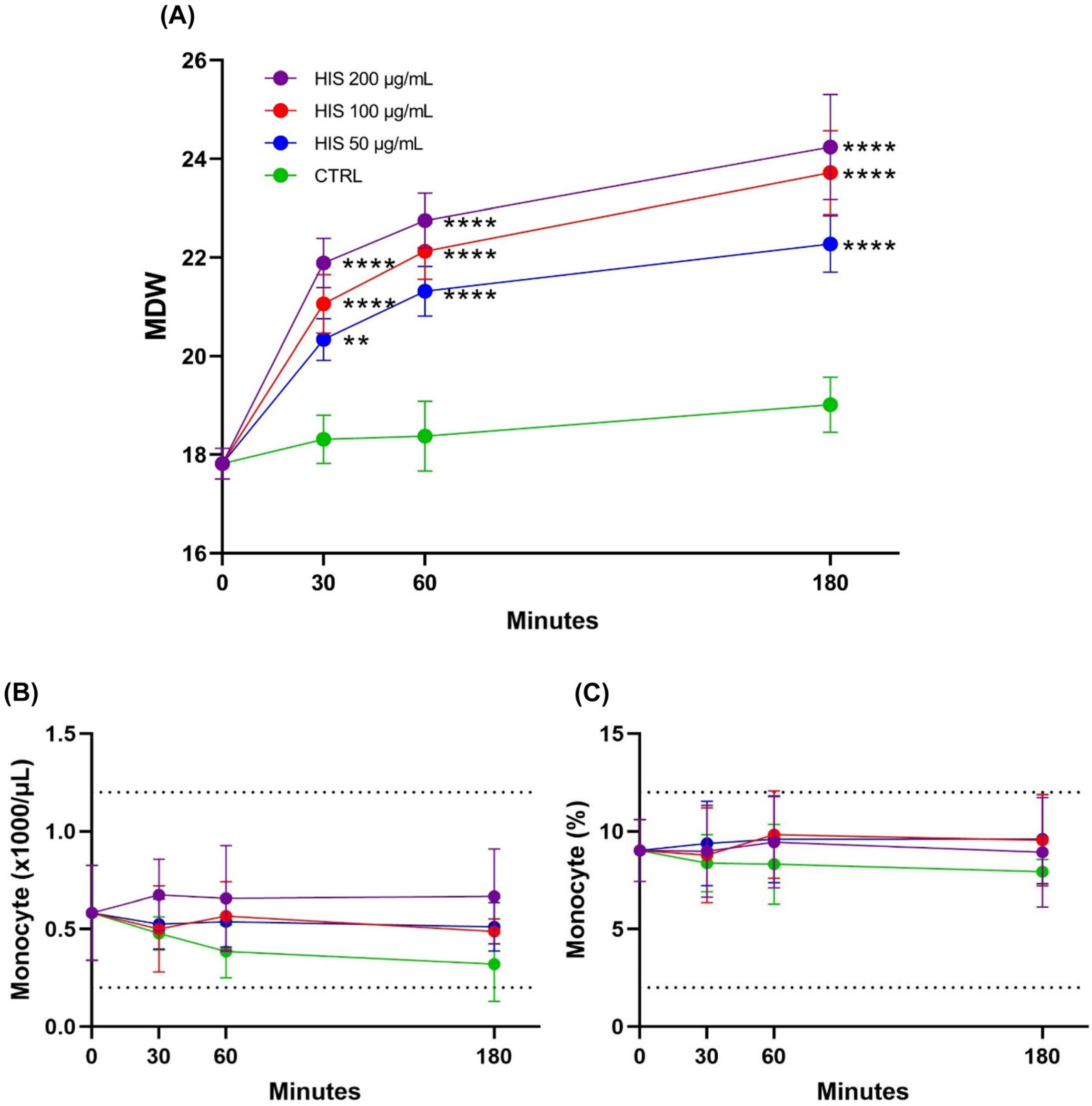

Figure 2: Time- and dose-dependent MDW and monocyte count variations in histone-treated whole blood samples. (A) Time- and dose-dependent MDW index modifications in whole blood samples collected from healthy subjects treated *in vitro* with 0, 50, 100, and 200 µg/mL of histone mixture. Values are expressed as mean ± SEM. (Two-way ANOVA: ** = p: 0.001–0.01; **** = p<0.0001; the comparisons were calculated among treatments and respective controls at each time). (B) Time- and dose-dependent monocyte count (×1.000/µL) and (C) monocyte percentage modifications in whole blood samples collected from healthy subjects treated *in vitro* with 0, 50, 100, and 200 µg/mL of histone mixture. (Two-way ANOVA). The dotted line indicates the cut-off levels.

Table 1: Simple linear regressions exploring dose-dependent MDW changes (time: dependent variable).

	Equation	R^2	p-Value
30 min	Y=0.01649 × X + 18.95	0.4196	<0.0001
60 min	Y=0.01982 × X + 19.44	0.4264	0.0002
180 min	Y=0.02378 × X + 20.37	0.3674	0.0006

After 3 h of treatment with 50, 100 and 200 µg/mL of histones we observed a dose-dependent increase of all cytokines (except for IL-13), as shown in Figure 3 and detailed in Supplemental Table 2.

In particular, we highlighted that G-CSF showed the strongest response to histone treatment; in fact, the levels of G-CSF were significantly increased by 9-fold (p<0.05) after

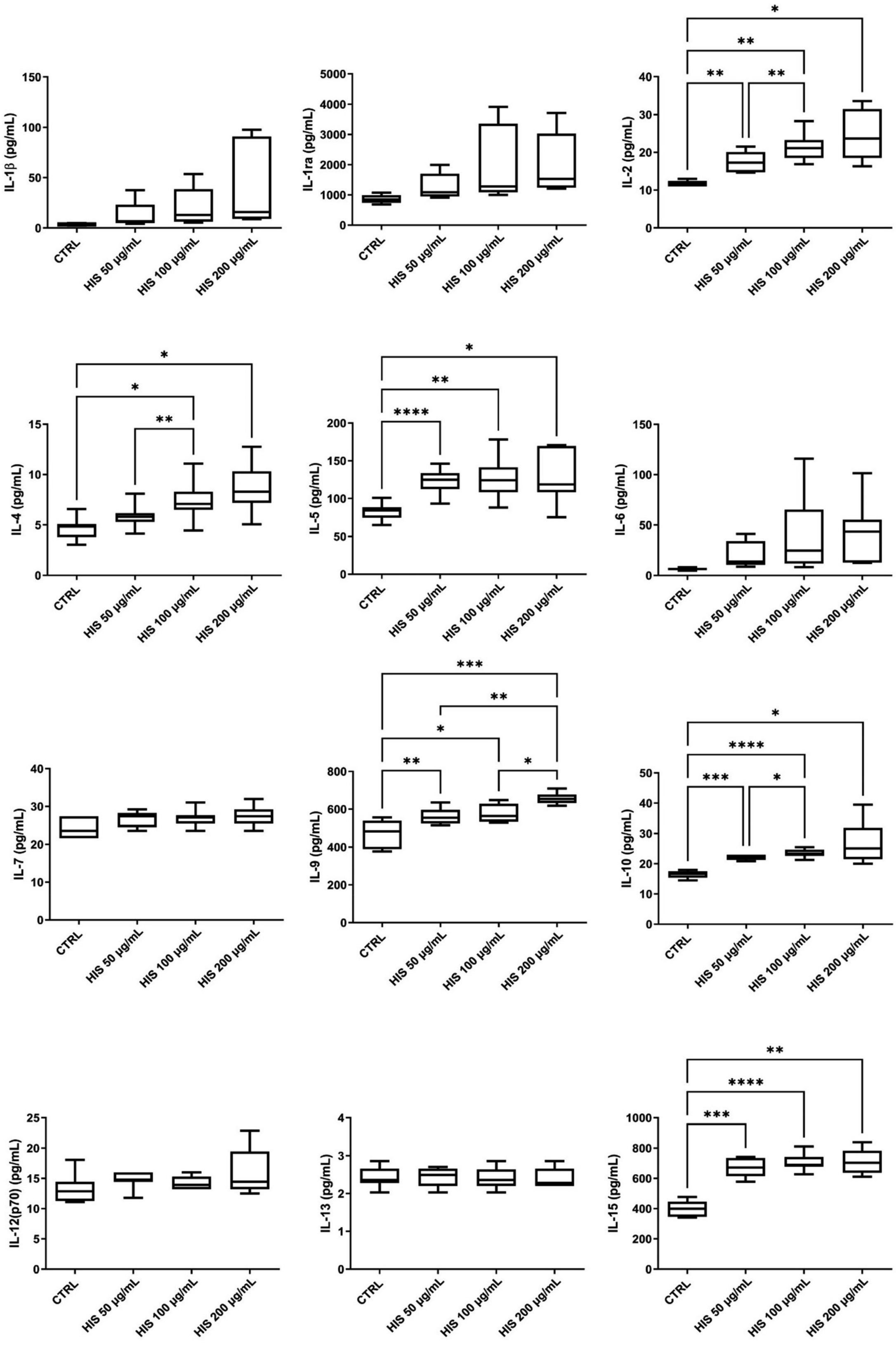

Figure 3: Cytokine values (mean ± SEM) after 3 h of treatment *in vitro* with 0, 50, 100, and 200 µg/mL of histone mixture (one-way ANOVA: *= p: <0.05; **= p: 0.001–0.01; *** =p: 0.001–0.0001; ****= p: <0.0001; comparisons were calculated among treatments and respective controls and among each treatment). Values are expressed as pg/mL.

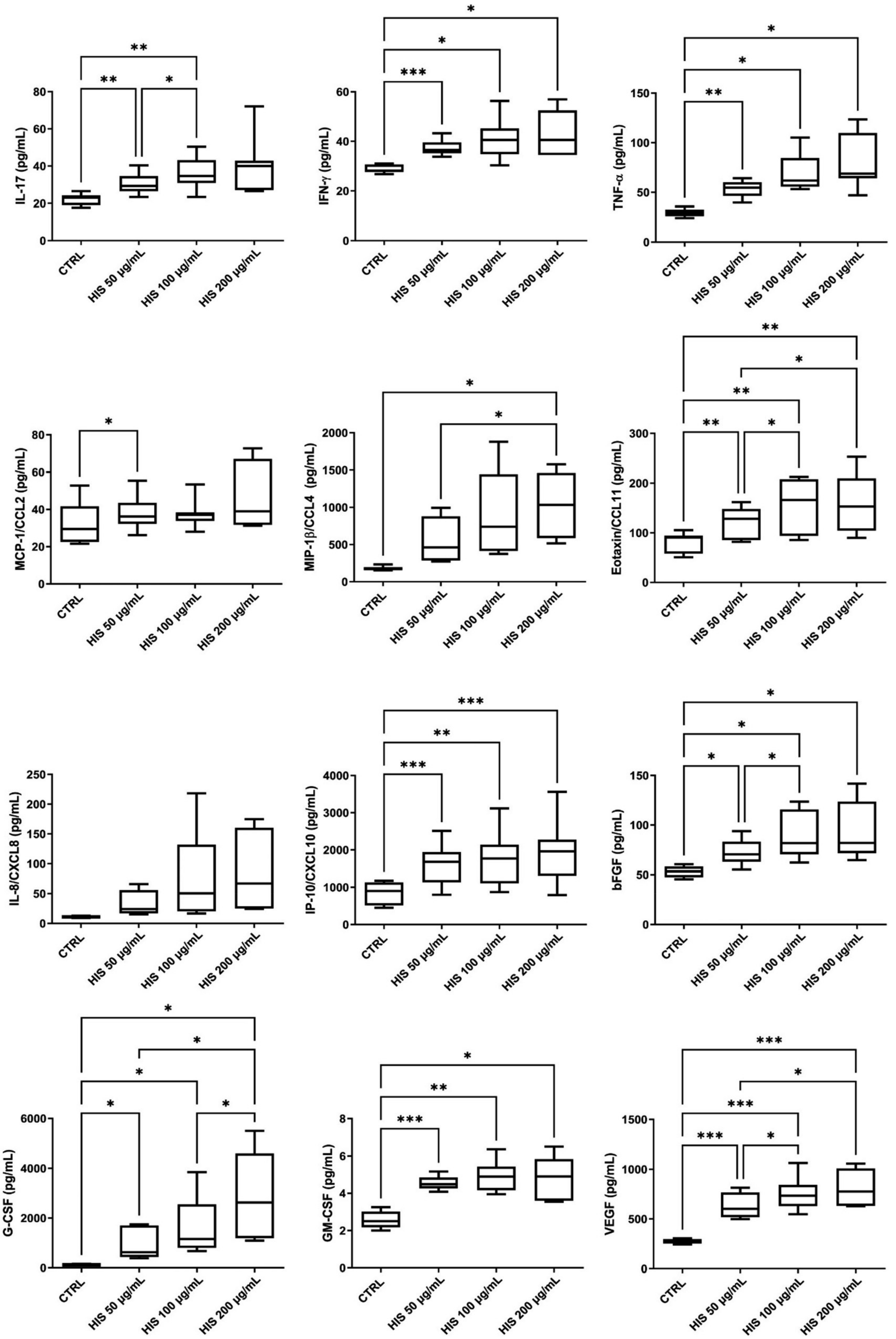

Figure 3: Continued.

treatment with 50 µg/mL of histones, and this trend was almost doubled (about 15-fold, p<0.05) with the doubling of the treatment. By using the highest dose of histones, we detected a 27-fold of increase vs. CTR (p<0.05). This dose-dependent behaviour is furthermore confirmed by the significant linear regression (Supplemental Table 3).

IL-1β, IL-6, MIP-1β/CCL4, and IL-8/CXCL8 showed a considerable increase of about 3-fold, 6-fold, and 8-fold at 50, 100 and 200 µg/mL, respectively. A significant increase was observed for VEGF, IP-10/CXCL10, GM-CSF, TNF-α, EOTAXIN/CCL11, IL-1Ra, IL-2, and IL-4, after histone treatment with all doses (excepted for IL-1Ra and IL-4 at 50 µg/mL), showing an up-regulation of 1.7-fold, 2.1-fold, 2.4-fold, at 50, 100 and 200 µg/2mL, respectively. A lower but significant increase, ranging from 1.4-fold to 1.6-fold, was observed for IL-15, IL-5, IL-17, bFGF, IL-10, IFN-γ, MCP-1/CCL2, and IL-9. These results are furtherly proven by linear regression analyses, showing significant trends (Figure 3 and Supplemental Table 3).

We furtherly correlated values of each inflammatory cytokine, chemokine, and growth factor measured in plasma samples obtained after 3 h of treatments by Pearson correlation analysis. A plot of all parameters is shown in Figure 4. We found that IL-4, IL-5, IL-6, bFGF, G-CSF, IFN-γ, MIP-1β/CCL4, VEGF, IL-1β, IL-1ra, IL-2, IL-8/CXCL8, and GM-CSF were the biomarkers showing the higher number of significantly positive correlations with other cytokines at the histone dose of 100 µg/mL (Table 2), confirming the presence of an intricate picture of interconnections among proinflammatory and anti-inflammatory cytokines, chemokine and growth factor response induced by histones (further details on the correlations among cytokines in controls, and histone-treated samples are provided in Supplemental Table 4).

Discussion

We demonstrated that extracellular histones can early and increasingly promote morphological changes in the circulating monocyte population similar to those found *in vivo* in classic [21, 22] and viral sepsis [23–25] patients. These alterations are accurately quantified and mirrored by MDW

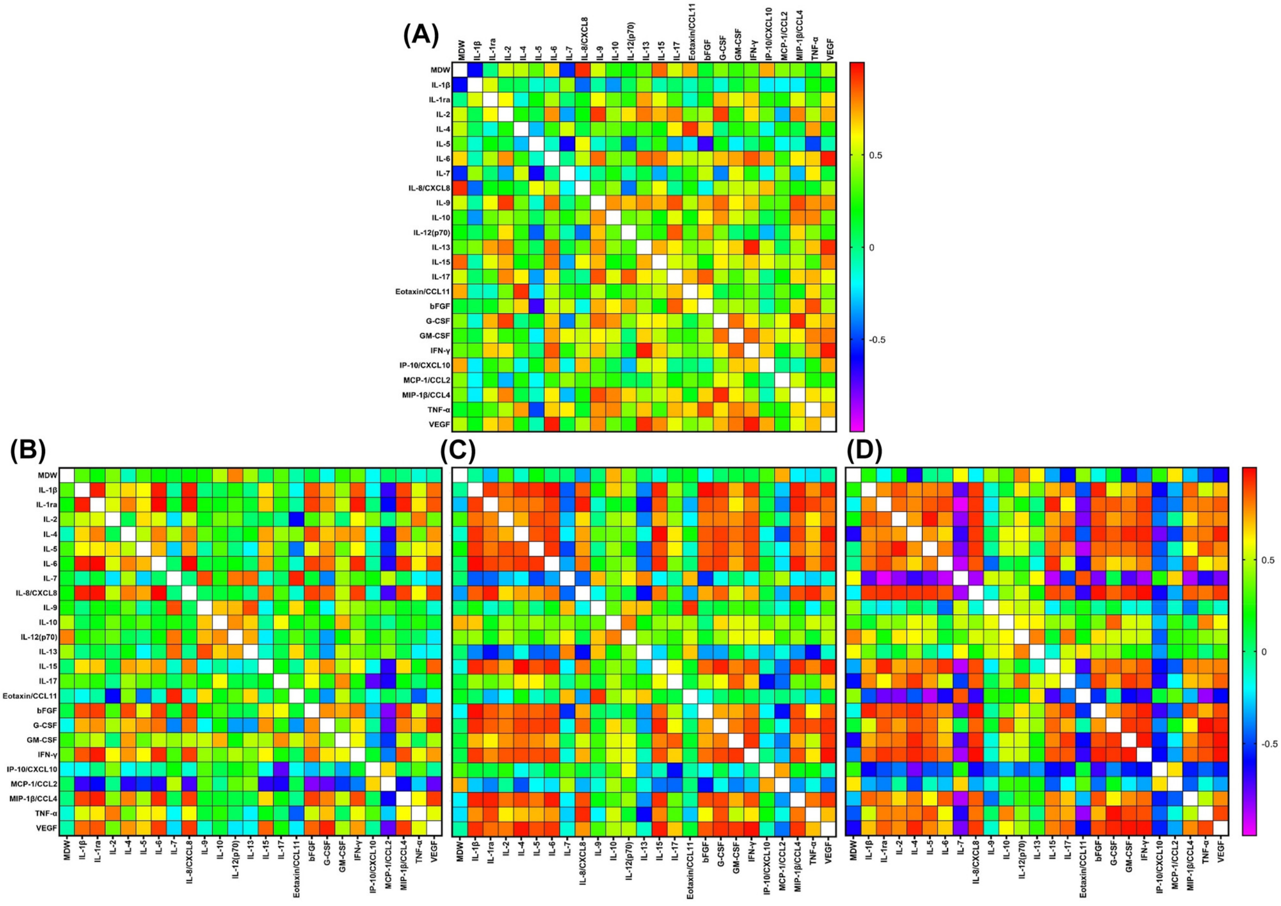

Figure 4: Correlation plot of all parameters determined in this study in controls (A), HIS 50 µg/mL (B), 100 µg/mL (C), and HIS 200 µg/mL (D) of histone-treated samples at 3 h. The plot is based on the Pearson correlation between each pair of biomolecules. On the right, the Pearson correlation coefficient is indicated by the colour gradient.

