

UNIVERSITY OF BIRMINGHAM

INVESTIGATION OF THE MECHANISMS DRIVING

ENDOTHELIAL DYSFUNCTION IN PATIENTS WITH SEPSIS AND COVID-19

by

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Abstract:

Endothelial cells play a crucial role in the active regulation of a quiescent anti-inflammatory and anti-thrombotic vascular surface. Controlled activation is also vital for mounting an appropriate immune response during inflammation. However, systemic endothelial activation can lead to an exaggerated immune response and thrombosis, independent of the original stimulus. We aimed to assess endothelial cell activation markers in the plasma of patients with sepsis and COVID-19 and to explore possible mechanisms driving endothelial dysfunction in patients. We also investigated the impact of two key damage-associated molecular patterns released during neutrophil extracellular trap formation, histone H4 and S100A8/A9, on endothelial cells under hyperglycaemic conditions and in the plasma of patients with sepsis. We showed that systemic endothelial cell activation is present in patients with both sepsis and COVID-19 as assessed by the increased levels of markers regulating immune recruitment, immunothrombosis and vascular permeability. Markers of NETosis were also observed in patients and these levels correlate with disease severity. Mechanistically, we show that hyperglycaemia alters the response of endothelial cells to histone 4 and increases its binding. Endothelial cell activation by histone 4 is significantly increased in the plasma of patients with sepsis, in particular patients who developed acute respiratory distress syndrome (ARDS) compared to healthy controls. In conclusion, endothelial activation in patients with sepsis and COVID-19 is a hallmark of disease severity. Immune cell activation, through the release of histone H4, increases endothelial cell activation and exacerbates immunothrombosis.

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Chapter 1: Introduction

1.1 Endothelium in health

The endothelium consists of a monolayer of endothelial cells (ECs) which separates the blood from the tissues (1). Endothelial cells control oxygen, nutrient and cell trafficking (2); support haemostasis and thrombosis (3); control the permeability of the vessel (4) and help regulate vascular tone (5). In the absence of inflammation, the endothelium maintains a quiescent state by the active regulation of an anti-inflammatory and anti-thrombotic surface. Many factors contribute to this state, including laminar shear flow, which leads to secretion of the nitric oxide (NO) and prostacyclin (PG), the sequestration of EC surface receptors and pro-thrombotic factors and the presence of the glycocalyx (6).

Laminar shear flow and production of NO and PG

Under physiological conditions, NO plays a crucial role in the maintenance of EC quiescence. Endothelial nitric oxide synthase (eNOS) produces the majority of NO (7) and its activation can be constitutive or induced via either calcium-calmodulin-dependent pathways (for example, histamine and bradykinin) and calcium-independent pathways (8-10). Nitric oxide induces s-nitrosylation of cysteine residues in multiple EC proteins, such as the transcription factor 'nuclear factor kappa-light-chain-enhancer of activated B cells' (NF-κB), which limits functional activity. This contributes to quiescence in a variety of ways, including anti-inflammatory effects via interference with cell adhesion molecules (CAM) expression and inhibition of chemokines (11-13); anti-thrombotic effects via inhibition of platelet adhesion and aggregation (14, 15) and regulation of vasomotor tone via cyclic guanosine monophosphate cGMP (16).

Sequestration of endothelial surface receptors and prothrombotic factors

Another key function of NO is the inhibition of the exocytosis of the rod-shaped, EC secretory organelles, called weibel-palade bodies (WPB) (17, 18), likely via blocking granule membrane fusion or inhibiting calcium mobilization (19, 20). Weibel-palade bodies sequester pro-inflammatory and pro-coagulant molecules, the most important of which are ultra-large multimers of von Willebrand's Factor (UL-VWF) and the proinflammatory molecule P-selectin (21).

The glycocalyx

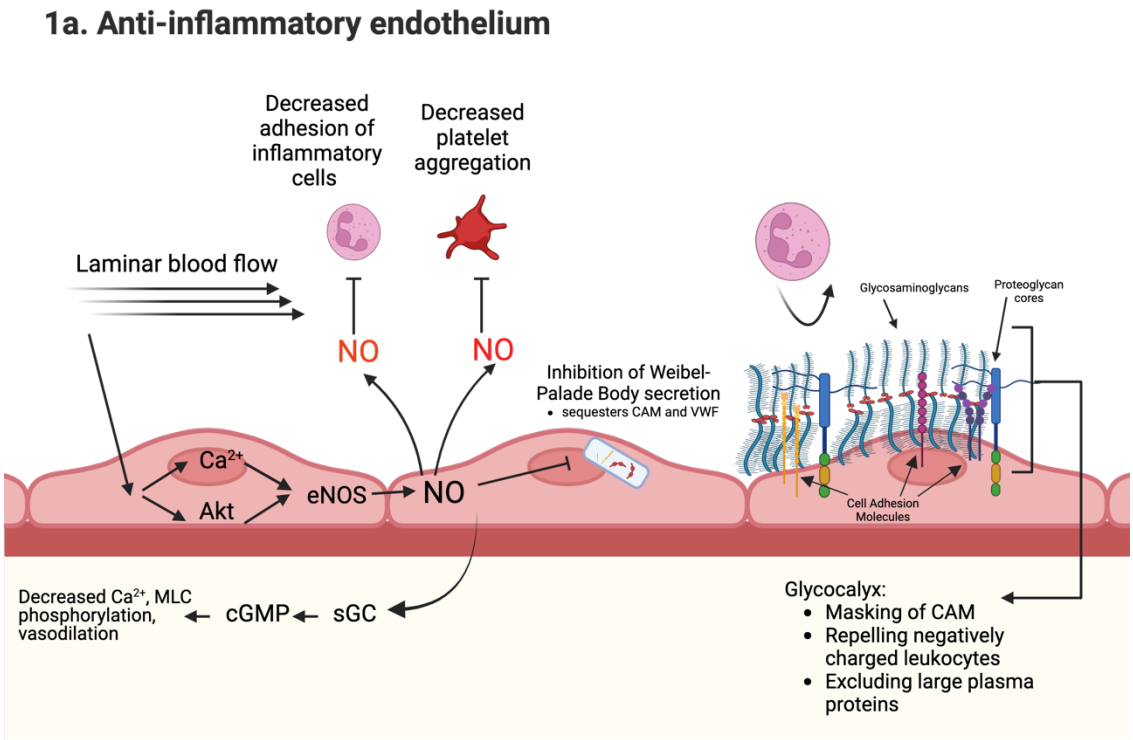
The glycocalyx is a negatively charged, heavily hydrated layer of glycosaminoglycans (GAGs) (comprising 50-90% heparan sulphate (HS) with smaller amounts of hyaluronic acid (HA)) that attach to membrane spanning proteoglycan cores (primarily syndecans and glypicans) which line the luminal endothelial surface (22). The structure is dynamic with all components being continuously shed and replaced (23). This luminal mesh binds plasma proteins and soluble GAGs to form a physiologically active endothelial surface layer which can extend 500nm into the microcirculation (24, 25) and up to 4.5µm into the macrocirculation (26). Different capillary phenotypes (continuous (e.g., lungs), fenestrated (e.g. kidneys) and sinusoidal (e.g. liver) have different glycocalyx morphology which contributes to their specific functions (27). The healthy endothelial surface layer contributes to the endothelium's barrier functions by masking CAM (28); repelling negatively charged leukocytes (29, 30) and excluding large protein molecules such as albumin to generate a protein free sub-glycocalyx space and to help maintain intravascular

volume (31). It also contributes to mechanosensory pathways and the calcium independent secretion of NO (10, 32).

The endothelium also exhibits many other specific characteristics and functions to maintain an anti-inflammatory, anti-thrombotic and selective barrier vascular surface:

Maintenance of an anti-inflammatory surface

In addition to the endothelial surface layer masking CAM, immune cell recruitment is regulated by sequestering CAM in WPB (33) or via suppressing their transcription (34, 35). Endothelial cells have also been shown to synthesis anti-inflammatory cytokines such as Transforming Growth Factor-β1 (TGF-β1) which downregulate CAMs (36, 37) (figure 1a).



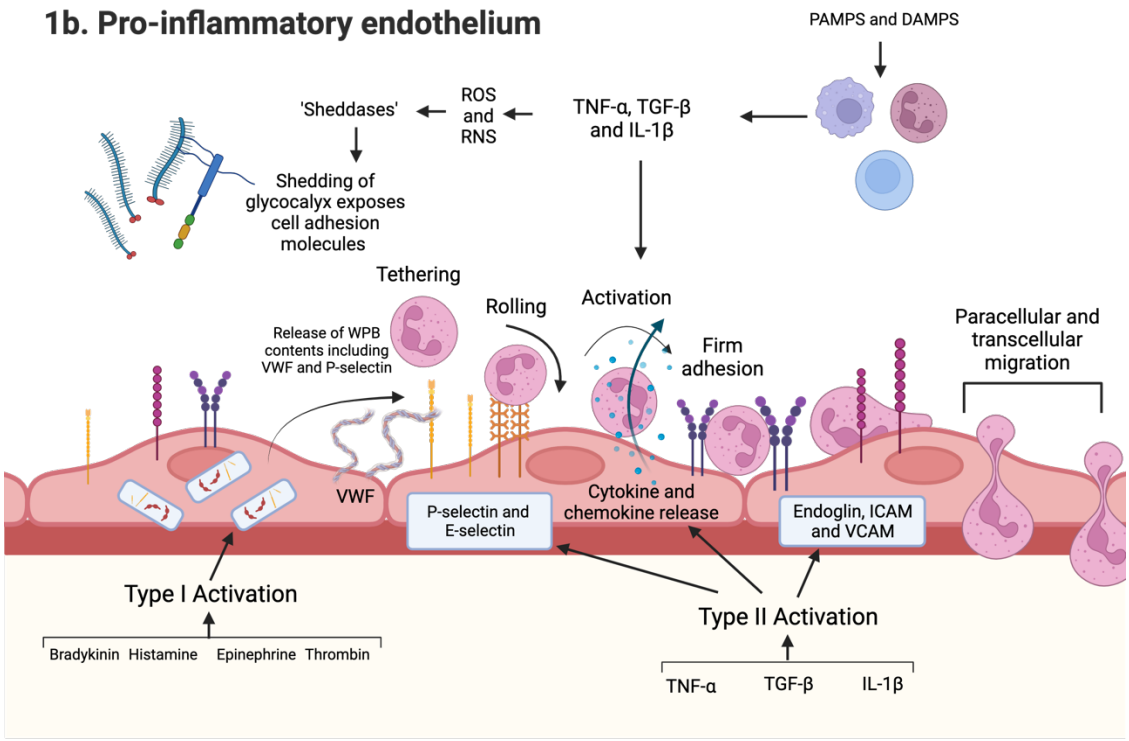


Figure 1: Factors contributing to the anti-inflammatory and pro-inflammatory nature of the endothelium

