**Recent advances in pathophysiology of disseminated intravascular coagulation: the role of circulating histones and neutrophil extracellular traps**

Yasir Alhamdi1 and Cheng-Hock Toh 1,2 \*

1 Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, Liverpool L69 7BE, UK

2 Roald Dahl Haemostasis & Thrombosis Centre, Royal Liverpool University Hospital, Liverpool, UK

\* Correspondence: Professor Cheng-Hock Toh (Toh@liverpool.ac.uk)

# Abstract

# Disseminated intravascular coagulation (DIC) is an acquired condition that develops as a complication of systemic and sustained cell injury in conditions such as sepsis and trauma. It represents major dysregulation and increased thrombin generation in vivo. A poor understanding and recognition of the complex interactions in the coagulation, fibrinolytic, inflammatory and innate immune pathways has resulted in continued poor management and high mortality rates in DIC. This review focuses attention on significant recent advances in our understanding of DIC pathophysiology. In particular, circulating histones and neutrophil extracellular traps fulfil established criteria in DIC pathogenesis. Both are damaging to the vasculature and highly relevant to the cross-talk between coagulation and inflammation processes, which can culminate in adverse clinical outcomes. These molecules have a strong potential to be novel biomarkers and therapeutic targets in DIC, which is still considered as synonymous with “Death is Coming”.

# Introduction

Disseminated intravascular coagulation (DIC) represents a major dysfunction in the hemostatic system, which is a physiological response to vascular injury. Upon injury, immediate interactions between components of the vessel wall and circulating blood lead to activation of the extrinsic and intrinsic pathways of coagulation to generate a burst of thrombin. Thrombin is immediately pro-coagulant in converting fibrinogen to fibrin but also mediates the anti-coagulant pathway by interacting with thrombomodulin (TM) to activate the protein C (PC) pathway. This controls the extent of localized clot formation but when injury is of a systemic or sustained nature, regulation of thrombin generation is lost with adverse functional consequences. Due to the ubiquitous nature of thrombin in affecting coagulation, fibrinolysis and inflammation,1-3 this can result in DIC and cause organ failure from microvascular thrombosis and endothelial barrier disruption.

This dynamic complexity in DIC pathogenesis is clinically important because its presence is well-validated as an independent predictor of mortality4. Development of DIC significantly increases risk of death beyond that of the underlying pathology. For example, DIC development in septic and trauma patients doubles the risk of mortality.5 Despite this awareness, and the fact that its acronym could stand for “Death Is Coming”, it remains poorly recognized by critical care clinicians and poorly managed due to the lack of high quality evidence. DIC diagnosis is based on scoring a number of hemostatic parameters.6 Although measurements of the prothrombin time, fibrinogen, platelets and fibrin-related products are generally available, changes in all these parameters may not occur at the same time and can delay recognition and diagnosis. In some critical care settings, not all these tests might be requested. Together with the lack of understanding that the manifestation of DIC can vary depending on primary disease-specific drivers of thrombin generation in causing multi-organ failure, this has resulted in poor management and mortality rates of 50%.7

With the overall aim of improving our understanding of how DIC contributes to adverse clinical outcomes, this review will build upon key criteria in DIC. These were set out by the International Society on Thrombosis and Hemostasis (ISTH) Scientific and Standardization Sub-Committee (SSC) in describing how DIC can arise from the vasculature but also cause damage to the vasculature.4 Furthermore, the direct coupling of inflammation to coagulation processes progresses development of organ dysfunction. Specifically in this review, we will examine how extracellular histones and neutrophil extracellular traps (NETs), fit the DIC criteria and key principles in our understanding of DIC pathogenesis. Insight into this rapidly growing area of research could pave the way for improved clinician understanding and better approaches to manage the patient with DIC.

**Extracellular histones and Neutrophil Extracellular Traps (NETs)**

While the intra-nuclear function of histones as proteins that package DNA into nucleosomes has been well understood,7,8 it is their role when released extracellularly upon cellular damage or death that is of interest and relevance to DIC. The cytotoxicity of extracellular histones were first described in 2009, whereby their neutralization in sepsis models with anti-histone antibodies or activated protein C (APC) conveyed survival benefit.9 This discovery, followed by further in vitro and in vivo work, has translated into studies in patients with sepsis, trauma and pancreatitis to illustrate the clinical relevance of extracellular histones and histone-DNA complexes (nucleosomes) to systemic inflammation, microvascular thrombosis,10-14 organ injury10,11,15-18 and death. Extracellular histones may be found in the circulation (either free or complexed with DNA “nucleosomes”) or localized (and modified) as part of the extracellular traps released upon damage or activation of nucleated cells, primarily neutrophils. The various roles of neutrophils as key modulators of the complex interaction between innate immunity, inflammation and coagulation (also known as “immunothrombosis”) is increasingly recognized and has been recently well-reviewed by Stiel et al.19 The focus of this review is on one aspect of neutrophil contribution, which is mediation by NETs.