Table 2: p-Values resulting from Pearson correlation plots of all parameters determined in this study in samples treated 3 h with HIS 100 µg/mL. The dataset is based on the Pearson correlation between each pair of biomolecules.

	MDW	IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12(p70)	IL-13	IL-15	IL-17	Eotaxin	bFGF	G-CSF	GM-CSF	IFN-γ	IP-10	MCP-1	MIP-1β	TNF-α	VEGF
MDW		0.9418	0.4722	0.4723	0.7828	0.6244	0.9812	0.9412	0.3931	0.9569	0.0681	0.3774	0.9476	0.7319	0.7964	0.7103	0.6557	0.9425	0.7866	0.8488	0.4619	0.0644	0.9923	**0.0192**	0.6117
IL-1β			**0.0051**	**0.0226**	**0.0030**	**0.0038**	**0.0001**	0.3625	**0.0139**	0.6265	0.1365	0.3884	0.3887	**0.0018**	0.3234	0.9165	**0.0001**	**0.0022**	0.0739	**0.0046**	0.7956	0.5890	**0.0015**	0.7175	**0.0014**
IL-1ra				**0.0225**	**0.0154**	**0.0142**	**0.0025**	0.3226	**0.0002**	0.7776	0.7267	0.7354	0.1330	**0.0060**	0.4083	0.4424	**0.0052**	**0.0065**	0.1025	**0.0220**	0.8188	0.3835	**0.0051**	0.6213	**0.0003**
IL-2					**0.0463**	**0.0038**	**0.0048**	0.5259	0.0817	0.9875	0.3228	0.2603	0.3289	**0.0405**	0.2774	0.6432	**0.0177**	**0.0157**	**0.0256**	**0.0052**	0.5439	0.9451	**0.0208**	0.2443	**0.0127**
IL-4						**0.0053**	**0.0013**	0.6434	**0.0354**	0.4043	0.4079	0.4254	0.7062	**0.0000**	0.0959	0.6017	**0.0062**	**0.0021**	**0.0122**	**0.0032**	0.6820	0.2829	**0.0024**	0.8903	**0.0029**
IL-5							**0.0013**	0.2620	**0.0330**	0.9463	0.3217	0.5899	0.2599	**0.0049**	0.1081	0.7822	**0.0084**	**0.0024**	**0.0240**	**0.0043**	0.9143	0.6827	**0.0020**	0.3657	**0.0143**
IL-6								0.4650	**0.0132**	0.6713	0.4205	0.3520	0.2934	**0.0007**	0.3309	0.9797	**0.0038**	**0.0016**	**0.0462**	**0.0030**	0.7269	0.6066	**0.0017**	0.5581	**0.0002**
IL-7									0.3261	0.0767	0.4410	0.1854	**0.0342**	0.6577	0.9234	0.0780	0.2178	0.7230	0.9236	0.7523	0.4820	0.3451	0.6158	0.1205	0.6468
IL-8										0.6807	0.4967	0.8520	0.1251	**0.0139**	0.4315	0.4104	**0.0396**	**0.0083**	0.1813	**0.0428**	0.7965	0.4908	**0.0046**	0.6235	**0.0041**
IL-9											0.4425	0.0885	0.0553	0.5174	0.7758	**0.0012**	0.8948	0.7713	0.4815	0.5115	0.9748	0.7559	0.8460	0.1837	0.6632
IL-10												0.0560	0.6623	0.3507	0.2681	0.3279	0.9997	0.1122	0.1993	0.2103	0.4988	0.3480	0.1230	0.5608	0.5083
IL-12(p70)													0.3332	0.4313	0.5623	0.2210	0.7549	0.3360	0.1802	0.1923	0.1783	0.5890	0.4113	0.9507	0.3270
IL-13														0.5499	0.5719	0.0631	0.3925	0.4084	0.7736	0.8068	0.4428	0.7409	0.3515	**0.0267**	0.3706
IL-15															0.1297	0.7191	**0.0097**	**0.0004**	**0.0185**	**0.0042**	0.8450	0.3510	**0.0004**	0.9793	**0.0011**
IL-17																0.8081	0.2214	0.1249	**0.0066**	0.0692	0.1271	0.3235	0.1323	0.7202	0.3724
Eotaxin																	0.6980	0.9479	0.7458	0.8887	0.8918	0.9807	0.9806	0.2250	0.9184
bFGF																		**0.0387**	**0.0405**	**0.0121**	0.6458	0.1686	**0.0379**	0.8695	**0.0047**
G-CSF																			**0.0169**	**0.0031**	0.8196	0.7067	**0.0000**	0.6293	**0.0027**
GM-CSF																				**0.0014**	0.6027	0.3767	**0.0251**	0.9276	**0.0449**
IFN-γ																					0.9959	0.5319	**0.0038**	0.8276	**0.0043**
IP-10																						0.0582	0.8550	0.2024	0.6996
MCP-1																							0.6932	0.1310	0.4268
MIP-1β																								0.6108	**0.0031**
TNF-α																									0.8377
VEGF																									

Values in bold, in light grey cells, are referred to p-values <0.05, according to the Pearson correlation analysis.

index changes, whose values are equally increased in COVID-19 (recently overviewed in [26]) and sepsis patients [12, 27].

In particular, we observed a significant time- and dose-dependent enhancement of MDW, that was already significantly increased by the lowest histone dose of 50 μg/mL at the shortest time-point of 30 min. These results suggest that histones exert a potent and early effect on circulating monocyte population, affecting mainly their morphology, including cell volume, cytoplasmic granularity and vacuolization, and nuclear structure, features mirrored by the MDW parameter changes [20], without affecting monocyte count.

According to literature data [28], MDW values in healthy subjects could reach 23.5 when blood was obtained in EDTA tubes. In this respect, our experiments carried out in K_3-EDTA tubes showed that healthy controls had MDW values ranging from 14.3 to 20.7 during time, highlighting the stability of MDW measurements on this model over time.

Up to date, there are no single laboratory tests or specific stand-alone biomarkers with sufficient sensitivity and specificity to timely and accurately diagnose sepsis. In this respect, the measurement of MDW and histone plasma levels may provide useful biomarkers, with possible diagnostic and prognostic value to rule out infection and monocyte activation as critical players in classic and viral sepsis-related inflammation.

Our findings are consistent with the well-known ability of histones to modify cellular and biomolecular pathways of monocytes through direct translocation across cell plasma membrane, endocytosis-independent mechanisms, destabilization of plasma membrane, generation of extracellular vesicles and activation of TLR2/4/9, thus promoting inflammatory reactions and coagulative cascade [2, 29].

Acting as DAMPs, histones activate monocytes promoting not only morphological but also functional changes. In this scenario, we demonstrated that histones promoted a significant release of a wide panel of cytokines from peripheral blood cells. Of note, the cytokine-mediated inflammatory responses resulting from a whole-blood assay model, despite including all circulating blood cell types (WBC, RBC, PLT), has been reported to be representative of the monocyte population, providing better results compared to peripheral blood mononuclear cell and monocyte cultures [30].

In this respect, we observed that histones triggered a significant dose-dependent release of almost all cytokines. Interestingly, G-CSF showed the strongest response to histone treatments. This finding is crucial since G-CSF has been reported to be essential for the onset and amplification of the cytokine storm [31]. CSFs are growth factors implicated in the stimulation of myeloid cell differentiation and proliferation, that once activated synthesize and release further cytokines, thus inducing the cytokine storm [31].

Furthermore, our results highlighted that IL-1β, IL-6, MIP-1β/CCL4, and IL-8/CXCL8 exhibited a relevant dose-dependent increase after histone treatment and it is well-known that these parameters are increased in COVID-19 [32–34], and sepsis [35], suggesting them as crucial soluble mediators during disease progression. A significant increase of VEGF, IP-10/CXCL10, GM-CSF, TNF-α, EOTAXIN/CCL11, IL-1Ra, IL-2, and IL-4, and a lower but significant increase of IL-15, IL-5, IL-17, bFGF, IL-10, INF-γ, MCP-1/CCL2, and IL-9 was observed after histone treatment with almost all doses.

In sepsis conditions, nonsurvivors patients have been demonstrated to present increased levels of IL-2 (7.6-fold), IL-10 (3.1-fold), and MCP-1, IL-15, and GM-CSF (>2-fold) compared to survivors [35]. Moreover, levels of histones in non survivors have been found 28-fold higher compared to survivors' sepsis patients ($p=0.025$) [35].

During sepsis, levels of IL-1β and IL-6 have been reported higher in nonsurvivor vs. survivor patients, suggesting their potential involvement in predicting disease outcome [36–39]. IL-12 has also been reported elevated in sepsis [31].

In COVID-19 viral sepsis, IL-6, IL-2, IL-7, IL-10, G-CSF, IP-10, TNF-α, MCP-1, and MIP-1α have been reported upregulated and suggested to play a crucial role in the pathogenesis of the disease [40]. Similarly, Liu et al. by evaluating a panel of 48 cytokines in the plasma of COVID-19 patients concluded that 38 out of 48 cytokines were remarkably elevated in patients with COVID-19 and there was a strong linear association between severe lung injury and the level of 15 cytokines including: IFN-γ, IFN-α2, IL-1ra, IL-2, IL-4, IL-7, IL-10, IL-12, and IL-17, as well as IP-10, macrophage colony-stimulating factor (M-CSF) and G-CSF [41].

Data from a limited number of COVID-19 patients have shown a cytokine storm in critically ill patients. Here, levels of IL-2, IL-6, IL-10, and IFN- γ were found increased in severe cases of COVID-19 than in mild cases, and a strong inflammatory response during its clinical course was reported to be associated with high morbidity and mortality [42]. In this respect, recent findings indicated that G-CSF levels are also increased in plasma samples from COVID-19 and sepsis patients [40, 43], furtherly corroborating the notion on its fundamental involvement in cytokine storms associated with these conditions.

Interestingly, histones have been demonstrated able to modulate several cytokines including TNF-α, IL-6, IL-10, IL-1β, IL-8, CXCL9, IP-10, MIP-1α, MIP3A, and MCP3 in different experimental models [2, 35, 44–49].

However, no literature data reported such a wide panel of mediators as targets of histone treatment in whole blood model. Most of these mediators are deeply involved in the regulation of immune cell proliferation, differentiation, and production of new cytokines, leading to a vicious cycle which in turn causes cell and organ damage.

Although the size of the sample is limited, our whole blood assay model and findings demonstrate that histones, acting as DAMPs are potent inducers of a wide array of cytokines and chemokines, simulating *in vitro* the cytokine storm observed *in vivo* in both COVID-19 and sepsis patients, and highlighting that histones are major triggers and contributors during the clinical progression of these pathological conditions.

Being that whole blood assay model is an experimental model that closely and significantly mimes the monocyte responses, and that monocytes are largely implicated in high-inflammatory impact diseases, as classical and viral sepsis, with our findings on MDW changes and associated cytokine release we furthermore provide clear and increasing evidence on the critical roles of monocytes in these conditions.

On these bases, the identification of potential strategies to neutralize histones, and thus histone-mediated damages are warmly encouraged. Several studies suggest that both endogenous molecules (e.g., albumin, C Reactive Protein, Activated Protein C, polysialic acid) and pharmacological treatments (heparins and heparinoids) could directly bind histones, thus limiting their harmful effects on cells and tissues of several organs [6, 46, 50].

Further studies are ongoing to elucidate the involvement of histones in hypercoagulability events in classical and viral COVID-19 sepsis, as well as the possible modulation by heparin compounds.

In conclusion, in the light of the herein emerged new roles and functions of histones as inducers of hyperinflammatory responses and MDW modifiers, we suggest that monitoring MDW index and histone concentrations in patients with classic and COVID-19 sepsis both upon admission and throughout hospitalization may be a useful parameter to early predict higher risk of worst outcome.

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Informed consent: Not applicable.

Ethical approval: This study was approved by the Ethical Committee of University of Palermo (protocol: 07/2019) and Urbino. 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. 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.

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Chapter 8

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)

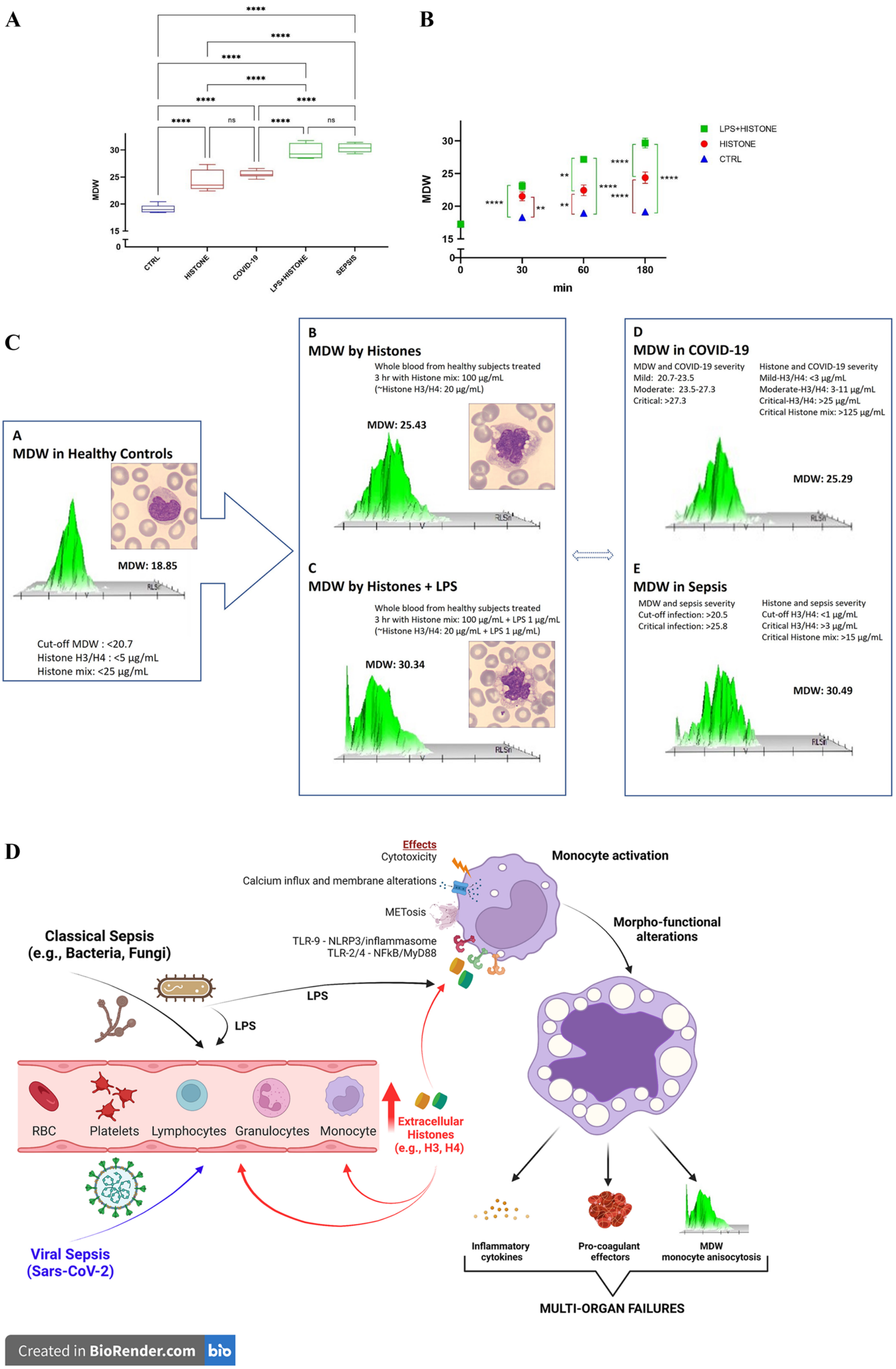

Fig. 1 (See legend on previous page.)

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

Chapter 9

General Discussion

Sepsis is a critical medical emergency characterized by a dysregulated host response to an infection. In both classical and viral sepsis, the innate immune response and haemostasis intertwine within the framework of immunothrombosis. In this scenario, accurately described and detailed in the chapter "General Introduction", the present PhD thesis aims to demonstrate and discuss the role of DAMPs, PAMPs, and live bacteria in sepsis, investigating their roles in the pathological alterations of both monocytes and platelets, driving the crosstalk between inflammation and coagulation.