1a. In health, laminar blood flow generates NO which decreases immune cell adhesion and platelet aggregation on EC and inhibits WPB exocytosis. It also contributes to vasodilation. The intact glycocalyx minimizes immune cell recruit by masking CAM and repelling negatively charged leukocytes and contributes to endothelial barrier function. 1b. During inflammation, type 1 EC activation leads to the rapid exocytosis of pre-synthesised molecules, such as VWF and P-selectin. PAMPS and DAMPs can increase inflammatory cytokine synthesis and lead to shedding of the glycocalyx which exposes CAM. Inflammatory cytokines led to type 2 EC activation which increases the ability of the endothelium to recruit immune cells by upregulating CAM and secreting cytokines and chemokines. CAM: Cell adhesion molecules; cGMP: cyclic guanosine monophosphate; DAMPs: Danger associated molecular patterns; eNOS: endothelial nitric oxide synthase; IL-1β – interleukin-1 β; MLC: myosin light chain; NO: nitric oxide; PAMPS: Pathogen associated molecular patterns; ROS: Reactive oxygen species; RNS: Reactive nitrogen species; sGS: soluble guanylate cyclase; TGF-B: Transforming growth factor- β; TNF-α: Tumour necrosis factor-α; VWF: Von Willebrand Factor. *Figure made using BioRender.*

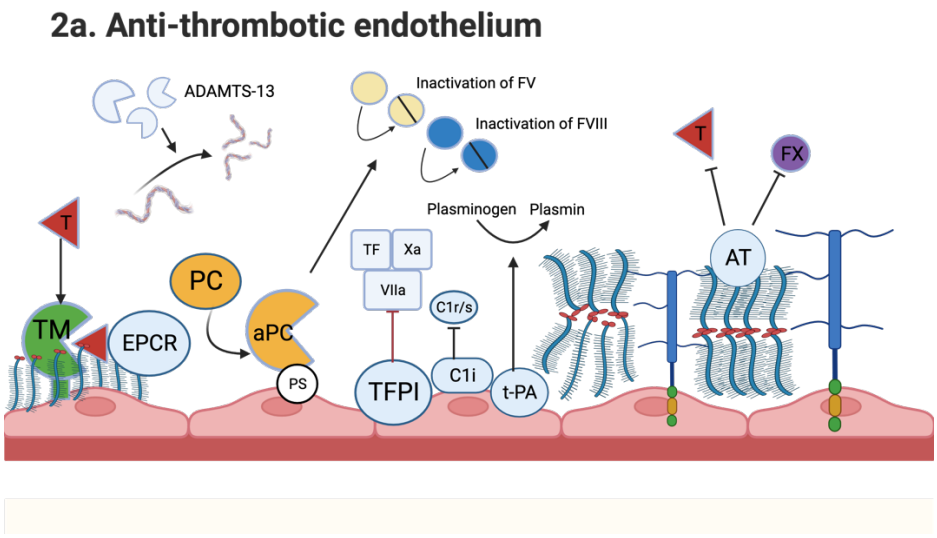
Maintenance of anti-thrombotic surface

The healthy endothelium inhibits coagulation in several ways: Firstly, a variety of coagulation factors are either constitutively secreted, localized or enhanced by the endothelium (table 1). Secondly, sequestering of ultra-large-VWF (UL-VWF) in WPB prevents unwanted UL-VWF expression but ensures a ready supply when required (38). Finally, prostacyclin release, alongside NO, limits platelet activation (39, 40) (figure 2a).

Endothelial anticoagulant mechanisms	
ADAMTS-13	Metalloenzyme primarily synthesised in the liver (41) but also by ECs (42). ADAMTS13 cleaves UL-VWF multimers under high shear conditions limiting its prothrombotic functions (43-45).
Antithrombin (AT)	SERPIN synthesised in the liver which inhibits thrombin and FXa and to a lesser extend FIXa and FXIa AT binds to and is enhanced by HS on ECs (46-49).
Complement inhibitors	C1 inhibitor is produced by ECs (49). It irreversibly binds to and inactivates C1r and C1s as well as mannan-binding lectin serine proteases. C1-inhibitor also inhibits the contact system (kallikrein, FXIa, and FXIIa)(50-52). Inhibition is enhanced by GAGs (52). C1 inhibitors limits complement activation and endothelial damage.
Heparan Cofactor II	SERPIN synthesised in the liver which inhibits thrombin. Endothelial cell binding is enhanced by GAGs (53-55).
Protein C (PC)/ activated protein C (aPC)	PC is a zymogen which is activated by thrombin on the surface of ECs in the presence of thrombomodulin. APC cleaves FVa and FVIIIa (56, 57). The EPCR augments protein C activation (58, 59). It is one of the major natural anti-coagulant systems.
Protein S	Synthesized by ECs (60) and acts as an anticoagulant by inhibiting FIXa, the prothrombinase complex (FV and FX) and acting as a cofactor for aPC and tissue factor pathway Inhibitor (61).
Tissue factor Pathway Inhibitor (TFPI)	Synthesised by ECs (62). Inhibits the TF-FVII and prothrombinase complex (63-65).
Tissue plasminogen activator (t-PA)	Synthesised by ECs and catalyses the activation of plasminogen to plasmin which is essential for clot lysis and fibrinolysis (65-67).
Thrombomodulin (TM)	Primarily synthesised and associated with the endothelial surface layer (68). Activates the PC pathway and TFPI (46, 48, 69).

Table 1: Coagulation factors involved in maintaining an anti-thrombotic endothelium

ADAMTS-13 - disintegrin and metalloproteinase with a thrombospondin type motif 13; ECs – Endothelial cells; EPCR – Endothelial Protein C Receptor; HS – heparan sulphate; SERPIN – serine protease inhibitor; TAFI - - Thrombin activatable fibrinolysis inhibitor UL-VWFs – Ultra-large Von Willebrand's Factor



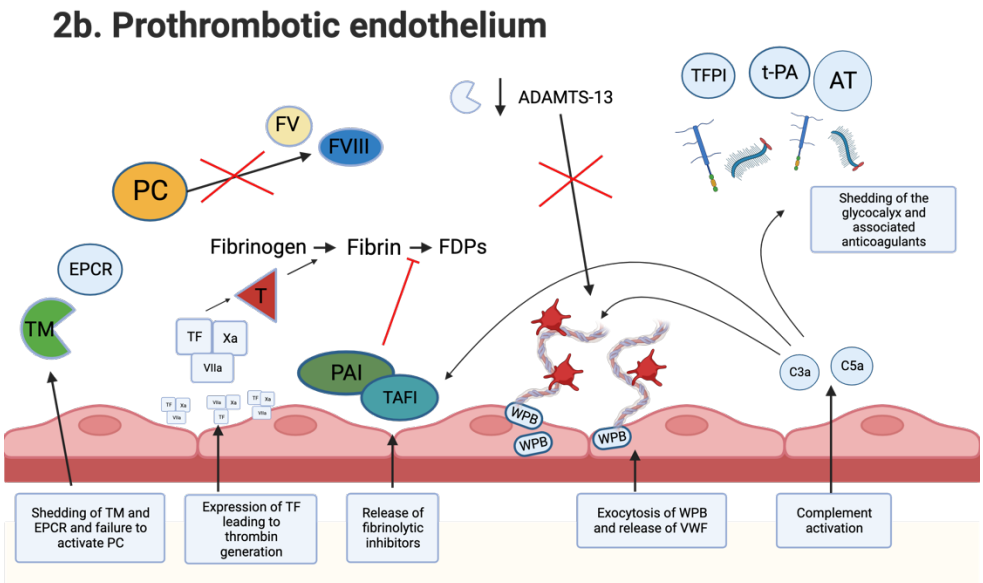


Figure 2: Factors contributing to the anti-thrombotic and pro-thrombotic nature of the endothelium

2a. In health, ECs and the liver synthesize anticoagulants, including those that inhibit the clotting cascade and complement activation. ECs also synthesize components of the protein C pathway and activators of fibrinolysis. The healthy glycocalyx localizes several anti-coagulants to the endothelium, including antithrombin and thrombomodulin. 2b. During inflammation, shedding of the glycocalyx and the EPCR results in failure to activate protein C and reduced inhibition of FV and FVIII. Tissue factor is expressed on activated ECs and there is exocytosis of EC WPB leading to VWF release alongside reduced synthesis of ADAMTS-13. This leads to persistent EC binding of UL-VWF multimers which readily capture platelets. EC also release fibrinolytic inhibitors. *Figure made using BioRender.*

aPC – activated protein C; AT: Anti-thrombin; ADAMTS-13 - disintegrin and metalloproteinase with a thrombospondin type motif 13; EPCR – Endothelial Protein C Receptor; FDPs – Fibrin degradation products; PAI – Plasminogen activator inhibitor; PC – Protein C; T: Thrombin; TAFI - Thrombin activatable fibrinolysis inhibitor; TF – Tissue Factor; TFPI: Tissue factor pathway inhibitor; t-PA – tissue plasminogen activator; TM – thrombomodulin; UL-VWFs – Ultra-large Von Willebrand's Factor; TF – Tissue Factor; WPB – Weibel Palade Bodies

Regulation of vascular permeability

The endothelial barrier is comprised of intercellular contacts and the sieve-like endothelial surface layer which repels negatively charged plasma proteins (48). Intercellular contacts include tight junctions (TJs), which are groups of transmembrane proteins (including

claudins and occludins) that associate with cytosolic Zonula Occludens (ZOs) proteins (70, 71), and adherens junctions (AJ), whose structure is based on cadherin (e.g. E- and N-cadherin) and catenins (72, 73). Both complexes are anchored to the EC actin cytoskeleton (74, 75). Tight junctions are more developed in small arterioles and regulate paracellular movement of water and solute, whereas AJs are more prominent in post capillary venules (76) and primarily determine permeability to large molecular weight plasma components (77, 78).