NETs were first described in 2004, as a neutrophil-derived amalgam of elastases, histones and DNA that collectively trap and kill bacteria.20 Like histones, NETs have important physiological but potential pathological manifestations. Their uncontrolled or inappropriate release by neutrophils can contribute to the pathogenesis of sepsis, micro- and macro-vascular thrombosis and multiple organ injury.21-26 Consequences of NETs are typically site-specific but breakdown products, such as cell free DNA (cfDNA) or DNA-myeloperoxidase (MPO) complexes, can be found in the circulation.27-31 However, cfDNA can arise from damaged cells and not from NETs only.32 As such, high circulating cfDNA levels should not be assumed to correlate directly with in vivo NETs formation. Furthermore, intact NETs are structurally different and functionally dependent on its associated contents both locally and when cleaved and present in the circulation.33,34

Importantly, there is a bi-directional relationship between NETs and histones (Figure 1). First, NETs bear exposed histones (and numerous potent enzymes, such as elastase) on their meshwork and therefore facilitate local histone-mediated cytotoxicity, pro-coagulant and pro-inflammatory effects. Histones can also be released from NETs into the circulation to disseminate its adverse effects.35,36 Secondly, histones can directly stimulate neutrophils to form NETs10,37,38 and there is therefore, a vicious circle triggered by cellular injury that is then propagated by this bi-directional relationship between histones and NETs to promote further thrombin generation and contribute to DIC pathogenesis. These will be specifically detailed below in addressing how the various pathophysiological aspects of DIC can be contributed to by circulating histones and NETs.

**Relevance of histones and NETs to DIC**

The discussion will be divided into how histones and NETs contribute to the initiation, amplification and propagation of coagulation activation (Table 1).

***Factors that trigger coagulation in DIC***

*Tissue factor expression*

It is widely acceptable that the most important trigger of coagulation in sepsis and trauma-associated DIC, is excessive tissue factor (TF) expression by circulating monocytes and its exposure from the vascular sub-endothelium following injury.39 This is supported by the observation that DIC patients have significantly higher levels of circulating TF compared to controls.40 Exaggerated TF expression in septic and trauma-DIC was historically attributed to systemic inflammation triggered by the invading micro-organisms and/or their toxins (such as LPS).41-43 However, two recent studies have shown that extracellular histones can directly induce TF expression in a dose and time-dependent manner on the surface of endothelial cells and macrophages via toll-like receptors (TLR)-4 and TLR-2 and activation of the NF-κB and AP-1 pathways.12,44 With regards to NETs, recent studies have reported that NETs bear TF and contribute to thrombosis in myocardial infarction45 and anti-neutrophil cytoplasmic antibody-associated vasculitis.46,47 One study in the cancer setting reported significant interactions and correlations between NET components (primarily circulating DNA and nucleosomes) and TF-bearing microparticles culminating in overt-DIC.48 Interestingly, both TF-bearing NETs and TF-bearing microparticles were found to be triggered by complement C5a,47 highlighting a significant interaction between the coagulant and innate immune systems that include complement in contributing to pathology.

*Pro-inflammatory cytokine involvement*

In sepsis and trauma, the DIC process is directly linked into, and triggered by, the systemic inflammatory host response, of which pro-inflammatory cytokines play critical roles beyond induction of TF expression.49 Excessive cytokine activities disrupt the fine balance and crosstalk between coagulant, anti-coagulant and inflammatory pathways to augment the pro-coagulant phenotype.49,50 Histones can directly induce the release of several pro-inflammatory cytokines including IL-6, IL-1β and TNF-α.10,51-53 NETs act as scaffolds to potentiate interleukin production and activation.54-56 Conversely, cytokines can induce NETosis57 in a bi-directional relationship akin to that between histones and NETs (Figure 1).