Monocyte and Platelet activation as hallmarks of systemic inflammation

Several studies in the last decade have recognized MDW as a valuable biomarker for sepsis diagnosis in the emergency department and intensive care units. MDW demonstrates a diagnostic performance comparable, or higher, to conventional biomarkers (1-5). The numerous advantages provided by MDW, which reflects the monocyte anisocytosis due to their early activation after massive infection, are the rapid availability, the low turn-around time, and the low-cost, being MDW part of a routine CBC panel (6). Our research studies on MDW, described in chapters 2,7, and 8 expand the investigation of the biochemical role of live bacteria, PAMPs, and DAMPs in monocyte morpho-functional alterations. We demonstrated, using an *ex vivo* whole blood model, that live *E. coli*, its associated PAMP (LPS), and a well-known DAMP (extracellular histones), induced an increase in MDW values in a dose- and time-dependent manner, without altering the monocyte count. In general, leucocytes and red blood cells were not affected by the above-described treatments. To compare MDW kinetics across stimuli, it is necessary to consider that two different anticoagulants were used in the studies conducted, K2EDTA and K3EDTA. Literature documented that the use of K3EDTA is associated with higher MDW values compared to K2EDTA, justifying two distinct diagnostic cut-offs: 21.5 and 20 respectively (7-9). The reason of this difference is in pre-analytical and morphological effects: blood collection in K3EDTA is associated with greater cellular volume changes, increased osmotic pressure and major cell size distribution, resulting in an higher MDW value (10, 11). Our findings underline differences in both timing and intensity of increase in MDW across stimuli. *E. coli* and LPS induced an earlier and higher increase in MDW than histone, according to the different activated cellular/molecular signalling. This observation highlights that whole bacteria provoke a broader and earlier activation, probably due to simultaneous activation of several PRRs, including TLR2, TLR4, and NOD-like receptors (12). Histones act as secondary DAMPs, that, once released, signal through TLRs, MyD88, NF-kB, and NLRP3 inflammasomes, amplifying inflammation and sustaining cellular morphological changes (13). The MDW enhancement mirrors and quantifies the morpho-functional alterations of monocytes, characterized by enlarged volume, cytoplasmic vacuolization, increased granularity, and nuclear deformation. Through digital morphology we confirmed these features: the 3-hour treatment of whole blood with live *E. coli*, LPS, and histones revealed significant changes in monocyte heterogeneity. From a round-shape of a typical monocyte, these treatments induced several pathological modifications, starting from an increased cell size, intracellular vacuolization and hyper-granularity, up to a significant membrane alteration. Moreover, the nucleus of treated monocytes lost the reniform structure and our smears clearly showed the phagocytosis of live *E. coli*. Therefore, our experimental studies provide an explanation of the biochemical mechanisms underlying the clinical evidence found in literature. Meta-analyses and multicentre cohorts demonstrate that MDW has equal and complementary performance to conventional biomarkers, such as CRP and PCT, in the early identification of sepsis, with the advantage of being part of the CBC, supporting the rapid triage and continue monitoring (5, 6, 14). Particularly, chapter 2 describes the experimental work in which we reproduced, through the treatment of whole blood with 3 increasing doses of *E. coli* (10^6-10^{10} CFU/mL) and its associated bacterial endotoxin, LPS (0.1-10 μg/mL), a septic condition, demonstrating that with both types of stimuli, MDW increases in a time- and dose- dependent manner, reaching levels significantly higher than controls as early as 30 minutes after treatment. Therefore, we demonstrated the ability of MDW to early identify the bacteria-activated monocytes, explaining why it moves before others biochemical markers, showing also a prognostic value. MDW values obtained in our *ex vivo* model overlap those found in septic patients, confirming the reliability of the *in vitro* whole blood model. This double approach reduces possible *ex vivo* model biases, showing that the morphological variation reflects real monocytic plasticity induced by the septic environment. Moreover, we observed that the increase in monocyte heterogeneity is accompanied by a significant increase in the release of proinflammatory mediators, such as Macrophage Inflammatory Protein 1-alpha (MIP-1α/CCL3), IL-8/CXCL8, TNF-α, IL-1β, and MIP-1β/CCL4, but also of IL-10, an anti-inflammatory cytokine, according to literature data (15). Interestingly, the morphological changes induced by *E. coli* and LPS are similar, except for phagocytosis observed only with *E. coli* treatment. The monocyte vacuolization after LPS exposure may indicate a priming-induced inflammatory activation, and not related to true phagocytosis, as the vacuoles are not of lysosomal or phagosomal origin (16). On the other hand, the inflammatory response induced by the two stimuli is different. Our results confirmed literature data, supporting that live *E. coli* and LPS trigger distinct inflammatory mediator’s release. The recognition of live bacteria involves multiple PRRs beyond TLR4, with consequent differences in cytokine kinetics (17, 18). Moreover, MIP-1α/CCL3, IL-8/CXCL8 and

Platelet-derived Growth Factor (PDGFbb) appeared to be sensitive to different age and gender groups, suggesting that these patient characteristics could affect the immune response. These data are preliminary, but in agreement with results reported in the literature (19), which should be confirmed in larger cohorts, for setting the basis of novel evidence of a gender-based laboratory medicine biomarker. The most comprehensive and detailed discussion is in chapter 2. As previously anticipated, we demonstrated that circulating histones are crucial MDW modifiers. Chapter 7 investigates how a mixture of histones (50-200 µg/mL) induces, up to 3-hours post-treatment MDW changes and an inflammatory response. Results showed a significant time- and dose- dependent increase in MDW values and a peculiar enhancement in cytokine release. G-CSF showed the highest response; it is a crucial mediator in the onset and amplification of inflammatory processes (20). Also IL-1β, IL-6, MIP-1β/CCL4, and IL-8/CXCL8 showed important and dose dependent increase after histone treatment; our data are in agreement with *in vivo* findings described in literature, emphasizing that these parameters are also risen in COVID-19 (21) and sepsis (22). These results highlighted that circulating histones promote functional changes in immune cells, amplifying disease progression by inducing a significant release of inflammatory cytokines which, in turn, extend the hyperinflammatory response. In chapter 8 we demonstrated for the first time that the *in vitro* treatment of whole blood with histones, at concentrations comparable to those released in viral (23) and bacterial (24) sepsis, induced significant monocyte morpho-functional modifications. These changes were reflected by an increase in MDW, reaching values similar to those observed *in vivo* in patients with sepsis (25) and COVID-19 (26). Assessed the biological impact of histones released during pathological conditions, this PhD thesis proposes a unifying framework in which they not only cause monocyte heterogeneity, evidenced by MDW, but also amplify cytokine release and lead to platelet activation and consumption, reflected by PDW and MPV. Our experimental data provide an integrated profile to understand sepsis biology and to advance a panel of laboratory biomarkers useful for early diagnosis. It’s well known in literature that circulating histones are major mediators of organ injury and death in sepsis via TLRs as described in the chapter *General Introduction*, fuelling the immunothrombosis (27-29). In chapter 5 is reported our paper which confirm the direct involvement of extracellular histones in platelet aggregation and platelet morphological changes, generating giant and ballooned platelets, reflected by PDW and MPV increase. Our results are in agreement with literature data, confirming that these phenotypes are also consistent with previous findings describing histone H4-induced platelet ballooning and microvesciculation (28, 30, 31). Specifically, we used a whole blood model treated with a mixture of histones (50-200 μg/mL) to evaluate, up to 3-hours, platelet indices variations and cytokine's release. Results showed that platelet count very significantly decreased already after 30 minutes from histone treatment, in a dose-dependent manner and both MPV and PDW very significantly increased with all doses and at all times considered. After 3 hours of treatment with all doses of histones we demonstrated a significant and dose-dependent increase of MIP-1α/CCL3, RANTES/CCL5, PDGFbb, TGF-β1, TGF-β2, and TGF-β3, supporting the role of histones in the hyperinflammation found in sepsis. In literature different studies highlighted the pivotal role of histones in activating platelets and promoting pro-coagulant activities (28), as well as causing the thrombocytopenia found in bacterial and viral sepsis (23, 32). This role has been confirmed in mice (33). Moreover, the increase in PDW is associated with disease severity in infectious conditions such as in COVID-19 (33, 34). Our review, in chapter 4, further established PDW as a correlate of severity in cases of SARS CoV-2 infection, supporting the hypothesis that platelet heterogeneity reflects both the inflammatory and thrombotic response. Therefore, integrating platelet indices with MDW values we can capture both aspects of the immunothrombosis, in which monocyte activation and platelet reactivity are driven by histones through TLR signalling. The *ex vivo* whole-blood approach has several important advantages. It mimics the interaction between leukocytes, platelets and others cell type similarly in blood, and reproduces cytokine patterns that are closer to what happens *in vivo* than those produced by isolated cell systems (35). On the other hand, the whole-blood approach presents also some limitations. Live bacteria and LPS can induce platelet number reduction *in vivo*, but through mechanisms that our *ex vivo* whole blood assay doesn't reproduce, due to the absence of biological conditions (e.g., lack of vessels and their flow, spleen and liver clearance, endothelial surface and the interaction between complement and endothelium, etc.)(36). Therefore, we observe thrombocytopenia *ex vivo* after histone treatment, due to platelet aggregation by consumption, but not after *E. coli* and LPS treatment. In a human endotoxemia model (36), LPS induced a significant decrease in platelet count and α-granule release, demonstrating its role in thrombocytopenia, via TLR4 (37, 38). Thrombocytopenia is very common condition in Intensive Care Unit (ICU) and there may be many causes: pseudo-thrombocytopenia, haemodilution, increased consumption, decreased production, increased sequestration, and immune-mediated destruction of platelets (39): complex conditions that *in vitro* can't be reproduced. Sepsis accounts for 50% of all thrombocytopenia cases in critically ill patients (39) and the mechanisms are mainly multifactorial, including decreased platelet production, increased platelet consumption and sequestration also by haemophagocytosis. In sepsis, continuous thrombin activity and intensified interactions between platelets and endothelium contribute to accelerate platelet consumption. The thrombocytopenia reflects a dysregulated host response to the infection and it is correlated with a poor prognosis (39).

Can MDW differentiate Gram-negative from Gram-positive sepsis?

Reviews and clinical studies in ED show elevated MDW levels in sepsis (40) with no significant differences in Gram-negative and Gram-positive bloodstream infections (6, 41) declaring that MDW is an early sepsis biomarker but not pathogen-specific. Our research has thoroughly investigated this aspect to understand whether the level of MDW could give additional information about the infective pathogen. Using our *ex vivo* model we compared 24 K2EDTA-anticoagulated blood samples from healthy donors with two increasing doses (10^6 and 10^8 CFU/mL) of live Gram-negative *E. coli* and Gram-positive *S. aureus* and with their associated PAMPs, respectively with 1 µg/mL of LPS and 200 µg/mL of LTA. We evaluated MDW at different time point through DxH690T Haematology Analyzer by Beckman Coulter. Interestingly, we observed that MDW values were early, significantly and dose- and time-dependently increased by both live bacteria. Particularly, after 3 hours of treatment with *E. coli* 10^6 and 10^8 CFU/mL MDW increased respectively to 21.7 and 26.1, while with *S. aureus* 10^6 and 10^8 CFU/mL respectively to 18.1 and 20.9, compared to respective controls. LPS and LTA, despite used at different concentrations, according to literature data and dose-response curves, clearly confirmed the species-specific difference, inducing significantly different MDW changes comparable to those obtained with *E. coli* and *S. aureus* 10^8 CFU/ml, respectively. These results, presented as poster abstracts at conferences and currently in preparation for a manuscript *in extenso*, emphasized that Gram-negative *E. coli* induced a greater increase in MDW than Gram-positive *S. aureus*, confirming the clinical usefulness of MDW in early sepsis diagnosis and hypothesising a species-specificity in MDW variations. Our experimental results are based on biological plausibility, as described below. Gram-negative bacteria signal via LPS, LPS-binding protein (LBP), CD14-TLR4 receptor with complement co-activation, and CD14+. Blocking both CD14 and complement significantly reduces *E. coli*-induced cytokine release, demonstrating the broad cascade of signals induced. On the other hand, Gram-positive ligands such as LTA, lipoproteins and peptidoglycan mainly act via TLR2 and TLR6 (35, 42). Moreover, TLR4 uniquely interacts with MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) generating a wider range of transcriptional and metabolic response, with higher release of IL-6, IL-1β, TNF-α, IL-8/CXCL8and G-CSF, than TLR2 signalling alone (29). The consequence of the stronger inflammatory signals on MDW enhancement is the more pronounced change in cell size, vacuolization, granularity and nuclear remodelling. In our *ex vivo* study we observed phagocytosis, and abundant vacuoles, with live *E. coli* treatment but not with LPS, despite both raising MDW. *E. coli* tends to produce the richer morphologic alterations and that fits with the classical observations: monocyte recognition and opsonization of Gram-negative bacteria is efficient in presence of complement, whereas some Gram-positive pathogens such as *S. aureus* use immune evasion strategies (*e.g.* Protein A and complement inhibitors) that reduce the effectiveness of opsono-phagocytosis, resulting in less consistent vacuolisation and anisocytosis (35, 42-44). On the other side, both models are characterized by a limited, despite still present, complement activation due to the anticoagulant used. Human monocytes can utilize adaptive condition to a specific stimulus. This is the peculiar case with *S. aureus* infection that, after an initial active phase, shows an attenuation of TNF/IL-1β signals. Differently, the binding of LPS with TLR4 primes cells for stronger response (45). Consequently, more phagosome and vesicles result in more cellular heterogeneity, clearly evidenced and quantified by higher MDW in *E. coli* samples than *S. aureus*. The limitation of our experimental tests lies in the whole blood model itself, which, although closely mimes the monocyte responses, cannot replace a clinical study. There are still no studies in the literature that prove bacterial specificity for MDW. In this respect, our future perspective is to evaluate the comparison between the data obtained in the *ex vivo* model with *S. aureus* treatment and the *in vivo* MDW data in patients with *S. aureus* sepsis, similarly to what already published and reported in Chapter 2 for *E. coli* sepsis. In this way, we could further confirm our model as valid methodology for mimicking a septic condition and comparing also MDW data from gram-negative and gram-positive sepsis. In parallel, our research group is currently expanding the frame of studies on CPD, additional promising class of haematological indices for early sepsis diagnosis, as carefully discussed in the *General Introduction*. This ongoing investigation consists of a retrospective analysis of CPD parameters in septic patients to assess their diagnostic accuracy and potential pathogen-specific patterns. Multiparametric analyses are being conducted to integrate CPD parameters with demographic data, clinical features, and CBC data to develop an algorithm that can accurately and quickly provide diagnostic information from a routine analysis. In addition to the retrospective *in vivo* study, we performed *ex vivo* tests involving the treatment of whole blood with live *E. coli* and *S. aureus*, as well as their respective PAMPs (LPS and LTA), to evaluate the modulation of morpho-functional modifications in neutrophils, monocytes and lymphocytes by several pathogens. These alterations are quantified by CPD parameters, specifically by the mean values and standard deviations of volume, conductivity and the 5 scatters of laser light. Integrating retrospective clinical data with experimental results will improve our understanding of the mechanistic biological basis of CPD alterations, enabling us to compare their diagnostic performance with that of other conventional biomarkers.

Extracellular histones and selectivity of compounds against histone toxicity

Histones, when extracellularly released by activated immune cells, are well recognized to exert strong biological effects, through established mechanisms (46), also during bacterial and viral infections. Their presence in the circulation promotes cytotoxic and pro-inflammatory responses, monocyte activation, platelet aggregation, finally contributing to the amplification of the inflammatory cascade. Our published results, as described in the previous chapters, showed that the exposure of blood cells to high concentration of free histones leads to the increase of MDW, reflecting monocyte heterogeneity and hyperactivation, but also to PDW, MPV, and PLT count significant changes, mirroring platelet activation and modification. All these alterations mirror the haematological patterns observed in bacterial and COVID-19 related sepsis and support the concept of free histones as crucial linker between inflammation and thrombosis. Our findings suggest that assessing MDW, platelet indices, and circulating histones, could provide a useful panel of early markers for identifying patients at higher risk of worst outcome in septic conditions. In this regard, a very useful future perspective and the subject of our ongoing studies is to find a pharmacological strategy to control both thrombotic and inflammatory components of sepsis disease. We focused the attention on the ability to neutralize histones and thereby prevent their interaction with platelets. Due to the positive charge, free histones exhibit high affinity for negatively charged heparin. The binding between histones and platelets could be inhibited by these type of molecules, preventing both thrombocytopenia and endothelial cytotoxicity (33). Our ongoing experimental studies aimed to test and compare two types of molecules, a non-heparin glycosaminoglycan anti-thrombotic (Danaparoid), and a non-heparin anticoagulant pentasaccharide (Fondaparinux), mainly in limiting the deleterious effects of free histones. Danaparoid sodium has a low molecular weight and is composed of linearly repeating disaccharide units of uronic acids: heparan sulfate (84%), dermatan sulfate (12%), and chondroitin sulfate (4%). The first mechanism of action described for Danaparoid consists in the inhibition of thrombin generation, necessary for the formation of fibrin clot. In particular, the heparan sulphate inhibit the factor Xa, a fundamental enzyme in the coagulation cascade, by binding antithrombin. Currently, this mixture of GAG is the unique FDA approved drug used to treat heparin-induced thrombocytopenia and deep vein thrombosis (47). On the contrary, Fondaparinux is a short synthetic pentasaccharide (1.7 kDa) which binds selectively and with high affinity to antithrombin, inactivating the Factor Xa (48). We used whole blood and Platelet Rich Plasma (PRP) as experimental models, and a mixture of histones (200 µg/mL) as pro-aggregating stimulus, and Danaparoid (0.5 and 1 U/mL) and Fondaparinux (2 µg/mL) as pharmacological molecules to be tested as anti-histone compounds. Our results, presented as poster abstracts at national and international conferences and actually in preparation for a manuscript *in extenso,* confirmed the role of histones in altering platelet indices in both whole blood and PRP samples (33). Specifically, we observed a rapid and massive platelet depletion, by consumption due to aggregation, and increased cell volume with giant platelet formation. These effects are significantly amplified in PRP, where the reduced amount of cells and plasma, and therefore of proteins such as albumin which has a protective effect against histones (49), and the presence of platelets as the only cell target type, significantly enhances the histone harmful effects. Interestingly, these significant alterations are prevented and reverted by the presence of Danaparoid sodium. We investigated the role of Danaparoid both in whole blood and PRP adding it simultaneously with histones and after 10-minutes of pre-incubation of histones alone.