Adherens junction integrity is the key mediator of vascular permeability (79) with stability maintained by the actin-myosin apparatus (80-82). Phosphorylation of the myosin light chain (MLC) causes actin filament contraction which, via attachment to AJ proteins, can open inter-endothelial gaps (4). Myosin Light Chain Phosphatase (MLCP) dephosphorylates MLC to maintain barrier function, whereas Myosin Light Chain Kinase (MLCK) phosphorylates MLC (83). Cytosolic calcium and the mutually inhibiting small GTPases RhoA and Rac1 are key regulators of MLCP and MLCK (84). Permeability is limited by Rac1 signaling which inhibits MLCK (80, 85, 86). This is supported via the endothelial tyrosine kinase receptor (Tie2) which is a strong phosphatidylinositol 3-kinase (PI3K)-dependent activator of Rac1 and deactivator of RhoA (87-89).

The angiopoietin system is the major regulator of vascular permeability. Angiopoietin-1 (Ang-1) is a glycoprotein for the Tie2 receptor (90) mainly expressed on ECs (91). It counteracts the permeability induced by a wide spectrum of mediators including bradykinin, Tumour Necrosis Factor-α (TNF-α), thrombin and lipopolysaccharide (LPS) (92, 93). Angiopoietin-1 is also reported to counteract neutrophil adherence to EC due to PI3-kinase activity decreasing expression of CAM (94) and inhibiting interleukin-8 (IL-8)

production by ECs (93). The balance between the protective factor Ang-1 and activator Angiopoietin 2 (Ang-2) determines EC permeability.

1.2 Role of endothelial cells during controlled inflammation

Activation of the endothelium is crucial for normal innate and adaptive immunity and ECs play an important role in dictating the location, extent, and duration of inflammation (95). There are two types of EC activation that occur (96) (summarized in table 2): Type I activation is typically mediated by agonists of G-protein coupled receptor (GPCR) pathways that either lead to increases in intracellular Ca^{2+}, such as thrombin, or increase intracellular levels of cyclic adenosine monophosphate (cAMP), such as epinephrine (97-99). This leads to the rapid release of pre-synthesised molecules, for example the WPB contents VWF, P-selectin and IL-8, which mediate initial leukocyte and platelet interactions with ECs. It also leads to release and synthesis of mediators, such as Ang-2 and NO, which contribute to increased endothelial permeability (primarily in the capillaries) and decreased vascular tone (primarily in the arteries) (100-103). Type II activation is mediated by agonists which stimulate gene transcription and protein translation. This leads to synthesis of cytokines, chemokines and CAM which further contribute to immune cell recruitment (104-106). It also leads to synthesis of procoagulant factors, such as tissue factor (TF). Type II activation principally occurs in venular ECs and leads to a more sustained inflammatory response (107, 108).

During inflammation, innate immune cells, in particular neutrophils and monocytes, are activated by pathogen-associated molecular patterns (PAMPs), such as bacterial LPS, and damage-associated molecular patterns (DAMPs), such as nuclear proteins and histones released from damaged cells. These PAMPS and DAMPs are recognized using pattern recognition receptors (PRRs)(109). Release of pro-inflammatory cytokines, such as TNF-α and interleukin-1β (IL-1β), from these activated neutrophils and monocytes will subsequently lead to type II activation of EC (110). Endothelial cells also form part of the innate immune system through their own PRRs, including Toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE) (111), a multiligand PRR that binds inflammatory mediators such as advanced glycation end products (AGEs) (112) and S100 proteins (113).

Endothelial activation	Timing	Classical agonists:	Mechanism
Type I	Rapid and transient (20 minutes)	Bradykinin, histamine, epinephrine, and thrombin	Activation of GPCR pathways which trigger cAMP and calcium signaling and lead to the release of pre-synthesised molecules and increases in NO.
Type II	Slower and sustained (hours to days)	TNF-α, TGF-β and IL-1β	Activation of transcription factors such as NF-κB which led to protein translation of CAM and synthesis of procoagulant factors.

Table 2. Type I and II endothelial cell activation

CAM – Cell adhesion molecules; GPCR – G-Protein coupled receptor; IL-1β – Interleukin 1 beta; NO – Nitric oxide; TNF-α – Tumour Necrosis Factor-a, TGF-β - Transforming Growth Factor β

Promotion of a pro-inflammatory surface for immune cell recruitment

Transendothelial migration requires interaction of leukocyte integrins with EC surface receptors (table 3)(114). The first stage of transmigration is low affinity tethering and endothelial rolling mediated by E-selectin, P-selectin and L-selectin (115-117) {Geng, 1990 #2866}. This rolling facilitates interaction with chemokines, for example C-X-C Motif Chemokine Ligand (CXCL) 1, 2, 8 (118) and Regulated on activation, normal T cell expressed and secreted (RANTES) (119), which are synthesised by ECs and immobilized within the endothelial surface layer (120-122). Rolling leukocytes are activated by these chemokines, which results in spreading and clustering of leukocyte integrins (e.g. β_1-integrin Very Late Activation Antigen-4 (VLA4) and β_2-integrin Lymphocyte Function-Associated Antigen-1 [LFA-1]). This increases their affinity and valency for their endothelial ligands (intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) respectively) which are induced following type II EC activation. Together, this results in firm leukocyte adhesion and extravasation to the site of inflammation (figure 1b) (123-125). Platelets have also been shown to trigger confirmational changes in leukocyte adhesion molecules which promote efficient extravasation (126).

Transendothelial migration can then occur either paracellularly through interendothelial junctions or transcellularly vis the formation of a transcellular pore (127). Neutrophils then reach the site of infection where they can either carry out phagocytosis, degranulate and release reactive oxygen species (ROS)(128, 129) or release neutrophil extracellular traps (NETs) (130). A fraction of neutrophils undergo reverse transmigration where they migrate to the lungs and bone marrow and undergo apoptosis, a mechanism that is considered to

support the resolution of inflammation. However, the recirculation of activated neutrophils can further disseminate inflammation and favour distal organ damage (131).

Membrane receptor	Synthesis / Expression	Ligands	Inflammatory mediators	Mode of expression	Specific function
Endoglin	ECs	Leukocyte α5β1 integrin (132)	TGF-β (133), and hypoxia (134)	Inducible	TEM (132)
E-selectin	ECs	ESLG-1, PSGL-1	TNFα, LPS, IL-1 β, G-CSF (135, 136)	Inducible	Low affinity binding for leukocyte tethering and rolling (117, 137)
P-selectin	EC WPBs and platelets	PSGL-1	TNFα, IL-4, IL-13, histamine, thrombin	Constitutive and inducible	Low affinity binding for leukocyte rolling (138)
ICAM-1	ECs	Mac-1, LFA-1	TNFα, LPS, IL-1	Constitutive, inducible	Firm adhesion, TEM (139)
VCAM-1	ECs	VLA-4	TNFα, LPS, IL-1	Constitutive, inducible	Firm adhesion, TEM (140)

Table 3. List of key endothelial cell adhesion receptors regulating immune cell trafficking

ECs – endothelial cells; ESLG-1 – E-selectin-ligand; G-CSF - Granulocyte-colony stimulating factor; LPS – lipopolysaccharide; IL-1 β – interleukin-1-Beta; IL-4 – interleukin-4; IL-13 – interleukin-13; LFA-1 - Lymphocyte function-associated antigen 1; Mac-1 - macrophage-1 antigen; PSGL-1 – P-selectin-ligand; TEM – transendothelial migration; TGF-β – transforming growth factor-beta; TNFα – tumour necrosis factor-alpha; VLA-4 - very late activation antigen-4, WPB – Weibel – Palade Bodies

The complement system and contribution to endothelial cell activation

The complement system is a collection of heat sensitive plasma and membrane-associated proteins that play a role in innate and humoral immunity(141). Endothelial cells have the ability to synthesize some of these proteins, including C1s, C4, (142, 143), C7(144) and, alongside hepatocytes, C3 and factor B (144, 145). Endothelial synthesis of complement proteins is upregulated by pro-inflammatory cytokines (146, 147).

The complement system is typically said to be activated in one of three ways: The classical pathway is activated by antigen-antibody complexes; the lectin pathway is activated by mannose-containing carbohydrates on the surface of bacteria and viruses and the constitutively active alternative pathway is spontaneously activated when complement binds to a pathogen surface. All proteolytic cascades converge to generate C3 convertase which generates the anaphylatoxins (C3a and C5a), opsonins (C3b) and the membrane attack complex (MAC) (C5b-9). A fourth pathway has been described in which thrombin cleaves and activates C5 independently of C3 convertase (148, 149).

Complement activation can occur on the surface of ECs undergoing apoptosis via the classical and potentially lectin pathways (150, 151) and on EC bound vWF (as complement factor B can bind the A2 domain and also bind C3b, providing a platform for alternative pathway activation (152). P-selectin has also been shown to support complement C3b binding, and favour complement deposition on ECs (153).

The anaphylatoxins, C3a and C5a, can also contribute to EC activation by up-regulation of CAM (154) and integrin dependent adhesion and transmigration (155). Endothelial cells also constitutively express complement receptors and physiological concentrations have

been shown to up-regulate TNF-α, IL-1β, IL-8 and RANTES (154, 156-159). C5a can also increase vascular permeability (160, 161) and may induce TF expression on ECs (162).