*Platelet activation*

Platelets are important for both the initial burst and further sustenance of thrombin generation by acting as scaffolds on which further coagulation activation takes place58 and through platelet-derived polyphosphate activation of factor XI.59 Platelets also promote a pro-coagulant phenotype via P-selectin expression which enables adherence to the vascular endothelium and leukocytes while also augmenting TF expression60 and phosphatidylserine exposure on monocytes61,62 which collectively enhances thrombin generation.62 Histones can directly induce platelet activation via calcium influx with subsequent platelet aggregation and consumption in vitro and in vivo.13,63,64 Histones can also promote thrombin generation in a platelet-dependent manner via P-selectin expression, phosphatidylserine exposure and FV/Va availability on platelet surfaces.65 Histone-induced TF expression and subsequent thrombin generation can further activate platelets. In terms of clinical relevance, a case-control study in intensive care patients has recently illustrated a strong association between high histone levels and subsequent platelet consumption and thrombocytopenia to translate the findings of histone-induced thrombocytopenia in animal models.66 In parallel, NETs can interact with platelets to induce platelet aggregation, polyphosphate release and subsequent thrombin generation to cause intravascular coagulation in septic mice.26 Conversely, platelets can directly induce NETosis33,67,68 (Figure 1) and contribute to pathology, including that of transfusion-associated acute lung injury.33,68

*Vascular endothelial injury*

The vascular endothelium, one of the biggest organs in the body, has a natural anticoagulant surface mediated by the generation of APC,69 tissue factor pathway inhibitor (TFPI)70 as well as expression of heparan sulfate and glycosaminoglycans that convey anti-thrombin activity.71 Endothelial cells also participate in fibrinolysis through release of tissue plasminogen activator (tPA) upon activation.72,73Therefore, damage or dysfunction of the vascular endothelium is another vital aspect of DIC pathogenesis. To this end, histone-induced toxicity on the vascular endothelium is well documented9 and further extended in clinical studies with strong correlations observed between levels of circulating histones and soluble thrombomodulin, a marker of endothelial cell injury, in critically ill patients.10 In addition, histones have also been reported to induce the release of ultra-large von Willebrand factor (vWF) multimers,74which are involved in platelet adhesion, platelet consumption and microvascular thrombosis. Reports in septic patients have indeed documented elevated levels of ultra-large multimers of vWF together with deficiency of a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS-13), which is responsible for degradation of these multimers,75,76 but the direct effect of histones on ADAMTS-13 is not currently known. As to NETs, a recent study has shown that vascular endothelial cells have limited phagocytic capacity for NETs, which could result in poor NETs clearance and subsequent damage to endothelial tight junctions to increase vascular permeability.77 Another study illustrated that matrix metalloproteinases within NETs contribute to endothelial dysfunction.78 Indeed, activated endothelial cells can also induce NETosis.79 As such, these histone and NETs-induced changes are also likely to play relevant roles in DIC pathogenesis.

A physical consequence of cellular injury induced by histones and NETs is the exposure of negatively-charged phospholipid surfaces from membrane disruption.80 Availability of such phospholipid surfaces is highly pro-coagulant and can accelerate the prothrombinase reaction by 250,000 fold.81 In addition, histones can directly bind prothrombin and cause its auto-activation into thrombin. The implications would be that histones can directly generate thrombin without requiring coagulation activation.82 Although this reaction takes ~8 hours and may therefore not be physiologically relevant, the available evidence points to the diversity in how histones directly contribute to thrombin generation and disseminate coagulation activation.

***Factors that amplify coagulation in DIC***

*Reduced endogenous anticoagulant activity*

The three endogenous anticoagulant pathways, anti-thrombin (AT), PC and TFPI, play a critical role in regulating the extent of clot generation. All of these pathways are significantly compromised in DIC. Declining trends in AT and PC levels can be used to identify patients in a non-overt stage of DIC before full decompensation.6 Low AT and PC levels have been validated as strong predictors of mortality in sepsis and DIC patients.83,84

Although low levels of endogenous anticoagulants can be due to pathological consumption by the excessive coagulopathy in DIC, other mechanisms also contribute to reduced activity and levels.85 Related to histones and NETs are two studies showing that histones can disrupt the PC pathway by downregulating TM,12 or dampening TM-dependent PC activation.86 This would impair APC ant-icoagulant, anti-inflammatory and cyto-protective functions,87 including its ability to proteolytically cleave histones.9 NETs-associated elastases can potently degrade AT and TFPI.88-90 In addition, two other potential mechanisms for endogenous anti-coagulant loss are reduced synthesis by the liver and loss to the extravascular space from enhanced vascular permeability.90 Histones can potentially contribute to both these two mechanisms by inducing liver injury and inflammation,16,91 and significantly increasing vascular permeability through endothelial damage.9,10