Results revealed that platelet count, PCT, MPV and PDW obtained by the simultaneous treatment of histone-Danaparoid did not significantly differ from control samples, emphasizing the protective role of Danaparoid. Moreover, this mixture of GAG also showed a strong ability to restore platelet count and morphology, perhaps due to the greater affinity of histones for Danaparoid than for platelets. Differently, Fondaparinux showed to be ineffective in protecting and restoring cellular homeostasis from histone effects in both whole blood and PRP models (50). Digital morphology approaches confirmed the different anti-histone activities of the two drugs. Moreover, we also measured the concentrations of cytokine released in plasma, revealing that peculiar mediators are modulated by Danaparoid. These findings suggest that not all anticoagulant molecules perform equally in protecting against harmful histone actions. The most widely accepted hypothesis is that the molecular size determines their ability in neutralizing histones. Molecules larger than 1.7 kDa are required to effectively neutralize the effects of histones and this ability is independent of the antithrombin-binding pentasaccharide (50). The higher molecular weight of Danaparoid, combined with its heterogeneous consistent sulfation, results in a multivalent electrostatic interface that is crucial for histone complexation and the effective protection of platelets. In contrast, Fondaparinux has insufficient chain length and charge to successfully bind histones (50, 51). Two further points are in favour of this hypothesis: firstly, heparins can promote the proteolysis of extracellular histones by neutrophil elastase, thus reducing cytotoxicity by histone fragmentation (52). Secondly, histones can bind vascular glycosaminoglycans, and the competition at these sites modulates coagulation and inflammation, reinforcing the concept that GAG sulphation determine histone sequestration (53). In classical bacterial and viral sepsis, high concentration of histones is associated with thrombocytopenia (54) and drugs which directly neutralize histones could be a promising strategy in histone-driven immunothrombosis for sepsis health care. Our in progress experimental study presents some limitations. Firstly, the cohort of healthy donors tested was small (n=25), and therefore, a future perspective will be to expand the case studies and distinguish the results by age and gender. Another limit consists in the model used, as the *ex vivo* whole blood model may not fully reproduce the complex biological *in vivo* condition. Regarding drug concentrations, although we tested the dose indicated by the pharmaceutical company, it may not reflect therapeutic levels in a patient. Our analyses were focused on platelet indices variations and inflammatory mediators release, without assessing complementary analysis (such as the coagulation parameter) leaving several clinical questions that should be answered in future investigations. Our group is currently conducting a study in collaboration with another University with the aim of developing a Point of Care testing (POCT) device based on functionalised gold nanoparticles and a colorimetric assay to detect circulating free H3 in biological fluids. Preliminary results were presented in a poster at a national conference. This study is of considerable clinical importance, considering the established pivotal role of histones in pathological conditions, as their measurement could be included in a panel of rapid biomarkers. Moreover, other future perspectives (most of them currently under investigation) are referred to the investigation of the specific molecular binding between histones and Danaparoid, to confirm the binding mechanisms, the study of aggregation kinetics, and finally a clinical investigation assessing circulating histone levels, platelet indices, MDW, and treatment response in septic patients who receive Danaparoid, to provide valuable translational evidence for potential therapeutic use.

Concluding Remarks

Across our experimental studies, all the data obtained converged on a crucial conclusion: extracellular histones are central DAMPs found in high concentrations in bacterial and viral sepsis (27, 55, 56), driving early monocyte activation (highlighted by MDW), platelet aggregation and heterogeneity, (evidenced by PDW), and consistent cytokine storm. These findings suggest that assessing MDW, platelet indices, and quantify circulating histones and peculiar cytokines, provide an integrated approach to understand the biology of sepsis and to pave the way for setting up a useful panel of early biomarkers to identify septic patients and facilitate fast risk stratification. Our integrated analysis of *in vivo* and *ex vivo* models provide a link between clinical findings and biochemical and cellular pathways. While bacterial components start the early inflammatory signalling, extracellular histones amplify the host response, causing further histone release fuelling a vicious cycle. This septic-related vicious cycle, characterized by NETosis, endothelial dysfunction, and immune cells activation, constitutes a key target for both biomarkers and potential therapeutic approaches. The combination of appropriate biomarkers with histone-neutralising strategies, particularly using glycosaminoglycan-based compounds, could lead to a precision-medicine approach able to improve both early identification and clinical outcomes of sepsis patients.

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Chapter 10

Scientific Curriculum

Publications

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2. Daniela Ligi, **Chiara Della Franca**, Ferdinando Mannello. Deciphering conundrums on heparins and circulating histones: hype or hope? Journal of Thrombosis and Haemostasis, Volume 23, Issue 8, 2025. DOI: 10.1016/j.jtha.2025.06.017. (IF:5 – Q1 Hematology).

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4. Daniela Ligi, **Chiara Della Franca**, Ferdinando Mannello. Capitolo 5b: Disfunzione endoteliale e biomarkers nelle malattie vascolari. Trattato di medicina vascolare, G. Camporese, P. Simioni, E. Bernardi. 2024. ISBN 9791298514515

5. Daniela Ligi, **Chiara Della Franca**, Kin Israel Notarte, Nathaniel Goldrich, David Kavteladze, Brandon Michael Henry, Ferdinando Mannello. Platelet Distribution With (PDW) as a significant correlate of COVID-19 infection severity and mortality: a systematic review. Clin Chem Lab Med. 2023. DOI: 10.1515/cclm-2023-0625. (IF: 8.49 – Q1 Medical Laboratory Technology).

6. Rosaria Vincenza Giglio, Daniela Ligi, **Chiara Della Franca,** Bruna Lo Sasso, Julia Zulema Rivas, Luisa Agnello, Ferdinando Mannello, Marcello Ciaccio. Thrombocytopenia and hyperinflammation are induced by extracellular histones circulating in blood. Clin Chem Lab Med 2023 Jun 21. doi: 10.1515/cclm-2023-0590. (IF:8.49 – Q1 Medical Laboratory Technology).

7. Daniela Ligi, **Chiara Della Franca**, Ferdinando Mannello F. Novel mechanisms of thrombo-inflammation during infection: the harmful impact of circulating histones. Res

Pract Thromb Haemost. 2023 Mar 30;7(3):100141. doi: 10.1016/j.rpth.2023.100141. eCollection 2023 Mar. (IF: 5.953 – Q1 Peripheral Vascular Disease).

8. Daniela Ligi, Bruna Lo Sasso, **Chiara Della Franca**, Rosaria Vincenza Giglio, Luisa Agnello, Marcello Ciaccio, Ferdinando Mannello. Monocyte distribution width alterations and cytokine storm are modulated by circulating histones. Clin Chem Lab Med. 2023 Feb 28. doi: 10.1515/cclm-2023-0093. Online ahead of print. (IF: 8.49 – Q1 Medical Laboratory Technology).

9. Daniela Ligi, Bruna Lo Sasso, Rosaria Vincenza Giglio, Rosanna Maniscalco, **Chiara Della Franca**, Luisa Agnello, Marcello Ciaccio, Ferdinando Mannello. Circulating histones contribute to monocyte and MDW alterations as common mediators in classical and COVID-19 sepsis. Crit Care. 2022 Aug 30;26(1):260. doi: 10.1186/s13054-022- 04138-2 (IF:19.344 – Q1 Critical Care Medicine).
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Conference Abstracts and Posters

1. "Role of sepsis-related Damage-Associated Molecular Patterns in the alteration of Monocyte Distribution Width" Daniela Ligi, **Chiara Della Franca**, Giulia Barchiesi, Francesca Salvatori, Marisol Huaman Palomino, Sofia Ranieri Chiatamone, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC –MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

2. "Role of extracellular histone fractions in thromboinflammation". Daniela Ligi, Francesca Salvatori, **Chiara Della Franca**, Giulia Barchiesi, Marisol Huaman Palomino, Sofia Ranieri Chiatamone, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC– MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025

3. "Neutrophil and monocyte Cell Population Data are altered by extracellular histone fractions: insights into sepsis diagnosis". Daniela Ligi, **Chiara Della Franca**, Giulia Barchiesi, Francesca Salvatori, Marisol Huaman Palomino, Sofia Ranieri Chiatamone, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

4. "Monocyte Distribution Width is differently altered by diverse Pathogen-Associated Molecular Patterns". Francesca Salvatori, **Chiara Della Franca**, Marisol Huaman Palomino, Ferdinando Mannello, Daniela Ligi. 57° Congresso Nazionale. SIBIOC – MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

5. "Pathogen-Associated Molecular Patterns in the alterations of blood cells: insight from digital morphology analysis". Francesca Salvatori, **Chiara Della Franca**, Marisol Huaman Palomino, Alicia Martinez-Iribarren, Cristian Morales Indiano, Ferdinando Mannello, Daniela Ligi. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

6. "Staphyloccoccus aureus-induced sepsis model: differences in Monocyte Distribution Width among live bacteria and Pathogen-Associated Molecular Patterns". Marisol Huaman Palomino, Francesca Salvatori, **Chiara Della Franca**, Daniela Ligi, Alicia Martinez-Iribarren, Cristian Morales Indiano, Brandi Giorgio, Schiavano Giuditta Fiorella, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025

7. "A novel poct assay for circulating histones in biological fluids: preliminary results and future perspectives". Margherita Borriello, Daniela Ligi, **Chiara Della Franca**, Diego Ingrosso, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

8. "Cell Population Data reveal monocyte activation in Gram-Positive and Gram-Negative Sepsis: a comparison between in vivo and ex vivo models". **Chiara Della Franca**, Daniela Ligi, Francesca Salvatori, Marisol Huaman Palomino, Giuditta Fiorella Schiavano, Giorgio Brandi, Marc Vasse, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025

9. "Toll-Like Receptors modulate Cell Population Data in response to Pathogen-associated molecular patterns: implications for early sepsis recognition". **Chiara Della Franca**, Daniela Ligi, Francesca Salvatori, Marisol Huaman Palomino, Marc Vasse, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC –MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

10. "Gram-negative and gram-positive bacteria-induced changes in Monocyte Cell Population Data: a comparison between in vivo and ex vivo models". **Chiara Della Franca**, Daniela Ligi, Francesca Salvatori, Marisol Huaman Palomino, Giuditta Fiorella Schiavano, Giorgio Brandi, Marc Vasse, Ferdinando Mannello. PhD Day 26/06/25, University of Urbino Carlo Bo.

11. "Role of different Pathogen-Associated Molecular Patterns in complete blood count parameters: insights of early sepsis diagnosis". Francesca Salvatori, **Chiara Della**

Franca, Daniela Ligi, Ferdinando Mannello. PhD Day 26/06/25, University of Urbino Carlo Bo.

12. “Age-dependent thrombocytopenia induced by histones in an ex-vivo whole blood model”. Francesca Salvatori, **Chiara Della Franca**, Daniela Ligi, Ferdinando Mannello. EuroMedLab Brussels 2025, Brussels, Belgium, May 18–22, 2025. DOI https://doi.org/10.1515/cclm-2025-8042 Clin Chem Lab Med 2025; 63, Special Suppl, pp S97 – S2842, May 2025.

13. “Effect of different blood collection anticoagulants on the variation of Complete Blood Count under physio-pathological conditions”. **Chiara Della Franca**, Francesca Salvatori, Daniela Ligi, Ferdinando Mannello. EuroMedLab Brussels 2025, Brussels, Belgium, May 18–22, 2025. DOI https://doi.org/10.1515/cclm-2025-8042 Clin Chem Lab Med 2025; 63, Special Suppl, pp S97 – S2842, May 2025.

14. "Variations of monocyte and neutrophil Cell Population Data by gram-negative and gram-positive bacteria: do they represent emerging early sepsis biomarkers?”. Daniela Ligi, **Chiara Della Franca**, Francesca Salvatori, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. EuroMedLab Brussels 2025, Brussels, Belgium, May 18–22, 2025. DOI https://doi.org/10.1515/cclm-2025-8042 Clin Chem Lab Med 2025; 63, Special Suppl, pp S97 – S2842, May 2025.

15. “Age-related variations in Monocyte Distribution Width to Lipopolysaccharide and histones in an ex vivo whole blood model”. **Chiara Della Franca**, Francesca Salvatori, Daniela Ligi, Ferdinando Mannello. EuroMedLab Brussels 2025, Brussels, Belgium, May 18–22, 2025. DOI https://doi.org/10.1515/cclm-2025-8042 Clin Chem Lab Med 2025; 63, Special Suppl, pp S97 – S2842, May 2025.

16. "Unraveling monocyte and neutrophil morphological alterations in sepsis condition by digital morphology and transmission electron microscopy". **Chiara Della Franca**, Sofia Ranieri Chiatamone, Michela Battistelli, Francesca Salvatori, Daniela Ligi, Elena A. Sukhacheva and Ferdinando Mannello. ISLH 2025- Halifax, May 7-9, 2025.

17. "Monocyte distribution width variations and inflammatory response induced by gram-negative and gram-positive live bacteria and their endotoxins". Daniela Ligi, **Chiara Della Franca**, Francesca Salvatori, Sofia Ranieri Chiatamone, Giorgio Brandi, Giuditta Fiorella Schiavano, Elena A. Sukhacheva and Ferdinando Mannello. ISLH 2025- Halifax, May 7-9, 2025.

18. “Escherichia coli and Staphylococcus aureus differently modulate Monocyte Distribution Width as an early sepsis biomarker”. Giuditta Fiorella Schiavano, **Chiara Della Franca**, Daniela Ligi, Erica Fabbri, Francesca Salvatori, Giorgio Brandi, Ferdinando Mannello. Poster Abstrac. 57°Congresso Nazionale SItI. Società Italiana di Igiene. Palermo. October 23-26, 2024.

19. "Monocyte Distribution Width in sepsis: differences among live bacteria, pathogen-and- damage associated molecular pattern proteins" **Chiara Della Franca**, Daniela Ligi, Francesca Salvatori, Erica Fabbri, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. 56° Congresso Nazionale SIBioC - Il ruolo clinico della Medicina di laboratorio nella sanità del terzo millennio. SIBioC, Bologna, October 8-10, 2024.

20. "Monocyte and neutrophil alteration in an in vitro sepsis model: insight from digital morphology analysis". Daniela Ligi, **Chiara Della Franca**, Michela Pelloso, Francesca Salvatori, Erica Fabbri, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello 56° Congresso Nazionale SIBioC. Bologna, October 8-10, 2024.

21. "Cell Population Data in sepsis diagnosis: E.coli and S.aureus live bacteria and endotoxins as monocyte and neutrophil activating trigger" Daniela Ligi, **Chiara Della Franca**, Francesca Salvatori, Erica Fabbri, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. 56° Congresso Nazionale SIBioC - Il ruolo clinico della Medicina di laboratorio nella sanità del terzo millennio. SIBioC, Bologna, October 8-10, 2024.

22. "Gram-negative and gram-positive bacteria-induced differential changes in Monocyte Distribution Width and Cell Population Data: insights into early sepsis diagnosis". **Chiara Della Franca**, Daniela Ligi, Erica Fabbri, Francesca Salvatori, Michela Pelloso, Giogio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. PhD day, University of Urbino Carlo Bo, 7/06/24.

23. "Platelet index changes induced by circulating histones: comparison between heparins as therapeutic approach" **Chiara Della Franca**, Daniela Ligi, Erica Fabbri, Ferdinando Mannello. ISLH Nantes, France, May 30- June 1, 2024.

24. "Monocyte Distribution Width and Cell Population Data: novel evidence on their role as sepsis biomarkers". Daniela Ligi, **Chiara Della Franca**, Erica Fabbri, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. ISLH Nantes, France, May 30- June 1, 2024

25. "Evaluation of Monocyte Distribution Width changes in whole blood samples treated with gram positive bacteria". Daniela Ligi, **Chiara Della Franca**, Erica Fabbri, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. ISLH Nantes, France, May 30- June 1, 2024

26. "Induction of platelet index alterations by circulating histones: heparinoid as a potential therapy". **Chiara Della Franca**, Daniela Ligi, Erica Fabbri, Ferdinando Mannello. PhD day 30/06/23 University of Urbino Carlo Bo.

27. "Monocyte Distribution Width and hyperinflammation: the impact of circulating histones". Daniela Ligi, Bruna Lo Sasso, **Chiara Della Franca**, Rosaria Vincenza Giglio, Luisa Agnello, Marcello Ciaccio, Ferdinando Mannello. WorldLab EuroMedLab Roma

2023 – Rome, Italy, May 21-25, 2023. DOI 10.1515/cclm-2023-7061. Clin Chem Lab Med 2023; 61, Special Suppl, pp S87 – S2222, May 2023

28. "The critical role of circulating histones as triggers of the cytokine storm in peripheral whole blood". Daniela Ligi, Marilisa Ferri, **Chiara Della Franca**, Julia Zulema Rivas, Simone Barocci, Ferdinando Mannello. WorldLab EuroMedLab Roma 2023– Rome, Italy, May 21-25, 2023. DOI 10.1515/cclm-2023-7061. Clin Chem Lab Med 2023; 61, Special Suppl, pp S87 – S2222, May 2023.

29. "Histone-induced alterations in human monocytes: down-regulation of hyper-inflammation by an heparinoid treatment". Daniela Ligi, **Chiara Della Franca**, Julia Zulema Rivas, Ferdinando Mannello. WorldLab EuroMedLab Roma 2023 – Rome, Italy, May 21-25, 2023. DOI 10.1515/cclm-2023-7041. Clin Chem Lab Med 2023; 61, Special Suppl, pp S87 – S2222, May 2023.

30. "Circulating histones induce platelet aggregation and trigger inflammatory responses". Rosaria Vincenza Giglio, Daniela Ligi, Bruna Lo Sasso, Luisa Agnello, **Chiara Della Franca**, Ferdinando Mannello, Marcello Ciaccio. WorldLab EuroMedLab Roma 2023 – Rome, Italy. May 21-25, 2023. DOI 10.1515/cclm-2023-7041. Clin Chem Lab Med 2023; 61, Special Suppl, pp S87 – S2222, May 2023.

31. "Histones as triggers of hyper- inflammation and vascular disorders". Daniela Ligi, **Chiara Della Franca**, Rosanna Maniscalco, Julia Zulema, Ferdinando Mannello. International Vascular Days (39° International Congress SIDV; 1° National Congress SIMV; 13° Congress EVF) Roma, 27-29/10/2022, Italy.

32. "Circulating histones as common mediators in classical and COVID-19 Sepsis: novel triggers for monocyte and MDW alterations". Daniela Ligi, Bruna Lo Sasso, Rosaria Vincenza Giglio, Rosanna Maniscalco, **Chiara Della Franca**, Luisa Agnello, Marcello Ciaccio, Ferdinando Mannello. 54° Congresso nazionale SIBIOC - MEDICINA DI LABORATORIO, Genova 5-7/10/2022, Italy.