Promotion of a pro-thrombotic surface on endothelial cells

Although thrombosis is generally considered detrimental due to compromised blood supply, host exposure to infection triggers both the innate immune system and coagulation, termed 'immunothrombosis', which is thought to contribute to the early immune defence against invading pathogens (163). Endothelial cell activation leads to a prothrombotic phenotype associated with the released of WBP; TF expression and initiation of the coagulation cascade; activation of the complement system; down-regulation of anti-thrombotic and anti-coagulant pathways and the release of anti-fibrinolytic proteins inhibiting clot resolution. The information related to the prothrombotic mechanisms is summarized below in table 4 and shown in figure 2b.

Endothelial factors contributing to immunothrombosis	
Activation of coagulation cascade	The expression of TF and its interaction with circulating activated FVII initiates thrombin production (164). TF is normally expressed perivascularly and exposed during injury but can be induced on ECs (165), neutrophils (166), monocytes (167, 168) and microparticles (169, 170) secondary to inflammation.
Exocytosis of WPB	EC WPB secretagogues include cytokines (IL-8 and TNF-α), hypoxia, heme, thrombin and epinephrine and can be released as therapeutic intervention by desmopressin (DDVAP) (96, 171). The key constituent is UL-VWF, which is pro-inflammatory and pro-thrombotic. WPBs also contain tPA (172), angiopoietin-2 (173), cytokines (174) and P-selectin (33). P-selectin comigrates with VWF and anchors UL-VWF on the EC surface (175). Anchoring is also facilitated by the glycocalyx (176).
Activation of the complement system	C3a and C5a anaphylatoxins are pro-coagulant by inducing EC TF expression (177), EC secretion of VWF (178) and contributing to loss of HS from the endothelial surface layer (179).

Downregulation of anticoagulant features	Down-regulation and proteolytic shedding of thrombomodulin and EPCR leads to impaired protein C activation (180, 181). Inflammation can also trigger degradation and shedding of the endothelial surface layer (182).
Up regulation of anti-fibrinolytic pathways	Endothelial cells produce the fibrinolytic inhibitors PAI-1 (183), PAI-2 (184), and TAFI (185) which are upregulated in response to inflammatory cytokines.

Table 4. Endothelial factors contributing to immunothrombosis

ECs – endothelial cells; NETS – Neutrophil extracellular traps; EPCR – endothelial protein C receptor; ESL – endothelial surface layer; PAI-1 – plasminogen activator inhibitor-1, PAI-2 – plasminogen activator inhibitor-2, TAFI – Thrombin activatable fibrinolytic inhibitor; TF – Tissue Factor; t-PA – tissue plasminogen activator; UL-VWF – Ultra-large Von Willebrand Factor multimers; WPB – Weibel Palade Bodies

Von Willebrand's Factor and immunothrombosis

The release of VWF from activated EC and its association with the endothelium has a myriad of functions. When at sites of high shear stress, UL-VWF multimers develop a fibrillar confirmation with subsequent exposure of the A1 domain which contains the GPIbα binding site required for initial platelet tethering (43, 186). This tethering activates the platelet αIIbβ3 integrin which is required for firm adhesion to fibrinogen, fibrin or VWF for aggregation (187). Aggregated platelets also create an 'activating surface' for the alternative complement pathway (188).

Von Willebrand's Factor also supports a pro-inflammatory endothelium as platelet-decorated UL-VWF strings form an adhesive matrix for leukocyte tethering and rolling under high shear stress in a P-selectin dependent manner (189). VWF-associated platelets can further support neutrophil extravasation (190) and extended UL-VWF can also bind and

immobilize NETs to increase platelet and leukocyte recruitment (191, 192). Under low shear stress, VWF provides both binding sites for transient leukocyte rolling (PSGL-1) and stable (β2-integrin) adhesion (193, 194).

Endothelial activation and promotion of endothelial hyperpermeability:

Disruption of AJs due to compromised integrity of VE-cadherin is the leading cause of hyperpermeability during inflammation (79, 195) with increases in cytosolic calcium and phosphorylation of the MLC both promoting hyperpermeability (4). Vessel permeability allows the extravasation of plasma proteins into the interstitial space to support an inflammatory matrix (196).

Activation of ECs by permeability mediators leads to generation of inositol triphosphate (IP_3) and diacylglycerol (DAG) which mediate increases in EC cytosolic calcium via transient receptor potential channels (TRPC): these differ between ECs and may account for some of the observed heterogeneity between different endothelium's (197, 198).

Calcium can bind calmodulin and this complex then activates MLCK which phosphorylates MLC. Calcium also activates RhoA signaling (4, 199). Activation of protein kinase C (PKC) (e.g. via thrombin or matrix metalloproteins (MMPs) acting on protease-activated receptor (PAR) (200)) and Src kinase (e.g. via thrombin or vascular endothelial growth factor (VEGF)) also disrupt AJs, either via phosphorylation or by increasing microtubule dynamics (198). Nitrosylation of β-catenin by NO also leads to dissociation of β-catenin from VE-cadherin and disrupts AJs (201).

Angiopoietin-2 can be released from EC WPBs during inflammation and, in this setting, can act as a non-signaling ligand to decrease Tie2 phosphorylation leading to increased RhoA

signaling and MLC phosphorylation (202, 203). Angiopoietin-2 is also reported to promote leukocyte adhesion by sensitizing ECs toward TNF- α and increasing expression of CAM (173), as well as disrupting the endothelial surface layer in a heparanase dependent manner (204).

Glycocalyx modulation of inflammation

The glycocalyx plays a crucial role in modulating the EC response during controlled inflammation. It captures chemokines from the circulation which support leukocyte activation and transmigration (120); stimulates TLR2 and 4 signaling on immune cells (205); competitively inhibits circulating inflammatory mediators, including histones, at endothelial binding sites (206) and binds antithrombin to elicit anti-inflammatory signaling.

1.3 Dysregulated inflammation and endothelial dysfunction

During severe inflammatory conditions, maladaptive, auto-amplifying cytokine production, known as 'cytokine release syndrome' (CRS), can lead to a dysregulated immune response and systemic inflammatory response syndrome (SIRS) (40, 207). Early deaths are often attributed to an overwhelming hyperinflammatory response (208) that can result in microvascular thrombosis, capillary permeability and tissue ischaemia and hypoxia (209, 210). Systemic endothelial activation is thought to be an important contributor to this progression and occurs independent of the original stimulus (211-213). Furthermore, the EC activation that can occur during an initial severe inflammatory event, can result in an exaggerated host immune response to subsequent, relatively trivial insults, the 'two-hit

hypothesis' (214, 215). Pre-existing endothelial dysfunction, for example due to dysglycaemias (34, 216) and glycocalyx perturbations (217), can also predispose patients to developing a systemic, dysregulated immune response.

Neutrophil extracellular traps and their impact on systemic endothelial cell activation

During severe inflammation, certain populations of neutrophils, known as low-density neutrophils, show increased longevity and impaired migration. This ensures the presence of primed neutrophils at the site of inflammation (218) and can contribute to resolution of inflammation. However, persistence may also lead to EC cytotoxicity due to an enhanced proinflammatory phenotype, ROS production, and spontaneous formation of intravascular neutrophil extracellular traps (NETs) (219, 220).

Neutrophil extracellular traps are extracellular, web-like structures of decondensed chromatin fibres studded with cytotoxic cytosolic and granular proteins such as histones, S100A8A9 and enzymes with microbicidal activity (130, 221). The formation of NETs, termed 'NETosis', occurs in one of two ways: suicidal NETosis refers to programmed cell death with associated loss of membrane integrity, whereas in vital NETosis and mitochondrial NETosis, membrane integrity and neutrophil function are preserved (222, 223). NETosis has been reported following stimulation with certain cytotoxins (IL-8), bacterial products (e.g. LPS) and specific pathogens as well as protein kinase C activators such as phorbol-12-myristate-13-acetate (PMA).

NETs can immobilize pathogens, facilitate phagocytosis or directly kill pathogens (224). However, NETs can also cause EC activation and dysfunction. Firstly, many NET components, such as histones and S100A8/A9, can signal via TLRs, RAGE, and CD36

pathways leading to an increase in CAM expression and leukocyte recruitment (111, 225-227). They can also increase cytokine production via activation of the NLRP3 inflammasome (227). Furthermore, NETs can concentrate enzymes, such as neutrophil elastase, cathepsin G and myeloperoxidase in the vasculature. These can degrade the glycocalyx resulting in increased permeability (228) and loss of endogenous anti-coagulants, such as antithrombin, APC, TFPI and thrombomodulin (229-232). A procoagulant phenotype is induced by TF upregulation and reduced thrombomodulin expression (230, 233) and induction of phosphatidylserine and protein-disulfide isomerase expression (233). Permeability is also increased by destabilization of VE-cadherin (234, 235). In some cases, EC are directly damaged via pore formation (214, 236) or activation of caspase binding and pyroptosis via NOD2 and NLRP3 pathways (237) or PKC-δ pathways (225).

Histones make up around 70% of NET-associated proteins and themselves have specific actions on ECs (238). They are able to stimulate WPB body release and therefore UL-VWF and Ang-2 secretion (225, 239) with subsequent platelet binding (240). Histones also bind directly to VWF (241), fibrinogen and fibrin (242), creating a prothrombotic scaffold in the vasculature. Endogenous and exogenous heparins will also be compromised by high affinity binding to histones (243).

S100A8A9, also known as calprotectin, is the main NET-bound cytosolic protein (238). Following NETosis, around 25% percent of total cytoplasmic S100A8/A9 remains bound to NETs whereas at least 25% is released as soluble protein (244). It is also deposited in venules via binding to GAGs and activates ECs to increase CAM expression (245), although this can be blocked via exogenous heparin (246). Pre-activation by AGEs may increase the

EC pro-inflammatory response to S100A8A9 (247). S100A8A9 can also directly disorganize actin to increase permeability (248).

Endothelial cell activation and contribution to uncontrolled immunothrombosis

Although immunothrombosis is thought to contribute to the early immune response, disseminated intravascular coagulation (DIC) refers to the activation of coagulation with loss of localization which leads to microvascular fibrin deposition; consumption of clotting factors and platelets and subsequent life-threatening organ dysfunction and haemorrhage (249).