 *Intrinsic pathway activation*

In physiological terms, the intrinsic pathway of coagulation plays an important role in increasing thrombin generation and accelerating hemostatic clot formation. This is well exemplified by the significant bleeding issues in patients without Factor VIII or IX. cfDNA and NETs-bound DNA can exert pro-coagulant effects through activating the intrinsic pathway of coagulation via FXI and FXII.92,93 Likewise, histones can activate the intrinsic pathway through a FXII-dependent mechanism, with histone-DNA complexes significantly contributing to elevated FXII in overt DIC patients.94 Indirectly, histone-induced release of platelet polyphosphate can stimulate Factor XI auto-activation as well as accelerate its thrombin-mediated activation.95

 *Impaired fibrinolysis*

Impaired or excessive fibrinolysis is an important aspect of sepsis and trauma-induced DIC, respectively.96-98 All studies investigating the effects of histones, cfDNA and NETs on the fibrinolytic system have consistently shown an overwhelmingly anti-fibrinolytic effect.99-101 This effect is mediated by enhanced clot resistance to fibrinolysis by plasmin and downregulation of plasminogen activation by tPA.99-101 As such, it appears that the effects of histones and NETs on the fibrinolytic system are relevant for sepsis-induced DIC but may not directly account for the hyper-fibrinolytic phenotype in trauma-associated DIC although high histone levels in such patients may increase tPA release through significant endothelial stimulation and damage.102

***Factors that propagate coagulation in DIC***

The development of multiple organ injury further augments thrombin generation and dysfunction in DIC. Microvascular thrombosis can be triggered by the factors discussed above in leading to organ ischemia and failure. There is increasing understanding of the role mediated by circulating histones in particular. In addition to causing direct injury to endothelial and other hematopoietic cells,9,10,63,80 histones have been shown to mediate *distant* organ(s) injury and dysfunction. In mice models of trauma10 and sepsis15, histones are major mediators of lung and cardiac injury and dysfunction, respectively. The clinical relevance of these findings have also been demonstrated in cohorts of critically ill patients with trauma10 and sepsis.15 The evidence for the distant organ damaging properties of histones in trauma comes from the consequent development of acute lung injury after significant non-thoracic trauma. Translational relevance is supported by the increased development of acute lung injury in patients with severe non-thoracic trauma with high histone levels. Similarly, sepsis patients without pre-existing cardiac disease were significantly more likely to develop new-onset cardiac arrhythmia (9-fold increase) and left ventricular dysfunction (2-fold increase) if they had high histone levels.15 Equally, circulating histones can mediate renal,103, liver,91,104 and brain injury.104 Notably, the incubation of plasma or serum from critically ill patients (with sepsis, trauma or pancreatitis) with cultured endothelial cells or cardiomyocytes induces cell death, which can be prevented in the presence of an antibody to histones.10,15

Cellular damage is associated with microparticle formation and release.105,106 Their pro-coagulant properties include TF expression,46-48as discussed above. Circulating microparticles from damaged or activated haematopoietic cells also have exposed phosphatidylserine and these surfaces provide attachment sites for coagulation factors, which contribute to thrombotic complications in inflammatory disorders.107 Significantly high levels of microparticles from activated endothelial cells and neutrophils have been recently demonstrated in septic shock-induced DIC patients in whom elevated levels of NETs surrogate markers (e.g nucleosomes, circulating DNA-MPO complexes) were evident.108These microparticles may have synergistic pro-coagulant effect with NETs,107 and prime neutrophils to undergo NETosis by facilitating a pro-inflammatory environment, including the release of pro-inflammatory cytokines.109,110

These histone-induced cytotoxic effects are not only a manifestation of micro- and macro-vascular thrombosis due to the pro-coagulant effects described above, but are also from *direct* cytotoxicity mediated by histone binding to cellular membranes with consequent pore formation, calcium influx and overload.10,11,63,80,111 Fattahi et al. have demonstrated that after histone infusion into mice, histones localize (in order of concentration) in the lungs, spleen, kidneys, plasma, liver, heart and least in the brain.112 As histones unravel from DNA binding as part of nucleosomes, their cytotoxicity becomes apparent due to their ability to bind cell membrane phospholipids. However in intact nucleosomes, where histones binding sites are covered by DNA, no cytotoxicity could be elicited.113 Collectively, these data suggest that circulating histones in patients with sepsis or trauma-associated DIC are major mediators of distant organ injury and adverse clinical outcome.