33. "Emerging Mechanisms of Sulodexide As Anti-ETosis Agent In Vascular Biology: a Modulator Of The Thrombotic, Inflammatory And Proteolytic Processes" Ferdinando Mannello, Rosanna Maniscalco, Sabrina Burattini, **Chiara Della Franca**, Daniela Ligi. 22nd EVF Meeting 2022, 30/06/2022-02/07/2022, Venezia, Italy.

Awards and oral presentations

1. Best Oral Presentation Award 3rd Year, BHS PhD Day, University of Urbino Carlo Bo, 26th of June 2025. "Gram-negative and gram-positive bacteria-induced changes in Monocyte Cell Population Data: a comparison between in vivo and ex vivo models". **Chiara Della Franca**, Daniela Ligi, Francesca Salvatori, M.arisol Huaman Palomino, Giuditta Fiorella Schiavano, Giorgio Brandi, Marc Vasse, Ferdinando Mannello.

2. Prize and oral communication: 4° convegno SiBioC young scientists - Young scientists: il motore della medicina di laboratorio. "Morpho-functional and quantitative platelet alterations as potential early indicators of sepsis." **Chiara Della Franca**, Daniela Ligi, Erica Fabbri, Ferdinando Mannello

3. Invited Speaker 26° multidisciplinary conference Abruzzo and Molise: Emerging biomarkers in laboratory medicine, Chieti 5-6 ottobre 2023.

4. Prize "Poster" SIBioC 2022, for the abstract "Circulating histones as common mediators in classical and COVID-19 Sepsis: novel triggers for monocyte and MDW alterations". Daniela Ligi, Bruna Lo Sasso, Rosaria Vincenza Giglio, Rosanna Maniscalco, **Chiara Della Franca**, Luisa Agnello, Marcello Ciaccio, Ferdinando Mannello. 54° Congresso nazionale SIBIOC - MEDICINA DI LABORATORIO, Genova 5-7/10/2022, Italy.

5. "ePoster Prizes" of European Venous Forum (EVF) 2022, for the abstract "Emerging Mechanisms of Sulodexide As Anti-ETosis Agent In Vascular Biology: a Modulator Of The Thrombotic, Inflammatory And Proteolytic Processes" di Ferdinando Mannello, Rosanna Maniscalco, Sabrina Burattini, **Chiara Della Franca**, Daniela Ligi. 22nd EVF Meeting 2022, 30/06/2022-02/07/2022, Venezia, Italy.

Academic qualifications

1/11/22 - 31/10/25, University of Urbino Carlo Bo, Urbino, Italy. PhD Course in "Biomolecular and Health Sciences" Ciclo XXXVIII. Research Area 05 – Biology; BIOS/09A Clinical biochemistry and clinical molecular biology.

29/11/2022, University of Urbino Carlo Bo, Urbino, Italy. National Qualification as a Biologist Sec. A.

14/10/22, University of Urbino Carlo Bo, Urbino, Italy. Master's degree in molecular, Health and Nutritional Biology (LM-6). *110/110 cum Laude and Special Mention*.

7/07/2020, University of Urbino Carlo Bo, Urbino, Italy. Bachelor's degree in Biological Sciences (L-13).
2012 – 2017, Scientific high school Galileo Galilei, Pescara, Italy. Scientific High School graduate.

Conference Abstracts

“Role of sepsis-related Damage-Associated Molecular Patterns in the alteration of Monocyte Distribution Width”. Daniela Ligi, **Chiara Della Franca**, Giulia Barchiesi, Francesca Salvatori, Marisol Huaman Palomino, Sofia Ranieri Chiatamone, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC –MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA’ EFFICACE. Firenze 5-7 novembre 2025.

Sepsis is a critical medical emergency characterised by a dysregulated host response to infection. In recent years, Monocyte Distribution Width (MDW) emerged as a relevant FDA-approved early sepsis biomarker that quantifies monocyte heterogeneity, estimating their morpho-functional changes. Histones are interspecies conserved cationic intranuclear proteins acting as Damage-Associated-Molecular-Pattern proteins (DAMPs) when released extracellularly under pathological conditions, including sepsis, where they have been found at high levels. This study investigated the ability of different histone fractions to promote MDW alterations in an ex vivo whole blood model of sepsis. EDTA-K2 anticoagulated whole blood samples (n=51) were treated with a histone mixture (200 μg/ml) including H1, H2A, H2B, H3 and H4 fractions and with the human recombinant histone fractions H3 and H4 (10 μg/ml) alone. MDW was evaluated at 0, 30, 60, and 180 min through the DxH690T Haematology Analyser (Beckman Coulter). May-Grunwald-Giemsa-stained blood smears were also processed through the automated slide preparation system (Sysmex SP-50) for digital cell morphology analyses with the CellaVision DM software (DI-60). MDW values were early, significantly and dose- and time-dependently increased by histone mixture and histone H3 and H4 fractions, exceeding the threshold value of 20 as early as 30 minutes ($p < 0.0001$) with the fractions. Independent of the type and dose of stimulation, MDW reached mean values of 20.3±0.3 at T30, 21.2±0.1 at T60, and 21.6±0.2 at T180. Significant morphological changes in the monocyte population have been observed, with monocytes which lose their classical round shape in favour of a disruption of the cytoplasmic membrane, nuclear alteration, and a high level of vacuolation at the cytoplasmic level. Here we report that specific histone fractions and the post-translational modifications are critical for inducing MDW modifications. Our findings confirmed the clinical usefulness of MDW in early sepsis diagnosis, suggesting the crucial involvement of DAMP as contributors in monocyte activation during sepsis besides the pathogen itself.

“Role of extracellular histone fractions in thromboinflammation”. Daniela Ligi, Francesca Salvatori, **Chiara Della Franca**, Giulia Barchiesi, Marisol Huaman Palomino, Sofia Ranieri Chiatamone, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA’ EFFICACE. Firenze 5-7 novembre 2025

Thromboinflammation is a key component of sepsis pathology involving complex interactions between coagulation and immune responses. Histones are extracellularly released under pathological and infective conditions (e.g., sepsis) and act as Damage-Associated-Molecular-Pattern (DAMPs). Circulating histones promote platelet aggregation, increase Ca^{2+} influx and thrombin generation. H3 and H4 are the most investigated fractions in sepsis conditions, but the specific roles of individual histone fractions in platelet behaviour remain unclear. This study explored how different histone fractions affect platelet aggregation in an ex vivo whole blood model, in platelet rich plasma (PRP) and washed platelets (WP). EDTA-K2 anticoagulated whole blood samples (n=51), PRP (n=5), and WP (n=8) were treated with a histone mixture (200 μg/ml) and with the human recombinant histone fraction H3 and H4 (10 μg/ml). Platelet counts and indices were registered at multiple time-points up to 3h, 1h, and 15 min in whole blood, PRP, and WP, respectively, through the DxH690T Haematology Analyser (Beckman Coulter). May-Grunwald-Giemsa-stained blood smears were analysed through the automated slide preparation system (Sysmex SP-50) for digital cell morphology analyses with the CellaVision DM software (DI-60) and Scopio LabsX100. Platelet count in whole blood model was significantly and early reduced by histone mixture treatment, reaching levels (x10^3/μL) of 139.3±43.5 (T30), 146.4±41.2 (T60), and 151.5±45.0 (T180) ($p<0.0001$), compared to controls (211.1±48.7). H3 and H4 fractions did not induce a thrombocytopenic effect in whole blood. The mean platelet volume (MPV) was increased by the histone mixture to values of 10.8±0.8 (T30), 10.7±0.8 (T60), and 10.6±0.8 (T180) (fL; $p < 0.0001$) compared to controls (T0: 9.0±1.0). Similar findings were observed in the PRP model, which emphasises thrombocytopenia due to the absence of other cell types. In the WP model, all histone treatments, including the H3 and H4 fractions, induced an immediate, significant and maximum thrombocytopenic effect. These findings highlight histones as critical mediators linking inflammation and thrombosis in sepsis by activating immune cells and platelets, whose activation could depend on the specific histone fractions.

"Neutrophil and monocyte Cell Population Data are altered by extracellular histone fractions: insights into sepsis diagnosis". Daniela Ligi, **Chiara Della Franca**, Giulia Barchiesi, Francesca Salvatori, Marisol Huaman Palomino, Sofia Ranieri Chiatamone, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

Sepsis is a life-threatening condition caused by an excessive immune response to infection. Early diagnosis poses a significant challenge for patients in emergency departments. Monocyte Distribution Width (MDW), included in the Complete Blood Count (CBC) by Beckman Coulter analysers, is an early sepsis biomarker with a high negative predictive value. Cell population data (CPD), available with CBC, has also emerged as potential time- and cost-saving biomarkers of sepsis. This study investigated changes in Neutrophil (NE) and Monocyte (MO) CPD, referred to Volume (V), Conductivity (C) and Axial Light Loss (AL2) alterations induced by different histone fractions in an ex vivo whole blood model of sepsis. EDTA-K2 anticoagulated whole blood samples (n=51) were treated with a histone mixture (200 μg/ml) and with the human recombinant histone fraction H3 and H4 (10 μg/ml). CBC and CPD were evaluated up to 3h through DxH690T Hematology Analyzer (Beckman Coulter). May-Grunwald-Giemsa-Stained blood smears were also processed through the automated slide preparation system (Sysmex SP-50) for digital cell morphology analyses with the CellaVision DM software (DI-60). Whole blood treated with both histone fractions was characterized by a significant time-dependent decrease in MN-V-NE and increase SD-V-NE ($p<0.01$). The MN-C-NE and associated SD were significantly increased in a time-dependent manner with the fractions (T3h: $p<0.0001$). The MN-AL2-NE showed an opposite behavior regarding histone fraction and histone mixture stimulation, whereas its SD resulted significantly and time-dependently increase only by histone fractions (T3h: $p<0.0001$). On the other hand, the MN-V-MO was significantly and time-dependently increased mainly by histone fractions ($p<0.0001$), whereas its SD was significantly increased by all histone treatments ($p<0.01$-$p<0.0001$). The MN-AL2-MO follows a trend similar to those of neutrophils, with no differences in related SD. CPD analyses provided novel insights into the peculiar behaviour and morphofunctional alterations that monocytes and neutrophils, main effectors during infectious diseases, underwent by histone fractions. This evidence sustains the role of CPD as potential biomarkers to be included as part of an integrated sepsis screening approach.

"Monocyte Distribution Width is differently altered by diverse Pathogen-Associated Molecular Patterns". Francesca Salvatori, **Chiara Della Franca**, Marisol Huaman Palomino, Ferdinando Mannello, Daniela Ligi. 57° Congresso Nazionale. SIBIOC –MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

Innate immune responses vary across pathogens and by host genetics. Monocytes are pivotal in activating first-line defense mechanisms such as phagocytosis, inflammatory mediator release, and antigen presentation. Pathogen-associated molecular patterns (PAMPs) are conserved triggers of monocyte pattern recognition receptors (PRRs), with some of them specific for pathogen groups. This study aims to analyze the ability of different PAMP to elicit monocyte activation, as measured by Monocyte Distribution Width variation, and FDA approved early sepsis biomarker. EDTA-K2 anticoagulated whole blood samples (n=94) were treated with increasing doses of Gram negative bacterial lipopolysaccharide (LPS; 0.1-10 µg/ml), Gram-positive bacterial lipoteichoic acid (LTA; 20-200 µg/ml), fungal β-1,3-glucan polysaccharide from Saccharomyces cerevisiae (Zymosan A; 10-100 µg/ml), a synthetic analog of double-stranded RNA as PAMP associated with viral infections (Poly(I:C); 1-100 µg/ml) and a short synthetic single-stranded DNA molecule containing unmethylated CpG dinucleotides mimicking microbial DNA (ODN2216; 50-200 µg/ml). Complete blood Counts (CBC), including MDW, were evaluated at 0, 30, 60, and 180 min through DxH690T Hematology Analyzer (Beckman Coulter). MDW values were differently modulated by pathogen specific stimuli. All stimuli evoked dose- and time-dependent responses. After 3h of stimulation, we registered mean MDW values ranging from 23.0±2.7 to 47.4±12.5 with the lowest (10 µg/ml; p=0.0017) and highest (100 µg/ml; p<0.0001) doses of Zymosan A, respectively. MDW values range from 20.4±1.6 (LPS 0.1 µg/ml) to 26.8±4.1 (LPS 10 µg/ml) after 3h of stimulation. LTA increased MDW to values ranging from 19.8±0.6 (20 µg/ml) to 22.0±2.4 (200 µg/ml; p=0.0008) at 3h. The POLY (I:C) treatment induced monocyte anisocytosis with MDW values ranging from 18.9±1.7 with the dose of 1 µg/ml to 21.5±2.1 with 100 µg/ml (p<0.0001) after 5h. ODN2216 did not modify significantly MDW levels up to 5h. These findings provide novel evidence on the signalling pathways driving MDW variations on the basis of the sepsis-associated pathogen, paving the way for a potential application of MDW in early discrimination of the causative group of pathogens in sepsis diagnosis.

"Pathogen-Associated Molecular Patterns in the alterations of blood cells: insight from digital morphology analysis". Francesca Salvatori, **Chiara Della Franca**, Marisol Huaman Palomino, Alicia Martinez-Iribarren, Cristian Morales Indiano, Ferdinando Mannello, Daniela Ligi. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

Sepsis is characterized by a dysregulated immune response to infections causing life-threatening organ dysfunction and even death. Pathogen-Associated Molecular Patterns (PAMPs) are crucial triggers in initiating and modulating immune responses. PAMPs, originating from bacteria, fungi, parasites and viruses, include essential components of the pathogen (e.g., carbohydrates, nucleic acids, peptides, and wall molecules). This study aimed at investigating the role of different PAMP from Gram negative (lypopolysaccharide, LPS) and Gram positive (lypotheicoic acid, LTA) bacteria, yeast (Zymosan A) and viral origin (POLY I:C) to induce morphological changes of blood cells in an ex vivo sepsis model.EDTA-K2 anticoagulated whole blood samples were treated with increasing doses of Gramnegative bacterial lipopolysaccharide (LPS; 0.1-10 µg/ml), Gram-positive bacterial lipoteichoic acid (LTA; 20-200 µg/ml), fungal β-1,3-glucan polysaccharide from Saccharomyces cerevisiae (Zymosan A; 10-100 µg/ml) and a synthetic analog of double-stranded RNA as PAMP associated with viral infections (Poly(I:C); 1-100 µg/ml). Blood smears were manually prepared after 3h of treatment and processed through the automated slide preparation system (DxH SlideMaker Stainer II) for digital cell morphology after May-Grunwald- Giemsa-Staining and analyzed at the Cella Vision DM9600. Blood treatment with different PAMP was associated with a differentiated increase in cell size, activation of phagocytic mechanisms, cytoplasmatic vacuolization, coalescent granules, nuclear and membrane structural alterations, including chromatin decondensation in monocytes. Neutrophils were also differently activated, as highlighted by the appearance of vacuoles, phagosomes, and nuclear abnormalities. Abnormal neutrophils, classified by CellaVision as "smudge cells" due to their lack of a discernible plasma membrane, intact cytoplasm, and presence of dispersed granules and decondensed nuclei, were also identified, providing evidence for the release of Neutrophil Extracellular Traps in these settings. In conclusion, different PAMPs induce diverse monocyte and neutrophil morphological changes, highlighting a promising role for digital morphology as a potential additional tool for the early recognition of sepsis patients.

“Staphyloccoccus aureus-induced sepsis model: differences in Monocyte Distribution Width among live bacteria and Pathogen-Associated Molecular Patterns”. Marisol Huaman Palomino, Francesca Salvatori, **Chiara Della Franca**, Daniela Ligi, Alicia Martinez-Iribarren, Cristian Morales Indiano, Brandi Giorgio, Schiavano Giuditta Fiorella, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA’ EFFICACE. Firenze 5-7 novembre 2025.

Sepsis from Staphylococcus aureus is a severe, potentially life-threatening condition provoked by the gram-positive bacterium S. aureus, which invades the bloodstream and triggers a systemic inflammatory reaction, also releasing several virulence factors and toxins stimulating the immune system. Its main Pathogen-associated molecular patterns (PAMPs) are represented by lipoteichoic acid (LTA) and Peptidoglycan (PGN) which are recognized by the host’s immune system and initiate the inflammatory cascade. EDTA-K2 anticoagulated whole blood samples (n=71) were treated with increasing doses of live Gram-positive S. aureus (10^6 -10^8 CFU/mL), LTA from S. aureus (20-200 μg/ml), and PGN from S. aureus (1-200 μg/ml). Complete blood Counts (CBC), including MDW, were evaluated at 0, 30, 60, and 180 min through DxH690T Hematology Analyzer (Beckman Coulter). May-Grunwald-Giemsa- Stained blood smears were also processed through the automated slide preparation system (Sysmex SP-50) for digital cell morphology analyses with the CellaVision DM software. Plasma samples obtained after treatments were assayed to evaluate a panel of 27 inflammatory mediators (Bio-Plex 200; Bio-Rad). All stimuli promoted dose- and time-dependent responses, with MDW values differently modulated by live pathogen and related PAMPs. Live S. aureus induced MDW changes reaching a mean value of 20.9±1.9 after 3h of stimulation with 10^8 CFU/ml. With LTA we registered mean MDW values ranges from 19.8±0.6 (20 μg/ml) to 22.0±2.4 (200 μg/ml; p=0.0008) at 3h. PGN-treated samples were characterized by MDW values ranging from 19.0±1.8 with the dose of 1 μg/ml (plt;0.0001) to 30.4±1.3 with the dose of 200 μg/ml (plt;0.0001) after 3h. These findings were confirmed by digital imaging of monocytes engulfed with bacteria, hypervacuolized, and showing nuclear and membrane abnormalities. Peculiar cytokine profiles revealed different expressions of inflammatory mediator according to the triggering with live pathogens or related PAMPs. Overall, these findings reinforce the clinical usefulness of MDW in early sepsis diagnosis, underlying that MDW variations are early detected in case of S. aureus infection, and that MDW changes could be differently sustained by specific signaling pathway activated from each PAMP.