Endothelial dysfunction has been associated with the severity of coagulation and mortality in patients with DIC (250). This is attributed to loss of endogenous anticoagulants (such as protein C, thrombomodulin and antithrombin), due to suppression of synthesis (251) or loss of the glycocalyx; EC expression of TF (252); and reduced levels of the MMP ADAMTS-13 which leads to UL-VWF remaining uncleaved (253). IL-8 and TNF-α also significantly stimulate the release of UL-VWF by ECs, whereas IL-6 inhibits the UL-VWF cleavage by ADAMTS13. The acute phase response also results in EC release of PAI-1 which inhibits tPA (254).

Dysglycaemia and endothelial dysfunction

Under normoglycaemia, glucose transporters (GLUT) and sodium-glucose cotransporters (SGLT) take up glucose into ECs for use primarily in glycolysis, during which glucose is metabolized to pyruvate (255). During hyperglycaemia, increased rates of glycolysis result in excess glycolytic intermediates. These are shunted down glycolytic side branches and

contribute to endothelial dysfunction, either via reducing ROS scavenging (polyol pathway reactions) (256) or forming AGEs which bind RAGEs to activate inflammatory signaling pathways (257, 258). Accumulation of AGEs are also thought to upregulate RAGE expression, which bind and mediate cellular responses to a range of DAMPs, including histones and S100A8A9 (259).

Excess glucose also increases protein kinase C (PKC) activation, leading to reduced eNOS and enhancing oxidative stress (260, 261) and EC extracellular vesicle (EV) release (262, 263). Hyperglycaemia and advanced glycosylation products have also been shown to induce GAGs changes and lead to ESL degradation (264) (265, 266). The resultant endothelial dysfunction is characterized by reduced production of endogenous vasodilators (260), increased expression of inflammatory adhesion molecules (267, 268) and altered permeability (269).

Disruption of the glycocalyx and endothelial dysfunction

Disruption of the glycocalyx can occur in a myriad of ways: Systemic inflammation can result in excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which can activate 'sheddases' such as MMPs and inactivate endogenous protease inhibitors which leads to degradation of the glycocalyx (270, 271). NETosis also releases proteolytic enzymes (272, 273) and heparanases are upregulated at sites of inflammation by ROS (274), cytokines (275), hyperglycaemia (276), AGE products (265) and can be produced by immune cells and platelets (277). The loss of heparan sulfate enhances the susceptibility of the core proteins to proteolytic cleavage by matrix metalloproteinases (278). Heparanase also has non-direct enzymatic activity on ECs by upregulating expression

of enzymes that accelerate syndecan shedding and decrease nuclear syndecan-1 (275) . Other reported factors that can contribute to glycocalyx shedding include blood flow perturbations which increase hyaluronidase expression (271); cardiovascular diseases which cause thinning and altered sulfation patterns and altered NO production (279). Hyperglycaemia also results in marked reduction in the biosynthesis of GAGs, specifically heparan sulfate (264).

Shedding of the glycocalyx can further perpetuate systemic inflammation due to GAGs directly stimulating pro-inflammatory TLR-4 signaling (206, 280), as well as activating leukocytes. Glycocalyx degradation also exposes CAM, facilitating leukocyte rolling and adhesion (280-284). An altered endothelial surface layer also increases vascular permeability by changing Starling's forces (285, 286). The effects on coagulation are varied with glycocalyx components able to trigger thrombin generation and activation of protein C, alongside loss of anticoagulants ATIII, heparan cofactor II and thrombomodulin which culminates in excess coagulation and hyperfibrinolysis (287, 288). Heparanase also up-regulates and enhances TF and interacts with TFPI on the surface of ECs, leading to increased factor Xa production (289). There is also impaired mechanosensing due to inhibition of NO synthesis (10, 290) and impaired NO synthesis also increases WPB exocytosis and VWF release (18, 291) which further exacerbates hypercoagulability.

1.4 Specific endothelial dysfunction phenotypes: bacterial sepsis and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and ARDS

Sepsis

Sepsis is currently defined by the 2016 Sepsis 3.0 criteria as life-threatening organ dysfunction attributed to a dysregulated host immune response. Organ dysfunction is predominantly scored using the Sequential Organ Failure Assessment (SOFA) which assigns a score of 0-4 to the respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems (292). A score of 2 or more is associated with an in-hospital mortality of greater than 10%. Septic shock as defined sepsis with a serum lactate greater than 2mmol/L in the absence of hypovolaemia with vasopressors being required to maintain a mean arterial blood pressure of 65mmHg. This is associated with mortality rates greater than 40% (292).

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

In December 2019, a novel pneumonia condition emerged, termed coronavirus disease 2019 (COVID-19). This was a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), caused by a single-stranded RNA-enveloped virus from the β coronavirus family (293, 294).

Both sepsis and SARS-CoV-2 have been associated with systemic endothelial activation, coagulopathy and endothelial barrier dysfunction as summarized in table 5:

	Sepsis	SARS-CoV-2
Systemic endothelial activation	Direct activation via PAMPs and indirect activation by CRS, thrombin (295, 296) , complement, NETs and platelets (111-113, 297, 298).	Direct viral infection of ECs via spike or nucleocapsid glycoproteins (18, 299-301) vs solely indirect activation due to CRS (302-304), complement (305),

		NETs (306, 307) or adjacent pericyte damage (308)
Coagulopathy	Increased TF expression leads to thrombin production with secondary thrombocytopenia and consumption of clotting factors, AT and protein C (309). AT is also degraded and production impaired (310). Reduced EC TFPI and t-PA synthesis and increased release of PAI-1 and TAFI results in suppression of fibrinolysis with initially only mild Increases in FDPs (311-313). ADAMTS-13 deficiency increases UL-VWF levels (253, 314) Glycocalyx shedding also displaces TFPI (315).	Severe disease is associated with marked increases in local pulmonary and circulating TF on monocyte and EV-TF (316, 317). High proportion of low-density neutrophils and subsequent NET release contribute to this (318). Increased local pulmonary fibrin deposition with increased EC PAI-1 results in increased FDPs alongside a systemic hypofibrinolytic state (319, 320). This is associated with normal or only mild changes to platelet count and antithrombin (321, 322). VWF antigen and FVIII are also increased (41, 323, 324).
Barrier dysfunction	AJ disruption due to complement (160, 161), thrombin(325, 326), CRS (327), angiopoietin imbalance (328), MMPs (329) and NO (330). Glycocalyx shedding due to MMPs, heparanases (331, 332).	AJ disruption due to complement, CRS and angiopoietin imbalance (330, 333, 334). Glycocalyx shedding due to MMPs and heparanases (334, 335)

Table 5. Comparison of endothelial activation, coagulopathy and barrier dysfunction in sepsis and SARS-CoV-19 infection

ADAMTS-13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13); AT: Antithrombin; CRS: Cytokine Release Syndrome; EC: Endothelial cell; EV – extracellular vesicles; FDPs: Fibrin degradation products; NETS: Neutrophils Extracellular Traps; PAMPs: Pathogen Associated Molecular Patterns; PAI-1: Plasminogen-activator inhibitor-1; MMPs: Matrix Metalloproteinases; NO: Nitric oxide; TAFI: Thrombin activatable Fibrinolysis Inhibitor; TF: Tissue Factor; TFPI: Tissue Factor Pathway Inhibitor; UL-VWF: Ultra-large Von Willebrand's Factor

Sepsis-induced coagulopathy (SIC) and disseminated intravascular coagulation (DIC)

The sepsis-induced coagulopathy (SIC) scoring system was introduced in 2019 to detect the compensated phase of DIC in sepsis, which can lead to overt DIC with disease progression (336). The SIC score uses platelet count, PT ratio and SOFA score whereas the DIC score uses platelet count, PT, fibrinogen and fibrin degradation products (FDPs). The progression of impaired fibrinolysis is a key feature of the systemic microvascular thrombosis seen in DIC (337). Rapidly progressive DIC can led to hypocoagulability and bleeding due to consumption of platelets and clotting factors (338). DIC itself is a strong predictor of mortality in sepsis, independent of sepsis severity (339).

Sepsis, COVID-19, and acute respiratory distress syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) is a recognized complication of sepsis and SARS-CoV-2 infection associated with high mortality rates (34-62%) (340-342). Most severely affected COVID-19 patients meet the Sepsis 3.0 criteria and ARDS is the most frequent organ dysfunction reported (88%) (343). Direct (insult to lung epithelium) or indirect injury (systemic endothelial damage due to inflammatory mediators) results in increased permeability, protein rich oedema and diffuse alveolar infiltrates with subsequent loss of aerated lung tissue and severe hypoxaemia (344). However, COVID-19 associated ARDS also features a prominent alveolar thrombosis with excessive fibro-proliferative lung tissue remodeling and a degree of hypoxaemia that is not necessarily proportional to changes in lung mechanics or radiographic changes (345, 346). Patients with ARDS usually require mechanical ventilation and these patients are at increased risk of acquiring potentially fatal secondary bacterial infections (347-349).

1.5 Hypothesis and aims.

Hypotheses:

Endothelial cell activation (expression of cell-surface adhesion and coagulation molecules and increased junctional permeability) is indirectly triggered and augmented by endogenous DAMPs, such as those released during netosis and those occurring secondary to viral and bacterial PAMP exposure. Endothelial modulation due to hyperglycaemia or glycocalyx disruption further augments endothelial cell activation secondary to DAMPS.

Aims:

- Measure markers associated with endothelial cell activation in patients hospitalised with sepsis and COVID-19.
- Quantify changes in heparan and vWF expression on HUVEC monolayers exposed to plasma from patients hospitalised with COVID-19 and sepsis.
- Determine if COVID-19 spike and nucleocapsid glycoproteins can directly activate endothelial cells or complement.
- Measure markers of endothelial activation in response to NETosis components S100A8A9 and histones following modulation by hyperglycaemia and heparanase / hyaluronidase.
- Measure markers of EC activation in response to NETosis components S100A8A9 and histones following exposure to healthy plasma and plasma from patients hospitalised with sepsis of different severities.