**Summary and insights for the future**

In this review, we have highlighted how extracellular histones and NETs fulfil the most important principles of DIC pathophysiology, as established by the ISTH-SSC.4 Firstly, histones can arise from endothelial cells following damage or from an exaggerated inflammatory response, and in turn can mediate further significant damage to vascular endothelial cells. Directly and indirectly, histones can cause pro-inflammatory cytokine release and contribute towards “inflammation gone amok”, as described in the ISTH communication. With the bi-directional relationship between histones and NETs along with their functional consequences (Figure 2), histones can be considered as mediators of distant organ injury with NETs being the effectors of multi-organ failure.

As to how these findings can translate into better recognition of DIC, there are a number of studies showing histone-DNA complexes as important prognosticators in DIC patients,114 with their levels correlating with increasing DIC scores.115 As such, there appears to be potential in using these molecules as biomarkers in early DIC before full decompensation occurs. This could be a major advancement in the diagnosis and management of critically ill patient at risk of DIC. One reason for this is that the current recommendations rely on a panel of coagulation tests collectively forming the “DIC score”, which signifies that the phenomenon is already underway or indeed advanced (overt DIC) rather than presents a target for early therapeutic intervention. Furthermore, the scoring system can vary between different societies and countries (e.g. ISTH score, Japanese Association for Acute Medicine criteria, Japanese Ministry of Health and Welfare score), and this impacts considerably on specificities/sensitivities of detection, stage of DIC (e.g. overt or non-overt) identification as well as prognostic values.90 Therefore, there is an area of unmet need for biomarkers that can better improve standardization in diagnosing DIC.

One difficulty facing the implementation of such biomarkers is that there is no simple, rapid test for quantifying histones that could be suitable for the acute hospital setting. Furthermore, there is controversy regarding the level of circulating histones with some papers quoting levels in the µg/ml range using Western blotting quantification,10,15,66whereas others suggest levels are in the ng/ml or - pg/ml range using ELISA.13,116,117 From our experience, current ELISAs are not sufficiently specific for histone measurement in clinical samples due to interference from other plasma proteins. The same issue applies to NETs measurement in patients’ samples. Currently, most studies rely on measuring cfDNA, histone-DNA and DNA-MPO complexes as surrogate markers of NETs formation.27-31 While these assays are a good development, they are also associated with problems relating to specificity especially when the cfDNA may be released from other dying cells and not necessarily from NETs. Recent studies have illustrated promising potential for the use of neutrophil side-fluorescence as a marker of neutrophil chromatin decondensation (hence NETosis) in predicting DIC development in septic shock patients.118This new marker correlated significantly (yet with weak correlation coefficient) with circulating nucleosomes and DNA-MPO complexes in DIC patients.108 Standardization for histone and NETs measurements using accurate high output techniques is therefore a pressing need.

Nonetheless, these are exciting challenges to overcome. There is the potential for novel therapeutic approaches using modalities that neutralize histones (APC,9 anti-histone antibodies,10,11,15 recombinant thrombomodulin,13 heparin119) and/or NETs (DNase20, ADAMTS-13,120 PAD4-targeted therapy34). Many of these interventions are clearly anti-coagulant and could convey bleeding risk in DIC patients if not used with caution. These would require well-designed randomized control trials using appropriate DIC patient populations, e.g. with high circulating histones and/or NETs, as well as the potential for modified non-anti-coagulant versions as is the case with non-anti-coagulant heparins, which can neutralize circulating histones.119

**Abbreviations**

# DIC, disseminated intravascular coagulation; TM, thrombomodulin; PC, protein C; APC, activated protein C; NETs, neutrophil extracellular traps; DAMPs, damage-associated molecular patterns; cfDNA, cell-free DNA; TF, tissue factor; TLR, toll-like receptor; TFPA, tissue factor pathway inhibitor; AT, anti-thrombin; vWF, von Willibrand factor; ADAMTS-13, disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13

# Disclosure

 The author(s) declare that they have no disclosures.

# FIGURE LEGEND(S)

## Figure 1. Bi-directional relationship between histones and NETs

Cell damage releases histones which trigger NETs formation and the formed NETs are a source for both localized and systemic histone release. The increased thrombin generation, which is the hallmark of DIC, simultaneously affect coagulation, fibrinolysis and inflammation processes to amplify the reciprocal relationship between histones and NETs.

## Figure 2. Functional consequences of circulating histones and NETs

Summary of pro-coagulant, anti-fibrinolytic, pro-inflammatory and cytotoxic effects of histones and NETs.

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