“A novel poct assay for circulating histones in biological fluids: preliminary results and future perspectives”. Margherita Borriello, Daniela Ligi, **Chiara Della Franca**, Diego Ingrosso, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA’ EFFICACE. Firenze 5-7 novembre 2025.

Extracellular histones are a group of Damage-Associated Molecular Pattern molecules, mainly derived from neutrophil extracellular traps and dying cells, which play a crucial role in physiopathological processes, including sepsis. Free histones promote inflammatory response, cell damage, organ injury, and death through the activation of Toll-like receptors, inflammasome pathways, and platelet aggregation. The measurement of free histones is gaining increasing interest, also due to the absence of reliable quantitative methods, able to discriminate among DNA-free histones, nucleosomal histones, histone fractions and fragments. Accurate detection of circulating free H3, the main fraction detected in blood from sepsis patients, is crucial for improving the laboratory procedures available for the early sepsis diagnosis and prognosis. In this regard, we are developing a colorimetric biosensor Point of care testing (POCT), based on the presence of functionalized gold nanoparticles (AuNPs), to detect and quantify free H3. Although no reference range has yet been established for H3 monitoring in sepsis, from literature studies, it has emerged that a concentration range between 100 and 1500 ng/ml is useful to monitor sepsis patients. So, the device was tested in the presence of human recombinant H3, in the selected concentration range, firstly in water thus determining the responsivity of the device toward H3. Then, the AuNP-based biosensor was tested in serum and urine. The main results are: i) The newly designed POCT is responsive to free human H3 in water, serum, and urine in the tested concentration range; ii) From these preliminary results it has emerged that the suitable biological matrix to quantify H3 by using our assay is serum. Despite these being preliminary results, to be further confirmed, they are strongly encouraging. Furtherly, there is no available assay able to detect free H3. The employed technology will be integrated with a connectivity process, supported by an accredited laboratory, that allows the quality assurance and the management of these services which are intimately linked to operator training and competency. Our results represent the starting point for the application of promising and innovative POCTs in the detection and clinical evaluation of H3 in sepsis and inflammo-degenerative diseases.

"Cell Population Data reveal monocyte activation in Gram-Positive and Gram-Negative Sepsis: a comparison between in vivo and ex vivo models". **Chiara Della Franca**, Daniela Ligi, Francesca Salvatori, Marisol Huaman Palomino, Giuditta Fiorella Schiavano, Giorgio Brandi, Marc Vasse, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC–MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

Sepsis is a leading cause of death, driven by a dysregulated host immune response to infection. Its variable clinical manifestation delays diagnosis. Cell Population Data (CPD) provide rapid insights into leukocyte changes, aiding early detection. This study investigated the variability in monocytes volume (SD_V_MO) following a 3-hour in vitro exposure of whole blood from healthy donors (EDTAK2, n=24) to E.coli (10^8 CFU/mL), S.aureus (10^8 CFU/mL), and their associated Pathogen-Associated-Molecular-Pattern proteins (PAMPs), Lipopolysaccharide (LPS, 1µg/mL) and Lipoteichoic Acid (LTA, 200µg/mL), using the DxH690T analyzer (Beckman Coulter). Results were compared to CPD from control individuals (EDTAK3, n=318) and from patients with culture-confirmed E.coli (n=10^8) and S.aureus (n=28) bloodstream infections. We observed a significant enhancement in SD_V_MO in both in vivo and ex vivo sepsis models. In vivo, SD_V_MO increased from 19.0±1.7 (mean±SD) in healthy controls to 26.3±4.8 in E.coli sepsis ($p<0.0001$) and to 25.7±3.8 in S.aureus sepsis ($p<0.0001$). In ex vivo, baseline SD_V_MO values increased from 17.5±1.3 to 26.1±3.4 with E.coli 10^8 CFU/mL ($p<0.0001$), and to 24.6±2.3 with LPS ($p<0.0001$). Exposure to S.aureus 10^8 CFU/mL resulted in an increase from 17.6±2.0 to 22.4±3.6 ($p<0.0001$) and to 23.5±4.2 with LTA ($p<0.0001$). Owing to the different anticoagulants used in sample collection, basal SD_V_MO values from control individuals differed significantly from experimental controls (p=0.0034-0.0169). To address this, all values were normalized as fold-changes relative to their respective controls. Kruskall-Wallis analysis showed no significant differences between normalized SD_V_MO values in E.coli sepsis and those observed in ex vivo E.coli and LPS exposure, nor between S.aureus sepsis and the corresponding S.aureus and LTA models. These findings demonstrated that whole blood assay reliably mirrors monocytes alterations observed in septic patients. The significant changes in SD_V_MO induced by both live bacteria and their PAMPs support its value in early sepsis detection and underscore the direct role of these stimuli in driving monocyte activation and morphological changes.

"Toll-Like Receptors modulate Cell Population Data in response to Pathogen-associated molecular patterns: implications for early sepsis recognition". **Chiara Della Franca**, Daniela Ligi, Francesca Salvatori, Marisol Huaman Palomino, Marc Vasse, Ferdinando Mannello. 57° Congresso Nazionale. SIBIOC –MEDICINA DI LABORATORIO E MEDICINA CLINICA: INTEGRAZIONE PER UNA SANITA' EFFICACE. Firenze 5-7 novembre 2025.

Sepsis is characterized by a dysregulated host immune response driven by pathogens and their Pathogen-associated molecular patterns (PAMPs) like Lipopolysaccharide (LPS) and Lipoteichoic Acid

(LTA). Cell population data (CPD), including monocyte volume variability (SD_V_MO), may reflect early immune activation. This study investigates how Toll-Like Receptor (TLR) pathways modulate CPD changes in response to PAMPs, through DxH800 haematology analyzer (Beckman Coulter). EDTA-K3 anticoagulated whole blood from healthy donors (n=12) was stimulated with LPS (1μg/mL) or LTA (200μg/mL) for 3 h. For inhibition assays, TAK-242 (TLR4 inhibitor, 10μM) and TLR2 IN C29 (TLR2 inhibitor, 25μM) were pre-incubated, alone or in combination, for 3h prior to LPS or LTA stimulation, followed by 3h incubation. Blood smears were prepared through DxH SlideMaker Stainer II and then analysed at Cella Vision DM9600. SD_V_MO remained stable in controls over 3 hours (18.7±1.4 at 180 min). LPS induced a significant increase at all time points: 24.2 ± 2.0 at 30 min, 23.7 ± 2.1 at 60 min, and 25.0±2.8 at 180 min ($p<0.0001$). LTA significantly increased SD_V_MO levels, at 20.0±1.2 ($p=0.0183$), 21.1±2.0 ($p=0.0028$), and 21.8±1.5 ($p=0.0463$) at 30, 60, and 180 minutes, respectively. TAK-242 and C29 significantly reduced LPS induced increases in SD_V_MO. TAK-242-treated samples showed values of 20.5±1.4 (30 min), 20.9±1.6 (60 min), and 20.9±2.4 (180 min) ($p<0.0380$), while C29-treated samples showed 20.6±1.3 (30 min), 19.6±1.7 (60 min), and 21.6±1.2 (180 min) ($p<0.0434$). The combined treatment with TAK-242 and C29 significantly inhibited SD_V_MO increase at 60 (20.7±2.1) and 180 minutes (20.8±2.5) ($p<0.0477$). No significant changes in LTA-treated samples after inhibitor pre-treatment, in comparison to LTA alone. Morphology of monocytes confirmed the protective effects of TLR inhibition, with reduced cytoplasmic vacuolization and preserved membrane and nuclear integrity compared to LPS-treated cells. Our study demonstrates that SD_V_MO is a promising biomarker for monitoring early immune responses. Moreover, its reduction after TLR inhibition highlights its potential to assess the efficacy of immunomodulatory interventions in sepsis and confirms the role of these pathways in regulating inflammation.

"Age-dependent thrombocytopenia induced by histones in an ex-vivo whole blood model". Francesca Salvatori, **Chiara Della Franca**, Daniela Ligi, Ferdinando Mannello. EuroMedLab Brussels 2025, Brussels, Belgium, May 18–22, 2025. DOI https://doi.org/10.1515/cclm-2025-8042 Clin Chem Lab Med 2025; 63, Special Suppl, pp S97 – S2842, May 2025.

Platelets are key regulators of thrombosis and inflammation. Thrombocytopenia is significantly correlated with the worst outcome in sepsis patients and platelet indices could be potentially useful biomarkers. Platelet count, already included in SOFA score, is influenced by gender, genetic predisposition and age. The aim of this study was to investigate the platelet response to histone treatment, well-known prothrombotic stimulus, in three different age groups: young subjects (<10 years, n=9), adult subjects (>18 years, n= 25) and elderly subjects (>75 years, n=28). Whole blood was collected in EDTA-K2 tubes, samples were treated with a mixture of histones (200 μg/mL) and Complete Blood Count (CBC) was measured by DxH 690T Beckman Coulter at 0, 30, 60 and 180 min. Firstly, our results highlighted significant differences in the control values of platelet counts between adults (215±49 x10^3/μl) and elderly (196±63) compared to the young (312±71) group ($p=0.001$-0.01). To avoid bias due to different baseline platelet values, we normalized values according to percentage changes. Our findings revealed that histones promoted a rapid platelet depletion, due to aggregation, at 30, 60 and 180 min in adults ($p<0.0001$), elderly ($p<0.0001$) and young (p:0.01-0.05) subjects. In elderly subjects the platelet count is reduced by 27% at 30 min, 22% at 60 min and 20% at 180 min; in adults histones induced a platelet decrease by 34% at 30 min, 30% at 60 min and 29% at 180 min. This percentage of depletion at 180 min in adults is significantly higher than in elderly (p:0.01-0.05). The young population showed a platelet count reduction by 16% at 30 min, 12% at 60 and 180 min, whose values were significantly lower at all times compared to adults (p:0.01-0.05). In conclusion our study emphasized that platelet baseline values and histone-induced platelet aggregation are agerelated. The adult population showed a more pronounced response in terms of platelet count reduction than the other populations. The youngest group is the least responsive one, followed by the elderly group which is probably protected using pharmacological therapies.

“Effect of different blood collection anticoagulants on the variation of Complete Blood Count under physio-pathological conditions”. Chiara Della Franca, Francesca Salvatori, Daniela Ligi, Ferdinando Mannello. EuroMedLab Brussels 2025, Brussels, Belgium, May 18–22, 2025. DOI https://doi.org/10.1515/cclm-2025-8042 Clin Chem Lab Med 2025; 63, Special Suppl, pp S97 – S2842, May 2025.

The Complete Blood Count (CBC) is a clinical analysis, performed on anticoagulated whole blood, that quantifies and characterizes all blood cells to assess the general state of health of individuals. Anticoagulants play a critical role in blood collection, and they are commercially available in different formulations: acid ethylenediaminetetraacetic (EDTA-K2 and EDTA-K3), Sodium Heparin (NH), Sodium Citrate (9NC), Citrate Dextrose (ACD). EDTA-K2 is defined by ICSH as the gold standard anticoagulant for CBC. The aim of our study was to compare the CBC obtained with the five different anticoagulants up to six hours at RT, and to investigate the cellular response to two proinflammatory and prothrombotic triggers, depending on the anticoagulant used. Whole blood from healthy volunteer donors (n=12) was collected in EDTA-K2, EDTA-K3, NH, 9NC and ACD and treated with LPS (1µg/mL) and a mixture of histones (200µg/mL). Readings were obtained at 0, 1, 2, 3, 4, 5 and 6 hours in controls and at 30 min, 1 and 3 hours in treated samples through Beckman Coulter DxH 690T. Results confirmed that EDTA-K2 is the most appropriate anticoagulant for CBC determination. Values obtained with EDTA-K3 were not significantly different from EDTA-K2. Liquid anticoagulants, 9NC and ACD, induced significantly lower CBC values than EDTA-K2 because of hemodilution; this difference is adjusted using conversion factors. NH was not eligible for platelet index analysis due to heparin-induced pseudo-thrombocytopenia. CBC is stable for up to 6 hours with all the anticoagulants. Histones induced rapid and significant platelet depletion (due to aggregation), mean platelet volume increase and Monocyte Distribution Width (MDW) enhancement in blood collected with EDTA-K2 and EDTA-K3, whereas significantly different results were observed with 9NC and ACD. LPS promoted a significant timedependent increase in MDW in all tubes, except for 9NC and ACD, where MDW decreases after 3 hours due to the possible anti-inflammatory effect of citrate. In conclusion, EDTA is the most suitable anticoagulants for CBC, also recommended by Beckman Coulter for the reliability of MDW. The potential use of selected liquid anticoagulants would require appropriate correction factors but needs further investigation.

"Variations of monocyte and neutrophil Cell Population Data by gram-negative and gram-positive bacteria: do they represent emerging early sepsis biomarkers?”. Daniela Ligi, **Chiara Della Franca**, Francesca Salvatori, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. EuroMedLab Brussels 2025, Brussels, Belgium, May 18–22, 2025. DOI https://doi.org/10.1515/cclm-2025-8042 Clin Chem Lab Med 2025; 63, Special Suppl, pp S97 – S2842, May 2025.

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to the infectious agent. Early diagnosis is a major challenge for patients in emergency departments. Monocyte Distribution Width (MDW), obtainable from hematological analyzer Beckman Coulter, is an early sepsis biomarker with high negative predictive value. MDW is included in Complete Blood Count (CBC) along with Cell Population Data (CPD) which are potential time and cost saving biomarkers obtained through Volume Conductivity Scatter technology. This study is focused on the investigation of Neutrophil (NE) and Monocyte (MO) activation during infection as reflected by Volume (V), Conductivity (C) and Axial Light Loss (AL2) alterations. Whole blood (n=24) was treated with live E. coli and S. aureus (10^6 and 10^8 CFU/ml), lipopolysaccharide (LPS) 1µg/ml, and lipoteichoic acid (LTA) 200µg/ml. CBC and CPDs were obtained at 0, 30, 60, and 180 min using DxH 690T (Beckman Coulter). Firstly, our results proved that E.coli, S.aureus and their PAMPs induced an early and significant time- and dosedependent increase in Mean Monocyte Volume (MN_V_MO) ($p<0.0001$). E.coli, LPS, S.aureus 10^8 CFU/ml and LTA also induced at each time a significant enhancement of Volume Standard Deviation (SD_V_MO) (p:0.001-0.01) and a significant decrease in

MN_C_MO. MN_AL2_MO significantly decreased with E.coli 10^6 and E.coli 10^8 CFU/ml (p:0.0001-0.001) while SD_AL2_MO was significantly increased by both live bacteria and LPS (p:0.001-0.01). The mean volume of neutrophils (MN_V_NE) is differently affected from that of monocytes, decreasing significantly after treatment with E.coli, LPS, S.aureus 10^8 CFU/ml, but accompanied by a significant increased anisocytosis reflected by SD_V_NE. Moreover, E.coli and LPS significantly increased MN_AL2_NE and decreased MN_C_NE values. SD of AL2 and C in neutrophils were significantly enhanced by both bacteria and PAMPs. In conclusion, since monocytes and neutrophils are the main actors during infectious diseases, CPDs characterising their morpho-functional changes could be potential biomarkers to discriminate sepsis patients according to the pathogen. Our findings highlighted CPDs' emerging role as part of an integrated sepsis screening approach

“Age-related variations in Monocyte Distribution Width to Lipopolysaccharide and histones in an ex vivo whole blood model”. Chiara Della Franca, Francesca Salvatori, Daniela Ligi, Ferdinando Mannello. EuroMedLab Brussels 2025, Brussels, Belgium, May 18–22, 2025. DOI https://doi.org/10.1515/cclm-2025-8042 Clin Chem Lab Med 2025; 63, Special Suppl, pp S97 – S2842, May 2025.

Monocyte Distribution Width (MDW) is an FDA-approved early diagnostic biomarker of sepsis, a clinical condition defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. Monocytes are crucially involved in the immune response against pathogens and MDW reflects monocyte anisocytosis. MDW is measured with the Beckman Coulter hematology analyzer as part of Complete Blood Count (CBC) with differential leucocyte formula. The focus of this study is to investigate the monocyte response to Lipopolysaccharide (LPS), a Pathogen-Associated-Molecular Pattern (PAMP) and to histone, a Damage-Associated Molecular Pattern (DAMP), in three different populations: young subjects (<10 years, n=8), adult subjects (>18 yrs, n= 25) and elderly subjects (>75 yrs, n=28). Whole blood samples collected in EDTA-K2 tubes were treated with LPS (1 µg/ml) and a mixture of histones (200 µg/ mL). CBC was performed at 0, 30, 60 and 180 min through DxH690T Beckman Coulter. Firstly, our results showed that MDW control values were not significantly different among the three age groups. LPS induced a rapid and extremely significant increase in MDW in adult and elderly populations at all times considered ($p<0.0001$) whereas in young subjects MDW variation was not significant. In particular, at 180 min LPS promoted MDW enhancement significantly higher in adults (25.4±3.1) compared to young subjects (21.5±1.7) ($p=0.0029$). Histone treatment caused a significant increase in MDW at 30 min in the elderly ($p=0.0209$) and both in adult and elderly populations at 60 min ($p<0.05$) and 180 min (p:0.01-0.001). Young subjects are not affected by significant changes in MDW with histone treatment. In conclusion our findings emphasize that the monocytic response to PAMP and DAMP is age-dependent. The significantly different increase in MDW implies distinct morpho-functional alterations of monocytes according to age, underlying that monocytes in young subjects underwent minor morphological changes in response to stresses compared to adults and elderly.

"Unraveling monocyte and neutrophil morphological alterations in sepsis condition by digital morphology and transmission electron microscopy". Chiara Della Franca, Sofia Ranieri Chiatamone, Michela Battistelli, Francesca Salvatori, Daniela Ligi, Elena A. Sukhacheva and Ferdinando Mannello. ISLH 2025- Halifax, May 7-9, 2025.