Chapter 2. Material and Methods:

2.1 Collection of Human blood

Venous blood was drawn from healthy, consenting, drug-free volunteers using a 25-gauge butterfly needle into 3.2% trisodium citrate BD (Becton Dickinson, Oxford, UK). BD Vacutainer EDTA and Lithium heparin blood collection tubes were used. Ethical approval for use of healthy human blood was granted by the University of Birmingham Research Ethics Committee to the Birmingham Platelet Group (ERN_11-0175). Plasma from patients with COVID-19 were collected through the Catalyst study. This trial was approved by the East Midlands Nottingham 2 Research Ethics Committee (20/EM/0115)(350). Blood from ventilated sepsis patients with and without ARDS was collated by Dr Rahul Mahida at the Queen Elizabeth hospital (REC 16/WA/0169).

2.2 Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs; Promocell) were cultured in a humidified incubator at 5% CO2 and 37°C using Endothelial Cell Growth Media (ECGM 2; Promocell) with 1% penicillin-streptomycin. Cells were used up to passage 6. All cells were passaged or detached for experiments using 0.04 % Trypsin / 0.03 % EDTA (Promocell) and trypsin was subsequently neutralized using an equivalent volume of Trypsin Neutralizing Solution (0.05 % trypsin inhibitor, 0.1 % BSA; Promocell). Cells were plated in either µ-Slide 18-well slides (Ibidi) at a density of 20,000 cells or 24-well plate at a density of 70,000 for 24 hours in ECGM2 before application of any endothelial agonists. All experiments included at least one condition cultured in ECGM 2 (Promocell) or reduced serum media (Opti-MEM, Thermofisher Scientific) as a negative control and an agonist known to stimulate endothelial cells as a positive control (see table 6).

	Action	Concentration	Time	Source
Positive controls for endothelial cell activation				
Histamine	Binds to H-1 receptor inducing eNOS (351), upregulation of adhesion receptors (352) (353) and coagulation and anti-coagulant factor expression (354) (355) and inducing endothelial barrier disruption (353, 356, 357)	50μM	24 hours	Sigma
Interleukin-1β	Binds to IL-1 β receptors to downregulate eNOS (358) and upregulate adhesion receptor and coagulation factor expression and cytokines and cytokine receptor expression (359, 360) and induce endothelial barrier disruption (361)	20ng/ml	24 hours	Peprotech
TNF-α	Binds to TNF-α receptors to induce either apoptosis (362), activation of eNOS (363) or upregulation of adhesion receptors (364) or coagulation factors (365){Esmon, 2003 #2532} and induce endothelial barrier disruption (366, 367)	10ng/ml	24 hours	Peprotech
TGF-β	Acts on TGF-β type I or II receptors and can inhibit leukocyte adhesion and activate eNOS (368). Possible role in priming EC to effects of TNF- α and other cytokines (369-371)	10ng/ml	24 hours	Peprotech
Endothelial cell modulators				
(D-)Glucose anhydrous	Taken up by endothelial GLUT and SGLT primarily for glycolysis. Hyperglycaemia can lead to excess glycolytic intermediates and endothelial dysfunction (255).	30mM	24 hours pre-treatment prior to other agonists	Fisher Scientific
(L-)Glucose anhydrous	Iso-osmotic control for D-Glucose. Taken up by endothelial GLUT and SGLT but unable to undergo glycolysis (372).	30mM	24 hours pre-treatment prior to other agonists	Sigma-Aldrich
Heparanase I	Cleaves heparin and HS at the linkages between hexosamines and O-sulfated iduronic acids, disrupting the glycocalyx (373).	10U/ml	Applied in Tris buffer 1 hour on shaker prior to other agonists	Sigma-Aldrich
Heparanase III	Cleaves HS at the linkages between hexosamine and glucuronic acid residues, disrupting the glycocalyx (373).	1U/ml	Applied in Tris buffer 1 hour on shaker prior to other agonists	Sigma-Aldrich
Hyaluronidase	Degrade HA into HA fragments by hydrolyzing the disaccharides at hexosaminidic β (1 to 4) linkages, disrupting the glycocalyx (205).	10U/ml	1 hour on shaker prior to other agonists	Sigma-Aldrich
Endothelial cell agonists				
COVID Spike protein	Binds to ACE2 on the host cell mediating viral cell entry (374).	100nM	24 hours	Professor Max Crispin
COVID Nucleocapsid protein	Protein responsible for viral replication, transcription, and virion assembly (375, 376).	100nM	24 hours	Dr Adam Cunningham
Histone 4	Endothelial dysfunction via direct pore-formation (214, 236) and EC PRR binding to upregulate adhesion receptors and coagulation factor expression and induce endothelial barrier dysfunction (111).	10μl/ml	24 hours	Abcam
Histone 3	As above.	10μl/ml	24 hours	New England Labs
S100 A8A9	EC PRR binding to induce transcription of pro-inflammatory cytokines and EC PRR binding to upregulate adhesion receptors and coagulation factor expression and induce endothelial barrier dysfunction (247, 248, 377-379).	20μl/ml	24 hours	PuriFiC 40-46
Plasma from patients hospitalised in ICU with COVID-19 or sepsis (with and without ARDS)	Plasma containing DAMPs to replicate the endothelial environment in systemic inflammatory conditions	10%	24 hours	See 'Collection of Human Blood'

Table 6. Mechanisms of actions of endothelial cell agonists

ACE2: Angiotensin converting enzyme 2; ARDS: acute respiratory distress syndrome; DAMPs: Danger Associated Molecule Patterns; EC: Endothelial cell; eNOS: endothelial nitric oxide synthase; GLUT: Glucose Transporter; HA: hyaluronic acid; HS: heparan sulfate; PRR: Pattern Recognition Receptors; SGLT: Sodium-glucose co-transporter.

2.3 HUVEC pre-treatment with D- and L- Glucose and / or heparanase and hyaluronidase

For conditions utilising HUVECs modulated with D- or L-Glucose, conditions were made up to 30mM in ECGM2 for 24 hours. Wells were then washed and replaced with reduced serum media (Opti-MEM, Thermofisher Scientific) before addition of further agonists.

For conditions utilising heparanase or hyaluronidase, ECGM2 was aspirated, and wells washed with reduced serum media (Opti-MEM, Thermofisher Scientific) before replacing with 37^{o}C Tris-HCl buffer and application of enzyme for 1 hour on a shaker. Wells were then washed and replaced with reduced serum media (Opti-MEM, Thermofisher Scientific) before addition of further agonists.

2.4 HUVEC activation with COVID proteins, histones and recombinant S100A8/A9

For experiments utilising COVID proteins, histones and S100A8/A9 as agonists, ECGM2 and any prior modulators were aspirated and wells washed and replaced with a reduced serum media (Opti-MEM, Thermofisher Scientific or Medium 199, Thermofisher Scientific containing 0.5% bovine serum Albumin (BSA)) before addition of agonist.

2.5 HUVEC treatment with patients' plasma

To assess the effect of patients' plasma samples on HUVEC activation, 10% plasma was added either in isolation or alongside histone 4 in reduced serum media (Opti-MEM, Thermofisher Scientific) and incubated for 24 hours.

2.6 Complement activation on endothelial cells

For experiments involving detection of complement proteins on HUVECs, wells were washed with PBS (Gibco) with additional 2mM Mg^{+} and Ca^{2+}. Healthy human AB serum containing complement was then added 1:2 with ECGM2 for 1 hour at 37^{o}C. De-complemented AB serum was obtained by heat inactivation of the serum at 56°C in a water bath for 60 minutes and the decomplemented serum used as negative control. Wells were then washed with PBS (Gibco) without Mg^{+} and Ca^{2+}. Non-enzymatic detachment was performed using 5% EDTA and 5mg/ml Lidocaine followed by aspiration using a pipette set to 200μl.

2.7 Endothelial Cell activation by Flow Cytometry

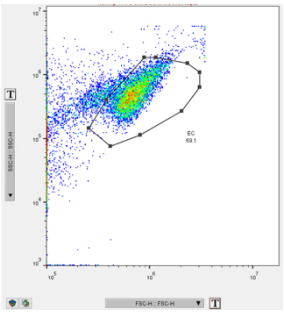
HUVECs were detached and transferred to round bottom 96-well plates and pelleted by centrifugation for 5 minutes (500xg, acceleration 9, deceleration 9, 22°C). The supernatant was discarded, and the pellet was resuspended in blocking buffer (PBS containing 10% foetal bovine serum [FBS]) and incubated for 20 minutes at 4°C. The plate was centrifuged for 5 minutes (500xg, acceleration 9, deceleration 9, 22°C) and the pellet resuspended in a primary antibody mix for 30 minutes at 4°C. Following incubation, cells were washed in PBS and centrifuged for 5 minutes (500xg, 22°C). The pellet was resuspended in a secondary

antibody mix for 30 minutes at 4°C in the dark and then washed and centrifuged as above. The final pellet was resuspended in 200µl PBS. Endothelial cell activation markers were assessed using a CytoFLEX Flow Cytometer (Beckman). Median fluorescence intensity (MFI) fold change over isotype control and the percentage of positive expression was analysed using FlowJo v10. Fluorescence compensation was calculated using fluorescence beads stained with single colours.

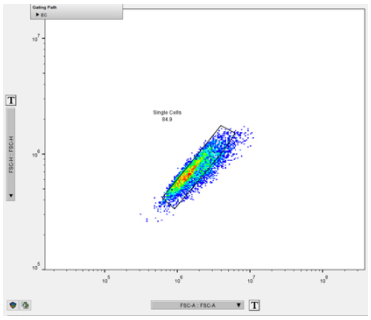
2.8 Gating strategy:

Endothelial cells were identified in a forward scatter area (FSC-A) versus a side scatter area (SSC-A) plot with axes set to logarithmic scale (Figure A). A total of ten thousand events were acquired for all conditions. Single cells were identified based on FSC-A versus forward scatter height (FSC-H) plot (Figure B). Experimental conditions were subsequently gated on single cells (Figure C, D, E).