Monocytes and neutrophils are the main effectors of the innate immune response, involved in rapid defense against pathogens through phagocytosis and the release of inflammatory mediators. In pathological conditions, such as bacterial and viral sepsis, monocytes and neutrophils undergo significant morphological and functional alterations. Escherichia coli is the most common gram-negative bacteria found in sepsis and lipopolysaccharide (LPS) is its associated endotoxin. Histones (HIS) are intranuclear cationic proteins acting as Damage-Associated Molecular Pattern (DAMPs) found extracellularly at high concentration in bacterial and viral sepsis. This study aimed to analyze morphological changes of monocytes and neutrophils after in vitro stimulation with endotoxin and DAMP. Whole blood from healthy donors was treated with LPS (1 µg/mL) and HIS (200 µg/mL) for 3h at RT. Manually prepared smears were stained with May-Grunwald-Giemsa and processed through the automated slide preparation system (Sysmex SP-50) for digital cell morphology analysis with the CellaVision DM software (DI-60). Further ultrastructural analyses were performed using Transmission Electron Microscopy (TEM). Images obtained through CellaVision showed that LPS and HIS induced monocyte morphological alterations characterized by extensive unusual cytoplasmic vacuolization, coalescent granules, an appreciable increase in volume and nuclear structural changes such as chromatin de-condensation. The rounded shape of the cell membrane is lost and becomes irregular. These findings are confirmed by TEM images that highlighted autophagic vacuoles and nuclear membrane detachment. Our stimuli promoted also neutrophil activation reflected by features like those described for monocytes. We identified abnormal neutrophils classified by CellaVision as smudge cells, with lack of discernable plasma membrane, no intact cytoplasm, dispersed granules and decondensed nuclei, structure recognized in literature as Neutrophil Extracellular Traps. Sepsis-associated triggers promoted monocyte and neutrophil activation in vitro as reflected by striking morphological changes identifiable with digital morphology and TEM. Detectable morphological changes of monocytes in the in vitro sepsis model provides additional proof points for the biological background of elevated monocytes distribution widths in sepsis.

"Monocyte distribution width variations and inflammatory response induced by gram-negative and gram-positive live bacteria and their endotoxins". Daniela Ligi, **Chiara Della Franca**, Francesca Salvatori, Sofia Ranieri Chiatamone, Giorgio Brandi, Giuditta Fiorella Schiavano, Elena A. Sukhacheva and Ferdinando Mannello. ISLH 2025- Halifax, May 7-9, 2025

Monocyte Distribution Width (MDW) is a sepsis biomarker—available on the Beckman Coulter DxH 690T hematology analyzer—that quantifies monocyte heterogeneity in response to bacterial, viral, or fungal infection. Sepsis is a critical medical emergency characterized by a dysregulated host response to infection: Escherichia coli and Staphylococcus aureus are the most commonly gram-negative and gram-positive infectious pathogens, respectively. Lipopolysaccharide (LPS) and Lipoteichoic acid (LTA) are their Pathogen-Associated Molecular Pattern proteins (PAMPs). The aim of this study was to investigate and compare the MDW increase in an ex-vivo whole blood model treated with live bacteria and PAMPs, evaluating the inflammatory response and monocyte morphological changes. Whole blood samples (EDTA-K2, n=24) were treated with live E. coli and S. aureus (10^6 and 10^8CFU/mL), LPS (1µg/mL) and LTA (200µg/mL). MDW was evaluated at 0, 30, 60, and 180 min on DxH 690T Hematology Analyzer (Beckman Coulter). Inflammatory response was evaluated through the Pro™ Human Cytokine 27-plex assay by Bio-Plex 200 system. Manually prepared smears were stained with May-Grunwald-Giemsa on Sysmex SP-50 for digital cell morphology analysis with CellaVision DM software (DI-60). E.coli and S. aureus induced early, significant, dose- and time-dependent MDW increase, reaching pathogen-specific values. In particular, at 180 min of treatment with E. coli 10^6 and 10^8 CFU/ml MDW increased to 21.7 ($p=0.0059$) and 26.1 ($p=0.0008$), while with S. aureus to 18.1 and 20.9 ($p<0.0001$), compared to respective controls. LPS and LTA also promoted significantly different bacterial species-specific MDW changes. E. coli induced a greater increase in MDW than S. aureus. Treatment of whole blood aliquots with increasing concentrations of E. coli, S. aureus, LPS and LTA was associated with the early appearance of monocyte anisocytosis. Monocytes lose their round shape with a reniform nucleus. We observed cell size increase, cytoplasmic vacuolization, hypergranulation, nuclear and membrane altered structure, together with clear evidence of monocytes phagocytizing live E. coli bacteria. Live bacteria and associated PAMPs induced selective production of inflammatory mediators. We observed significant increases in several proinflammatory cytokines, including MIP-1α/CCL3, IL-8/CXCL8, TNF-α, IL-1β and a minor significant up-regulation was observed for MIP-1β/CCL4. Interestingly, we also observed an increased level of anti-inflammatory cytokine IL-10 but only after treatment with the highest concentration of E. coli.

Despite that our results have been obtained in an in vitro whole blood model that cannot fully represent the complexity of multicellular and humoral responses attainable in vivo, our present findings further support and confirm the clinical utility of MDW in early sepsis diagnosis. Moreover, we reveal that monocyte morphological alterations, as assessed by digital microscopy and quantified by MDW variations, could be induced not only by live bacteria through the activation of phagocytic mechanisms, but also by pro-inflammatory pathways associated with the release of bacterial endotoxins. Significant up-regulation of pro-inflammatory cytokines and chemokines in sepsis monocytes, together with up-regulation of anti- inflammatory cytokine IL-10 support the possible functional heterogeneity of monocytes in sepsis, which can result in morphological variability, detected by elevated MDW.

"Escherichia coli and Staphylococcus aureus differently modulate Monocyte Distribution Width as an early sepsis biomarker". Giuditta Fiorella Schiavano, **Chiara Della Franca**, Daniela Ligi, Erica Fabbri, Francesca Salvatori, Giorgio Brandi, Ferdinando Mannello. Poster Abstrac. 57°Congresso Nazionale SItI. Società Italiana di Igiene. Palermo. October 23-26, 2024.

Sepsis is a life-threatening systemic inflammatory response syndrome with organ dysfunction due to a dysregulated host response to infections. Escherichia coli and Staphylococcus aureus are among the most diffused GRAM-negative and GRAM-positive bacteria found in pathogenic sepsis, respectively and their pathogen associated molecular pattern proteins (PAMP), lipopolysaccharide (LPS) and lipoteichoic acid (LTA), account for the most investigated triggers in innate immune cell responses. Monocytes are early involved in defending the host from invading pathogens, and the determination of the monocyte heterogeneity through the Monocyte Distribution Width (MDW) as part of the Complete Blood Count (CBC), has emerged as an early indicator of sepsis in emergency departments. This study aimed to investigate the ability of live E. coli, S. aureus, LPS, and LTA to induce early monocyte morpho-functional alterations and inflammatory responses in an ex-vivo whole blood model. EDTA-K2 anticoagulated whole blood collected from 24 healthy participants was in vitro stimulated with live E. coli and S. aureus (10^6, and 10^8 CFU/ml), LPS (1 µg/ml) and LTA (200 µg/ml) at RT for up-to 3h. CBC, including MDW, were evaluated at 0, 30, 60, and 180 min through DxH690T Hematology Analyzer (Beckman Coulter). A panel of 27 cytokines, chemokines, and growth factors was interrogated in plasma samples through Bio-Plex 200 System (Bio-Rad). MDW values were early and significantly increased by live E. coli and S. aureus treatments, with a dose- and time-dependent mechanism ($p<0.05$). In particular, MDW was increased at values of 21.7 ($p=0.0059$) and 26.1 ($p=0.0008$) after 180 min of treatment with E. coli 10^6 and 10^8 CFU/ml compared to respective controls, whereas MDW values of 18.1 and 20.9 ($p<0.0001$) were obtained at 180 min of treatment with S. aureus, showing a significantly enhanced response of E.coli compared to S. aureus at each dose and time point. Moreover, LPS 1 µg/ml and LTA 200 µg/ml treatments promoted a time-dependent MDW increase similar to those induced by live E. coli 10^8 CFU/ml and S. aureus 10^8 CFU/ml, respectively. Peculiar inflammatory mediators were significantly over-expressed by both GRAM-negative and GRAM-positive bacteria. Live E. coli and S. aureus early induce monocyte morpho-functional modifications, mirrored by high MDW values, greater for E. coli than S. aureus. These findings are corroborated by the different responses elicited by LPS and LTA. Overall, these data reinforce the clinical usefulness of MDW in early sepsis diagnosis, underlying possible species-specific differences in MDW variations.

"Monocyte Distribution Width in sepsis: differences among live bacteria, pathogen- and-damage associated molecular pattern proteins". Chiara Della Franca, Daniela Ligi, Francesca Salvatori, Erica Fabbri, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. 56° Congresso Nazionale SIBioC - Il ruolo clinico della Medicina di laboratorio nella sanità del terzo millennio. SIBioC, Bologna, October 8-10, 2024.

Monocyte Distribution Width (MDW) is an FDA-approved early sepsis biomarker that quantifies monocyte heterogeneity and mirrors their morpho-functional changes. Sepsis is a critical medical emergency characterized by a dysregulated host response to infection. Escherichia Coli and Staphylococcus Aureus are the most commonly gram-negative and gram-positive bacteria found in sepsis, respectively. Lipopolysaccharide (LPS) and Lipoteichoic acid (LTA) are the major bacterial endotoxins and represent the most studied Pathogen-Associated Molecular Pattern proteins (PAMPs). Histones are cationic intranuclear proteins acting as Damage-Associated- Molecular-Pattern proteins (DAMPs) when released extracellularly under pathological conditions, including sepsis, where they have been found at high levels. This study aimed to investigate the ability of live bacteria, PAMPs and DAMPs to induce MDW alterations in an ex vivo whole blood model od sepsis. EDTA-K2 anticoagulated whole blood samples (n=24) were treated with live E. coli and S. aureus (10^6 and 10^8CFU/ml), LPS (1µg/ml), LTA (200µg/ml) and a mixture of histones (200µg/ml). MDW was evaluated at 0, 30, 60, and 180 min through DxH690T Hematology Analyzer (Beckman Coulter). MDW values were early, significantly and dose- and time-dependently increased by live bacteria. At 180 min of treatment with E. coli 10^6 and 10^8 CFU/ml, MDW increased to 21.7 (p=0.0059) and 26.1 (p=0.0008), while with S. aureus to 18.1 and 20.9 (p<0.0001), compared to the respective controls. LPS and LTA effect confirmed this species-specific difference, inducing significantly different MDW changes overlapping those obtained with E. coli and S. aureus 10^8 CFU/ml, reaching 28.4 and 21.9, at 180 min respectively. Histone treatment induced MDW variations (22.2 at 180 min) similar to those obtained with LTA, at each time point, and significantly lower than LPS (p=0.05-0.001). Our findings confirmed the clinical usefulness of MDW in early sepsis diagnosis, suggesting time- and dose-dependent MDW increase and possible species-specific MDW variations, as proved by both live bacteria and associated PAMPs. Sepsis-associated DAMPs promoted significant MDW changes highlighting that histones are critically involved in morpho-functional alterations of monocytes.

"Monocyte and neutrophil alteration in an in vitro sepsis model: insight from digital morphology analysis". Daniela Ligi, **Chiara Della Franca**, Michela Pelloso, Francesca Salvatori, Erica Fabbri, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello 56° Congresso Nazionale SIBioC. Bologna, October 8-10, 2024.

Sepsis is a medical emergency caused by a dysregulated host response to infections. Monocyte and neutrophils are prominent effectors of the innate immune responses and undergo early and significant morphological changes after their activation. Escherichia Coli and Staphylococcus Aureus are the most commonly gram-negative and gram-positive bacteria found in sepsis, with Lipopolysaccharide (LPS) and Lipoteichoic acid (LTA) as their major bacterial endotoxins, respectively. Histones are intranuclear proteins acting as Damage- Associated-Molecular-Pattern proteins (DAMPs) when extracellularly extruded at high levels in sepsis conditions. This study aimed to analyze the morphological changes of monocytes and neutrophils after in vitro stimulation with sepsis triggers. EDTA-K2 anticoagulated whole blood samples (n=24) from healthy donors were incubated with live E. coli and S. aureus (10^6 and 10^8CFU/ml), LPS (1 µg/ ml), LTA (200 µg/ml) and a mixture of histones (200 µg/ml) for 3 h at 37 °C. Manually prepared smears were May-Grunwald-Giemsa stained and processed through the automated slide preparation system (Sysmex SP-50) for digital cell morphology analyses with the CellaVision DM software (DI-60).Blood treatment with live bacteria was associated with increased cell size, activation of phagocytic mechanisms, vacuolization, nuclear and membrane structural alterations, including chromatin decondensation in monocytes. Neutrophils were also activated, as highlighted by the presence of intracellular bacteria, vacuoles, nuclear abnormalities. Similar features of monocyte and neutrophil activation in LPS, LTA, and histone stimulated blood samples have been observed, excepted for the presence of phagosomes. Our findings demonstrated that sepsis triggers, including live bacteria, associated endotoxins, and histones, were able to early promote monocyte and neutrophil activation in vitro, leading to morphological changes which can be identified and monitored by digital morphology, providing additional tools for the early recognition of sepsis patients.

"Cell Population Data in sepsis diagnosis: E. coli and S. aureus live bacteria and endotoxins as monocyte and neutrophil activating trigger". Daniela Ligi, **Chiara Della Franca**, Francesca Salvatori, Erica Fabbri, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. 56° Congresso Nazionale SIBioC - Il ruolo clinico della Medicina di laboratorio nella sanità del terzo millennio. SIBioC, Bologna, October 8-10, 2024.

Sepsis is one of the most challenging health-care problem, representing a major cause of morbidity and mortality worldwide. Early biomarkers are currently being investigated for rapid identification of patients in intensive care unit. Hematology analyzers generate Complete Blood Count (CBC) along with Cell Population Data (CPD) which are obtained through Volume-Conductivity-Scatter technology. CPDs provide morphological information of immune cells through impedance, radio frequencies and light scattering, which are potential time- and cost-saving biomarkers. This study aimed to investigate neutrophil (NE) and monocyte (MO) activation during infection by Volume (V), Conductivity (C) and Axial Ligh Loss (AL2) alterations. EDTA-K2 anticoagulated whole blood (n=24) were treated with live E.coli and S.aureus (10^6 and 10^8 CFU/ml), LPS (1 µg/ml), and LTA (200 µg/ml). CBC was performed at 0, 30, 60, and 180 min through DxH690T (Beckman Coulter). Our results showed that live bacteria, LPS and LTA induced an early and significant increase in the mean monocyte volume (MN_V_MO) ($p<0.0001$) with a time- and dose-dependent mechanism and also accompained by an increased anisocytosis, as demonstrated by the significant higher values of volume standard deviation (SD_V_MO) induced by Gram-negative and LPS at each time and by S. aureus 10^8 CFU/ml and LTA ($p<0.01$). A significant decrease of MN_C_MO was observed with E. coli 10^6, 10^8 CFU/ml, LPS, S. aureus 10^8CFU/ml and LTA treatment; a similar trend was observed for the MN_AL2_MO parameter with E. coli 10^6 and 10^8 CFU/ml treatments, whereas SD_AL2_MO was significantly increased by both live bacteria and LPS ($p<0.01$). Conversely, neutrophil mean volume (MN_V_NE) was significantly decreased by E. coli 10^6, 10^8 CFU/ml, LPS and S. aureus 10^8 CFU/ml ($p<0.05$). E. coli and LPS significantly increased MN_AL2_NE and decreased MN_C_NE values. All neutrophil SD of V, C and AL2 were significantly enhanced by both bacteria and PAMPs. CPDs, characterizing different leukocyte populations, have emerged as promising biomarkers able to discriminate sepsis conditions according to the pathogen, that could be used as part of an integrated approach for the early diagnosis and management of sepsis, without any additional time and costs.

"Platelet index changes induced by circulating histones: comparison between heparins as therapeutic approach". Chiara Della Franca, Daniela Ligi, Erica Fabbri, Ferdinando Mannello. ISLH Nantes, France, May 30- June 1, 2024.

Platelets are key regulators of thrombosis, inflammation, and immunity, which play a prominent role in several diseases, including sepsis and COVID-19. Assessing the progress of the clinical condition by monitoring the variation in platelet indices may be a useful approach. It has also been demonstrated that histones, well-known cationic intranuclear proteins, when found in high concentrations in the bloodstream are drivers of coagulation by activating platelets, generating thrombin and damaging endothelial cells. On these bases, histones have emerged as potential players in thrombo-inflammatory processes. Here we tested the ability of Danaparoid and Fondaparinux to counteract the effect of histones on platelets. Whole blood samples (n=6) were treated in vitro with a mixture of histones, in the presence or absence of Danaparoid and Fondaparinux. Platelet indices were evaluated through DxH690T haematology analyzer (Beckman Coulter) at 0, 30, 60, and 180 minutes. Histones induced a rapid depletion of platelet count at 30 minutes compared to untreated controls (123±44 vs. 198±45 $x10^3/\mu L$; $p<0,0014$), which was maintained up to 3 hours. Danaparoid and Fondaparinux alone did not affect platelet counts. The simultaneous addition of histones and Danaparoid 0,5 U/mL or 1 U/mL significantly protect against platelet depletion already at 30 minutes, in a dose-dependent manner (177±38, $p<0.0097$ and 193±49, $p<0.0048$, respectively). A significant protective effect of Danaparoid has also been observed when the heparinoid was added after 10 min from histone stimulation of whole blood ($p<0.01$). The use of fondaparinux (2 µg/mL) showed no significant differences in platelet count compared to treatment with histones. Furthermore, histones induced an increase in platelet volume and heterogeneity, reflected by significant higher values of Mean Platelet Volume and Platelet Distribution Width, which are counteracted by danaparoid but not fondaparinux. Circulating histones are critically involved in the thrombocytopenia by consumption found in many diseases, promoting volumetric index increase that reflect platelet activation. Moreover, the low molecular weight heparinoid Danaparoid revealed a potent activity as histone-neutralizing agent, on the basis of their charge interaction.

"Monocyte Distribution Width and Cell Population Data: novel evidence on their role as sepsis biomarkers". Daniela Ligi, **Chiara Della Franca**, Erica Fabbri, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. ISLH Nantes, France, May 30- June 1, 2024.