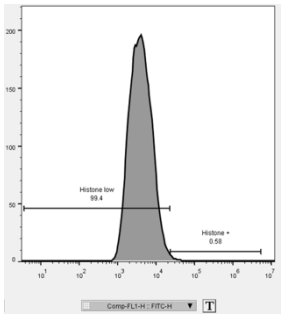
A.



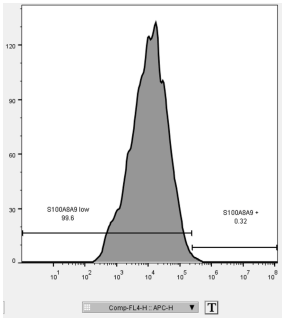
B.



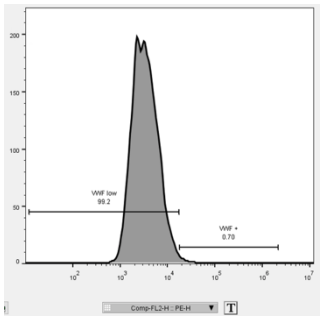
C.



D.



E.



Primary antibody (Clone number)	Host or conjugated fluorophore	Use: working concentration	Source
C3b	APC	1:300	Biolegend
CD142 (Tissue Factor)	PE	1:200	Biolegend
CD54 (I-CAM)	FITC	1:300	Thermofisher
CD62P (P-selectin)	PE Cy5.5	1:200	Thermofisher
CD106 (VCAM)	APC	1:200	Biolegend
Histone 4	Mouse	1:500	Sigma
Heparan	Mouse	1:1000	Amsbio
S100 A8A9	Mouse	1:100	BDBiosciences
Polyclonal vWF	Rabbit	1:500	DAKO

Table 7. Primary antibodies for flow cytometry

Secondary antibody (Clone number)	**Host or conjugated fluorophore**	**Use: working concentration**	**Source**
Anti-rabbit AF568	568 Goat	1:500	Invitrogen
Anti-mouse AF488	488 Goat	1:500-1000	invitrogen
Anti-mouse AF647	AF647 Goat	1:500	Invitrogen
Anti-mouse AF488	AF488 Goat	1:1000	Invitrogen

Table 8. Secondary antibodies for flow cytometry

2.9 Endothelial cell activation detection by immunofluorescence

HUVECs were cultured in µ-Slide 18-well slides (Ibidi) in 180µl of ECGM2. Following application of agonists, half of the supernatant was harvested and kept frozen for ELISA. Adherent HUVECs were then fixed by addition of equivalent volume of 4% Paraformaldehyde (PFA) for 5 minutes, followed by supernatant aspiration and the addition of 4% PFA for an additional 10 minutes. HUVECs were then permeabilized using 0.25% triton in PBS (10 minutes at RT). In some experiments, HUVECs were fixed using 16% Formaldehyde, Methanol-free (Thermo-Fisher) to limit cell permeabilization and 0.25% triton in PBS was not used. Auto-fluorescence was quenched using 20mM ammonium

chloride for 15 minutes at RT. Following washing with PBS Tween 0.05% (PBST), non-specific binding sites were blocked using PBS containing 1% BSA with 5% donkey or goat serum for 1 hour at RT. Cells were washed and then incubated with primary antibody mixture (table X) diluted in blocking solution for 1 hour at 22°C. Cells were then washed three times in PBST for 3 minutes on a rocker. The secondary antibody mixture was prepared in PBS and added for 45 minutes at RT in the dark. Slides were imaged directly using a Zeiss epi microscope (Zeiss) using a 1.49 NA 64 x oil-immersion with at least 5 images taken for each condition. Z-stack images were acquired were necessary.

Images were analysed using Image J Software. Macros to quantify total heparan and total von Willebrand Factor (VWF) staining were constructed using ImageJ.

Primary antibody (Clone number)	Host or conjugated fluorophore	Use: working concentration	Source
CD142 (tissue factor)	PE Biotinylated	1:200 1:100	Biolegend
CD54 (ICAM-1)	FITC	1:100	Thermofisher
CD62P (P-selectin)	PE Cy5.5	1:200	Biolegend
Histone 4	Mouse	1:500	Sigma
Heparan	Mouse	1:100-500	Amsbio
S100 A8A9	Sheep AF 647 Mouse	1:100 1:100	BD Biosciences
Von Willebrand's Factor	Rabbit	1:200-500	Sigma-Aldrich

Table 9. Primary antibodies for immunofluorescence

Secondary antibody (Clone number)	**Host or conjugated fluorophore**	**Use: working concentration**	**Source**
Anti-biotin 568	Streptavidin	1:500	Invitrogen
Anti-mouse 405	Goat	1:500	Invitrogen
Anti-mouse 488	Goat	1:500-1000	Invitrogen
Anti-rabbit 568	Goat	1:500	Invitrogen
Anti-rabbit AF647	Goat	1:500	Invitrogen

Anti-sheep 488	Donkey	1:500	Life technologies
Anti-sheep 568	Goat	1:500	Invitrogen
Calcein (platelets)	-	5mcg/ml	Thermofisher
DAPI / Hoescht 33342	-	1:10,000	Life technologies

Table 10. Secondary antibodies and stains for immunofluorescence

2.10 Detection of chemokines and cytokines by Luminex and ELISA

Cytokines, chemokines, and thrombotic markers were measured by Luminex following manufacture instructions. S100A8/A9 was detected by ELISA using a commercially available kit: Duo Set hu S100A8/A9 cat DY8226-05.

2.11 Statistical Analysis

Luminex data, flow cytometry (MFI fold change and percentage of positive expression) and ELISA data are presented as mean ±SD for patient groups using Prism 8 (GraphPad Software Inc, USA). Median total heparan and VWF IF staining across 5 images was obtained for each condition from Image J Software and the overall mean ±SD presented for each patient group using Prism 8. Statistical significance was represented by stars: *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$ ****<0.0001.

Chapter 3. Results

3.1 Endothelial cell activation markers are increased in patients hospitalized with sepsis and COVID-19

Endothelial dysfunction is well established in patients with sepsis and it has also been reported in patients with COVID-19 (302, 380). In this experiment, we compared endothelial activation markers from within the first 24 hours of admission in a cohort of patients with COVID-19 recruited from wards and from ICU and compared these to patients with sepsis. Using the Luminex assay, we compared soluble markers of endothelial cell activation (CAM, procoagulant molecules and markers of endothelial permeability) and inflammatory markers that are known to activate endothelial cells between these three cohorts and an age-matched control group.

3.1.A Soluble endothelial cell adhesion molecules are increased in patients with sepsis and severe COVID-19

We first assessed the levels of CAM, including endoglin, E-selectin, P-selectin, ICAM-1, VCAM-1. Compared to age-matched controls, plasma from patients with COVID-19 in ICU and with sepsis had increased levels of soluble E-selectin, P-selectin, ICAM-1, VCAM-1 and endoglin (Figure 3. A-E). In contrast, plasma from patients with COVID-19 on wards had increased levels of VCAM-1 and endoglin, suggesting an increase in mediators of firm leukocyte adhesion (Figure 3. D and E).

This data confirms that endothelial CAM are increased in patients with sepsis and in patients with COVID-19 admitted to ICU.

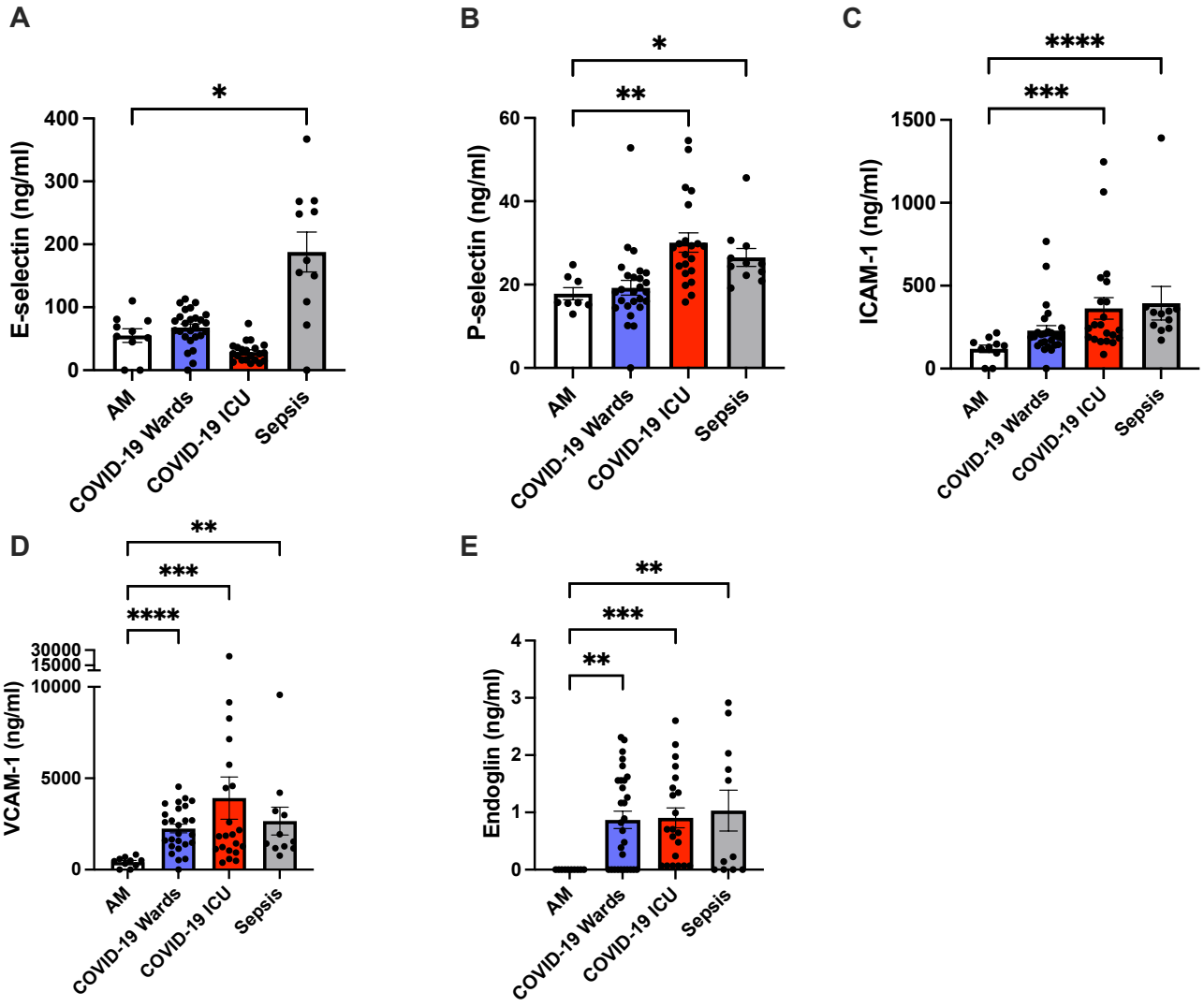


Figure 3. Soluble endothelial cell adhesion molecules are increased in patients with sepsis and severe COVID-19

Soluble CAM (A) E-selectin, (B) P-selectin, (C) ICAM-1, (D) VCAM-1 and (E) endoglin were quantified using the Luminex assay in patients with sepsis and COVID-19 hospitalized in wards and ICU within 24h of admission and compared to aged-matched (AM) control. The statistical difference between AM and multiple groups was analysed using a paired one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05 **p < 0.01 ***p < 0.001.