Sepsis is a life-threatening systemic inflammatory response syndrome with organ dysfunction due to a dysregulated host response to infections. Escherichia Coli is among the most diffused GRAM-negative bacteria found in pathogenic sepsis and its endotoxin lipopolysaccharide (LPS) represents one of the most investigated triggers able to activate innate immune cell responses. Monocytes are early involved in defending the host from invading pathogens, and the determination of the monocyte heterogeneity through the Monocyte Distribution Width (MDW) has emerged as an early indicator of sepsis in emergency departments. Recent findings highlighted that leukocyte Cell Population Data (CPD) are potentially useful for the early detection of sepsis. This study aimed to investigate the ability of live E. Coli and LPS from E. Coli to induce early monocyte morpho-functional alterations, MDW and CDP variations, in an ex vivo whole blood model. EDTA-K2 anticoagulated whole blood was collected from 25 healthy participants, and in vitro stimulated with live E. Coli (10^6, 10^8, 10^{10} CFU/ml) and LPS (1 µg/ml) at RT for up-to 3h. Complete Blood Counts, including MDW, and monocyte CPD were evaluated at 0, 30, 60, and 180 min through DxH690T Hematology Analyzer (Beckman Coulter). A panel of 27 cytokines, chemokines, and growth factors was interrogated in plasma samples through Bio-Plex 200 System (Bio-Rad). MDW values were early and significantly increased by live E. Coli in vitro treatments, with a dose- and time-dependent mechanism ($p<0.01$). LPS treatment promoted also a significant MDW increase ($p<0.0001$) overlapping those induced by live E. Coli 10^8 CFU/ml. Significant time- and dose-dependent increase of monocyte Mean Volume and decrease of monocyte Mean Conductivity and axial light loss after stimulation of whole blood with increasing concentrations of living E. Coli and LPS were observed. Peculiar inflammatory parameters, including IL-1β, IL-2, IL-6, TNF-α, IL-8, MIP1-α, MIP-1β, Eotaxin, G-CSF and GM-CSF were significantly up-regulated after treatments. Live E. Coli and LPS early induce monocyte morpho-functional modifications, mirrored by high MDW values, altered CPD and high levels of inflammatory mediators, underlying the clinical usefulness of MDW and the promising role of CPD in sepsis diagnosis.

"Evaluation of Monocyte Distribution Width changes in whole blood samples treated with gram positive bacteria". Daniela Ligi, **Chiara Della Franca**, Erica Fabbri, Giorgio Brandi, Giuditta Fiorella Schiavano, Ferdinando Mannello. ISLH Nantes, France, May 30- June 1, 2024.

Sepsis is a life-threatening condition caused by a dysregulated host response to infection, characterized by impaired balance of the immune and coagulation systems resulting in an overexpression of inflammatory mediators. Staphylococcus aureus is among the most diffused GRAM-positive bacteria found in pathogenic sepsis. Approximately 40% of bacterial sepsis cases are attributable to gram-positive. Monocytes are early involved in defending the host from invading pathogens, and the determination of the monocyte heterogeneity through the Monocyte Distribution

Width (MDW) as part of the Complete Blood Count, has emerged as an early indicator of sepsis in emergency departments. This study aimed to investigate the ability of live gram-positive bacteria, Staphylococcus aureus and Enterococcus faecalis, to induce early monocyte morpho-functional alterations in an ex vivo whole blood model.EDTA-K2 anticoagulated whole blood samples were collected from 7 healthy participants, and in vitro stimulated with live S. Aureus and E. Faecalis (10^6, 10^8, 10^{10} CFU/ml) at RT for up-to 3h. Complete Blood Counts, including MDW, were evaluated at 0, 30, 60, and 180 min through DxH690T Hematology Analyzer (Beckman Coulter). MDW values were early and significantly increased by in vitro treatments with GRAM positive bacteria, with a dose- and time-dependent mechanism. In detail, after 30 min from GRAM+ stimulation the highest concentration of bacteria induced a significant increase of MDW (MDW: 22.2; $p<0.05$). At 60 min significant increased MDW values were obtained by treating whole blood with GRAM + 10^8 CFU/ml and 10^{10} CFU/ml (21.0, $p<0.05$ and 28.9, $p<0.0001$, respectively). At 180 min, MDW reached mean values of 23.5 and 34.8 ($p<0.01$) with GRAM+ 10^8 CFU/ml and 10^{10} CFU/ml, respectively. Live GRAM-positive bacteria early induce monocyte morpho-functional alterations, reflected by increased values of MDW, reinforcing its clinical usefulness as early sepsis indicator, easily and rapidly obtainable with a complete blood count without additional cost and time.

"Monocyte Distribution Width and hyperinflammation: the impact of circulating histones". Daniela Ligi, Bruna Lo Sasso, **Chiara Della Franca**, Rosaria Vincenza Giglio, Luisa Agnello, Marcello Ciaccio, Ferdinando Mannello. WorldLab EuroMedLab Roma 2023 – Rome, Italy, May 21-25, 2023. DOI 10.1515/cclm-2023-7061. Clin Chem Lab Med 2023; 61, Special Suppl, pp S87 – S2222, May 2023.

Circulating histone levels increase with the severity of sepsis and COVID-19. This study aimed to investigate the role of circulating histones on Monocyte Distribution Width (MDW) and their effects on the release of cytokines by blood cells. Whole blood collected from 8 healthy subjects was treated with a histone mixture (0, 50, 100, 200 µg/mL) to analyze MDW modifications at 0, 30, 60, 180 min and digital microscopy of blood smears. Plasma obtained after 3 h of treatment was assayed to quantify 24 inflammatory markers. MDW values significantly increased in a time- and dose-dependent manner. These findings are associated with the histone- induced modifications of cell volume, cytoplasmic granularity, vacuolization, and nuclear structure of monocytes, promoting their heterogeneity without affecting their count. After 3h of treatment, almost all cytokines significantly increased in a dose-dependent manner. Circulating histones induce functional alterations of monocytes mirrored by MDW monocyte anisocytosis, and cytokine storm in sepsis and COVID-19. MDW and circulating histones mybe useful tools to predict higher risks of worst outcomes.

"The critical role of circulating histones as triggers of the cytokine storm in peripheral whole blood". Daniela Ligi, Marilisa Ferri, **Chiara Della Franca**, Julia Zulema Rivas, Simone Barocci, Ferdinando Mannello. WorldLab EuroMedLab Roma 2023– Rome, Italy, May 21-25, 2023. DOI 10.1515/cclm-2023-7061. Clin Chem Lab Med 2023; 61, Special Suppl, pp S87 – S2222, May 2023.

High circulating histone levels have been found in several pathological conditions, with critical inflammatory impact. Extracellular histones act as Damage-Associated Molecular Pattern proteins and exert a direct cytotoxic effect on several cell types. Here we investigated the ability of histones to modulate the release of inflammatory cytokines by blood cells. Peripheral whole blood was collected from healthy subjects, diluted in RPMI 1640, and in vitro treated with increasing doses of a histone mixture (0, 50, and 100 µg/mL). Whole blood was incubated in humified atmosphere, at 37°C, with 5% CO_2. After 24h, plasma samples were assayed to evaluate 27 inflammatory cytokines. The release of cytokines induced by histones was significantly increased in a dose- dependent manner. After stimulation with histone 50 and 100 µg/mL, we observed a very significant increase of IL-1β (29- and 43-fold vs. controls) and MIP-1α (21- and 35-fold vs. controls) ($p<0.05$). Levels of IL-1ra, IL-6, IL-8, G-

CSF, IP-10, PDGFbb, and MIP-1β were 6-fold and 8-fold increased when blood cells were stimulated with 50 and 100 μg/mL of histones, respectively. A significant increase was also highlighted for IL-2, -4, -5, -10, -12, -13, -15, - 17, eotaxin, bFGF, GM-CSF, IFN-γ, MCP-1, TNF-α, and VEGF which showed a 2-fold increase after stimulation with 50 and 100 μg/mL of histones. complex inflammatory responses in peripheral blood cells, mimicking the severe immune reaction named "cytokine storm" observed in vivo in classical sepsis and COVID-19 viral sepsis, characterized by a dysregulated release of various inflammatory cytokines produced at a much higher rate than physiological conditions. This hyper-cytokinemia exerts positive feedback on other immune cells, increasing their recruitment in the damaged site and contributing to tissue/organ injury. Thus, high circulating histone levels in septic conditions may contribute to the hyperinflammatory states and multi-organ failure of classical and viral sepsis.

"Histone-induced alterations in human monocytes: down-regulation of hyper-inflammation by an heparinoid treatment". Daniela Ligi, **Chiara Della Franca**, Julia Zulema Rivas, Ferdinando Mannello. WorldLab EuroMedLab Roma 2023 – Rome, Italy, May 21-25, 2023. DOI 10.1515/cclm-2023-7041. Clin Chem Lab Med 2023; 61, Special Suppl, pp S87 – S2222, May 2023.

Circulating histones (HIS) activate pro-inflammatory and pro-coagulative reactions, representing a potent immuno-thrombotic trigger. Heparins have the potential to counteract the dangerous effect of histones. We investigated the ability of the heparinoid Danaparoid (DP) to counteract the cytotoxic and pro-inflammatory effects induced by HIS in human monocytes. Human monocytic THP-1 cells were treated for 24h with a histone mixture (0, 25, 50, 100 μg/ml) in the presence or absence of DP (0.15, 0.3, 0.6, 1.2 U/mL). Monocytes were treated with either supernatant (sHIS) obtained from a histone-DP mixture previously incubated 60 min, RT, or directly added to cells. Cell viability, 27 inflammatory cytokines, and protein content were explored. HIS treatment significantly reduced the cell viability in a dose- and time- dependent manner and promoted a significantly increased release of most cytokines. The use of DP restored the cell viability at control levels and down-regulated the release of most cytokines with a dose- dependent mechanism against HIS 100 μg/ml, showing the strongest efficacy at 0.6-1.2 U/ml. The formation of HIS-DP complexes revealed that DP was both able to significantly reduce in a dose- dependent manner the % of free histones ($p<0.01$) and to revert the levels of almost all cytokines to those found in controls. Danaparoid is able to bind circulating histones and counteract the inflammatory responses mounted by histone-activated monocytes, providing novel evidence on the anti-inflammatory and histone-neutralizing activity of heparinoids.

"Circulating histones induce platelet aggregation and trigger inflammatory responses". Rosaria Vincenza Giglio, Daniela Ligi, Bruna Lo Sasso, Luisa Agnello, **Chiara Della Franca**, Ferdinando Mannello, Marcello Ciaccio. WorldLab EuroMedLab Roma 2023 – Rome, Italy. May 21-25, 2023. DOI 10.1515/cclm-2023-7041. Clin Chem Lab Med 2023; 61, Special Suppl, pp S87 – S2222, May 2023.

Circulating histones are involved in the alteration of the coagulative cascade found in several human diseases, including sepsis and COVID-19. Here we determined the role of circulating histones on the alteration of platelet (PLT) indices (count; mean platelet volume, MPV; platelet distribution width, PDW), and inflammatory cytokines released by blood cells. Whole blood was collected from healthy subjects and in vitro treated with increasing doses of a histone mixture (0-200 μg/mL) to evaluate PLT index changes at 0, 30, 60, and 180 min and digital microscopy of blood smears. Plasma samples obtained after 3h of treatment were used to quantify inflammatory cytokines. The treatment with increasing doses of histones reduced PLT count in a dose- and time-dependent manner. MPV and PDW increased of 10-13% ($p<0.05$) and 4-6%, respectively, in histone 50 μg\mL vs. controls. The dose of 100 μg\mL increased MPV of 15-20% ($p<0.0001$) and PDW of 69% vs. controls. These quantitative data were also supported by morphological changes highlighting PLT-PLT and PLTWBC aggregates. After 3h of treatment, we observed a significant dosede pendent increase of MIP-1α, PDGFbb, RANTES, TGF-β1, TGF-β2, and TGF-β3. These findings revealed that circulating histones may significantly contribute to the thrombocytopenia and cytokine storm observed in septic conditions (e.g. COVID-19 and classical sepsis).

"Histones as triggers of hyper- inflammation and vascular disorders". Daniela Ligi, **Chiara Della Franca**, Rosanna Maniscalco, Julia Zulema, Ferdinando Mannello. International Vascular Days (39° International Congress SIDV; 1° National Congress SIMV; 13° Congress EVF) Roma, 27-29/10/2022, Italy.

Histones are basic intranuclear proteins significantly mobilized in blood and body fluids during cell and tissue injuries in several pathological processes, where they activate inflammatory pathways and coagulative cascade. High circulating histone levels have been found in many human diseases (e.g., thrombosis, sepsis, COVID-19). We aimed at investigating the ability of histones to alter cell morphology and inflammatory phenotype in blood cells. We treated healthy human whole blood with increasing doses of histones, and we monitored the time-dependent blood cell changes through the hematology analyzer UniCell DxH900 (Beckman Coulter) and the automated analyzer for blood smears (Sysmex). A panel of 27 inflammatory mediators was investigated in plasma samples after 3h of histone treatments. Histones induced morphological modifications in the circulating monocytes, which are mirrored by significant and early changes of MDW (Monocyte Distribution Width), highlighting changes similar to those found in vivo in classic and viral sepsis. Platelet count was rapidly reduced after histone treatment, possibly due to platelet aggregation. Almost all cytokines were increased with a dose-dependent mechanism. Histones alter monocyte morphology and affect platelet count as observed in COVID-19 and sepsis, modulating thrombo-inflammation. Studies are ongoing to evaluate heparins as histone-blocking drugs.

"Circulating histones as common mediators in classical and COVID-19 Sepsis: novel triggers for monocyte and MDW alterations". Daniela Ligi, Bruna Lo Sasso, Rosaria Vincenza Giglio, Rosanna Maniscalco, **Chiara Della Franca**, Luisa Agnello, Marcello Ciaccio, Ferdinando Mannello. 54° Congresso nazionale SIBIOC - MEDICINA DI LABORATORIO, Genova 5-7/10/2022, Italy.

Histones (i.e., positively charged nuclear proteins) are key components in chromatin functions that under physiological conditions contribute to DNA packaging and regulate gene expression, but they are significantly mobilized in blood and body fluids during cell and tissue injuries in several pathological processes. Histones mediate both inflammatory pathways and coagulative cascade, crucially linked to the severity and mortality of many human pathologies (e.g., thrombosis, sepsis, COVID-19). SARS-CoV-2 and sepsis infections share common laboratory biomarkers, such as Monocyte Distribution Width (MDW), which is mainly linked to the heterogeneity of monocyte volume; these modifications, upon massive inflammatory activation, predict multiorgan dysfunction and increased mortality rate in several pathological conditions. No data are available on the roles of histones as MDW modifiers. Comparison of MDW index was undertaken by hematology analyzer UniCell DxH900 Hematology Analyzer (Beckman Coulter) 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 +LPS. We demonstrated the breadth of early, persistent, and significant increase of MDW index in whole blood from healthy subjects treated in vitro with histones, highlighting changes similar to those found in vivo in classic and viral sepsis patients. The findings of MDW changes are confirmed by digital microscopy of blood smears, highlighting the histone-induced modifications of cell volume, cytoplasmic vacuolization, and nuclear structure alterations of the circulating monocytes. Histones contribute to the pronounced and persistent monocyte alterations observed in classical and viral sepsis. Assessment of the biological impact of circulating histone released during COVID-19 and sepsis on monocytes should be considered as key factors 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).

"Emerging Mechanisms of Sulodexide As Anti-ETosis Agent In Vascular Biology: a Modulator Of The Thrombotic, Inflammatory And Proteolytic Processes" Ferdinando Mannello, Rosanna Maniscalco, Sabrina Burattini, **Chiara Della Franca**, Daniela Ligi. 22nd EVF Meeting 2022, bel30/06/2022-02/07/2022, Venezia, Italy.

Extracellular trap (ET) release is a mechanism used by blood cells to respond to different stimuli, such as infections, platelet activation, and inflammatory conditions. ETs are tridimensional web-like structures composed of extracellular DNA, histones, and enzymes, eliciting an innate human response. Scientific evidence proposed an emerging role of ETosis in several pathologies, including cardiovascular diseases and thrombotic events. Noteworthy, negative charged molecules (e.g. glycosaminoglycans) have been suggested as possible modulators of ET. We hypothesize that sulodexide, a glycosaminoglycan mixture used as an anti-thrombotic and profibrinolytic agent, could modulate the extrusion of ETs, the proteolytic network and the inflammatory storm orchestrated by human monocyte cells triggered with two main ETosis stimuli. Human THP-1 cells (monocytes and Phorbol Myristate Acetate (PMA)-differentiated macrophages) have been treated with increasing doses of calcium ionophore A23187 (5 and 25 μM) and PMA (25 and 250 ng/ml), in presence/absence of sulodexide (0.12 LSU/ml). After incubations, cells were stained with Acridine Orange (a fluorescent nucleic acid binding dye) and observed through confocal microscopy. Supernatants were used for the quantification of 27 inflammatory mediators and 8 Matrix Metalloproteases (MMP) through multiplex immunomagnetic assay. Statistical analyses were performed through Kruskal-Wallis test followed by post-hoc Dunn's multiple comparison test, and Mann-Whitney test according to variable characteristics. Morphological analyses showed a dose-dependent nucleic acid release following ETosis stimulation, suggesting the release of a more disorganized ET with the highest trigger dose. Sulodexide treatment was associated with a reduced number of monocytes that underwent ETosis and a compacting effect on ETs. The stimulation of monocytes and macrophages with increasing doses of both PMA and A23187 were able to significantly induce the release of both inflammatory and proteolytic mediators, jointly to the evidence of extracellular extrusion of dismantled nucleic acids. Sulodexide treatment was able to significantly reduce the inflammatory and proteolytic markers release, mainly when used in macrophages triggered by PMA and in monocytes stimulated with both A23187 and PMA. We demonstrated that human monocytes and macrophages stimulated with ETosis triggers produced a severe inflammatory and proteolytic response with consistent extracellular release of dismantled nucleic acids, which can be modulated by glycosaminoglycan sulodexide treatment, providing novel evidence on the potential mechanisms of sulodexide as anti-Etosis agent, with possible therapeutic effects on arterial and venous thrombosis and vascular inflammation.

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