3.1.B Soluble procoagulant and prothrombotic markers are increased in patients with sepsis and severe COVID-19

We next assessed the levels of soluble TF and VWF (as a marker of WPB release). Plasma from patients with COVID-19 in wards and ICU and with sepsis had increased levels of soluble TF (Figure 4-A) and VWF (Figure 4-B) compared to age-matched controls. Interestingly, the levels of TF and VWF were similar between patients with COVID-19 on ward and in ICU, suggesting that increased endothelial coagulopathy may be not associated with clinical severity scoring. Moreover, TF levels were even higher in patients with sepsis, suggesting a greater or more systemic activation of ECs and release of thrombotic markers in these patients.

Despite a significant increase in VWF, we did not observe a significant change in the levels of ADAMTS13, the MMP that cleaves VWF and reduces its thrombotic effect (Figure 4- B and C). We also did not observe a difference in the levels of the anticoagulant protein C.

These results show an increase in endothelial prothrombotic markers in COVID-19 patients irrespective of severity and without corresponding decreases in anticoagulants. Patients with sepsis have an even greater increase in prothrombotic markers.

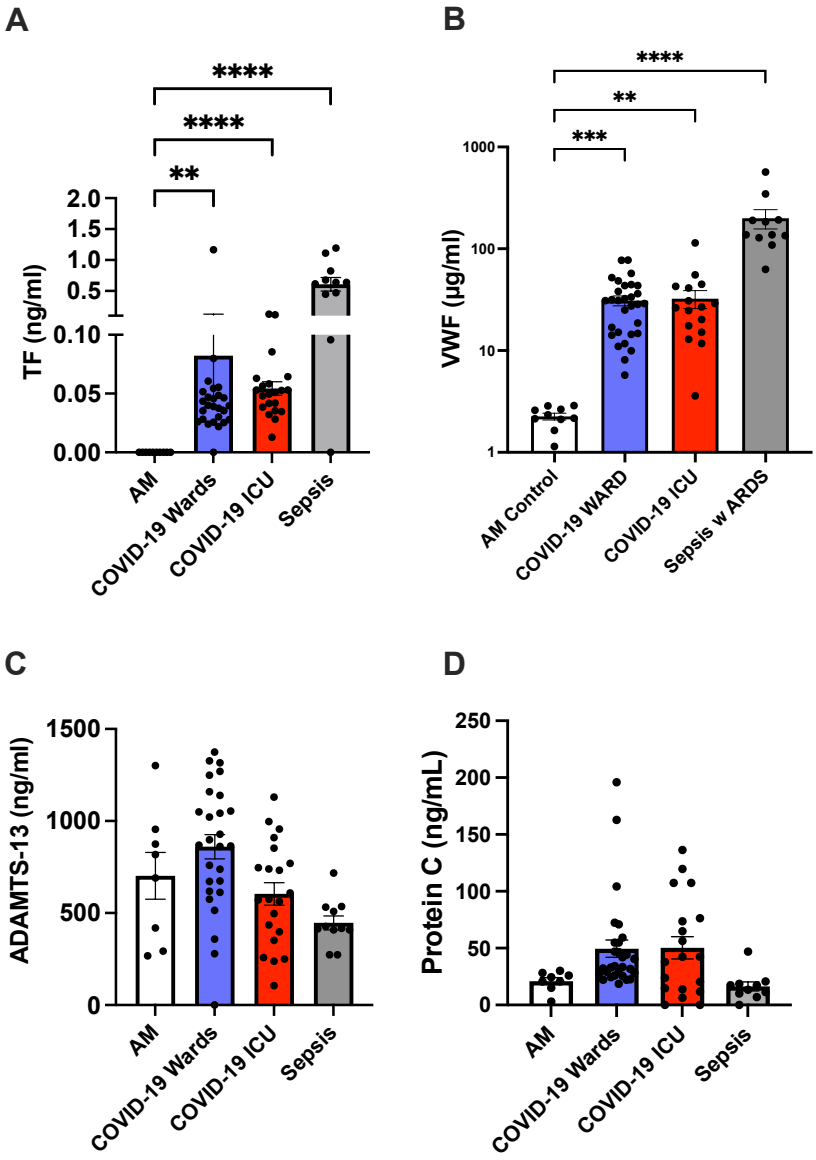


Figure 4. Soluble endothelial prothrombotic markers are increased in patients with sepsis and COVID-19 without corresponding decrease in anticoagulant markers

Soluble procoagulant molecules were quantified using the Luminex assay in patients with COVID-19 hospitalized in wards and ICU and with sepsis using (A) TF and (B) VWF, (C) ADAMTS13 and (D) protein C. The statistical difference between multiple groups was

analysed using a paired one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05 **p < 0.01 ***p < 0.001.

3.1.C. Endothelial permeability markers are increased in patients with sepsis and severe COVID-19

We next assessed EC permeability by measuring the levels of angiopoietin-1 and 2 and the levels of endothelial junctional protein N-cadherin. An impairment in endothelial permeability is associated with a decrease in the levels of angiopoietin-1 and an increase in the levels of angiopoietin-2. Our results show that plasma from patients with sepsis but not COVID-19 had decreased angiopoietin-1 (Figure 5-A) compared to age-matched controls. Patients with sepsis and COVID-19 in ICU but not in wards had increased angiopoietin-2 (Figure 5-B) compared to age-matched controls. There was no change in N-cadherin in any cohort. This data suggests a significant increase in endothelial permeability in patients with sepsis, and this effect was also observed in patients with COVID-19 in ICU, albeit less pronounced.

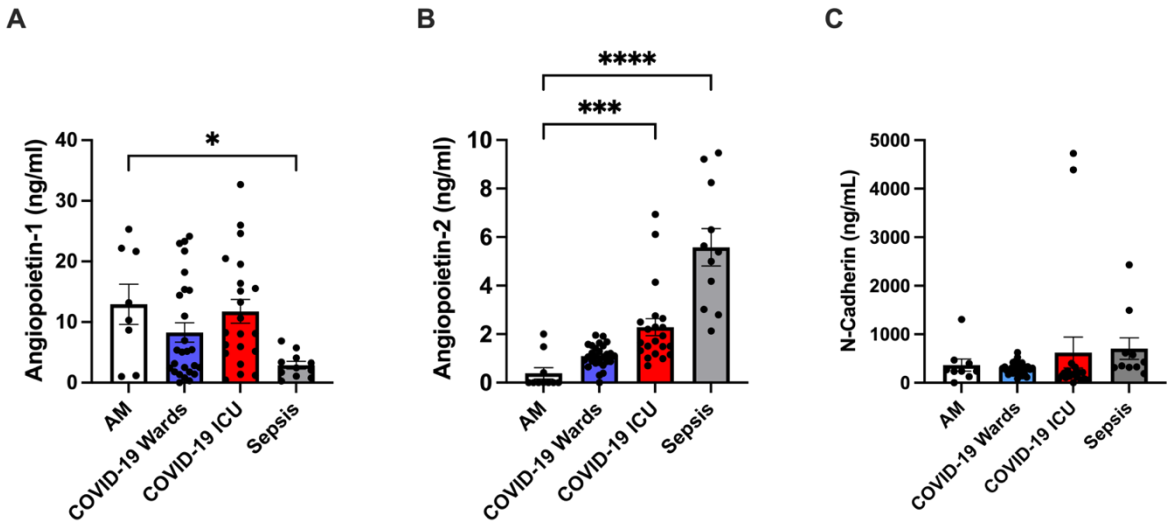


Figure 5. Increased endothelial permeability in patients with sepsis and severe COVID-19

Soluble mediators of EC barrier function (A) angiopoitein-1, (B) angiopeitin-2 and (C) N-cadherin were quantified using the Luminex assay in patients with COVID-19 hospitalized in wards and ICU and with sepsis. The statistical difference between AM and multiple groups was analysed using a paired one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05 **p < 0.01 ***p < 0.001.

3.1.D Markers of complement activation and endothelial cell injury are increased in the plasma of patients with sepsis and severe COVID-19

We next assessed the levels of complement C5a as a marker of complement activation as well as lipocalin-2 and RAGE as markers for kidney and lung injury, respectively. Plasma from patients with COVID-19 (on ward and in ICU) and with sepsis had increased levels of complement C5a compared to age-matched controls (Figure 6-A). Patients with sepsis had increased plasma Lipocalin-2 and RAGE suggesting significant EC injury in patients with sepsis (Figure 6-B and C). Patients with COVID-19 in ICU had increased Lipocalin-2 (Figure 6-B) but the levels of RAGE were not significantly different, despite an increase in the levels of some patients. These data show that complement activation and organ damage occur in patients with COVID-19 and sepsis and these markers increase with disease severity.

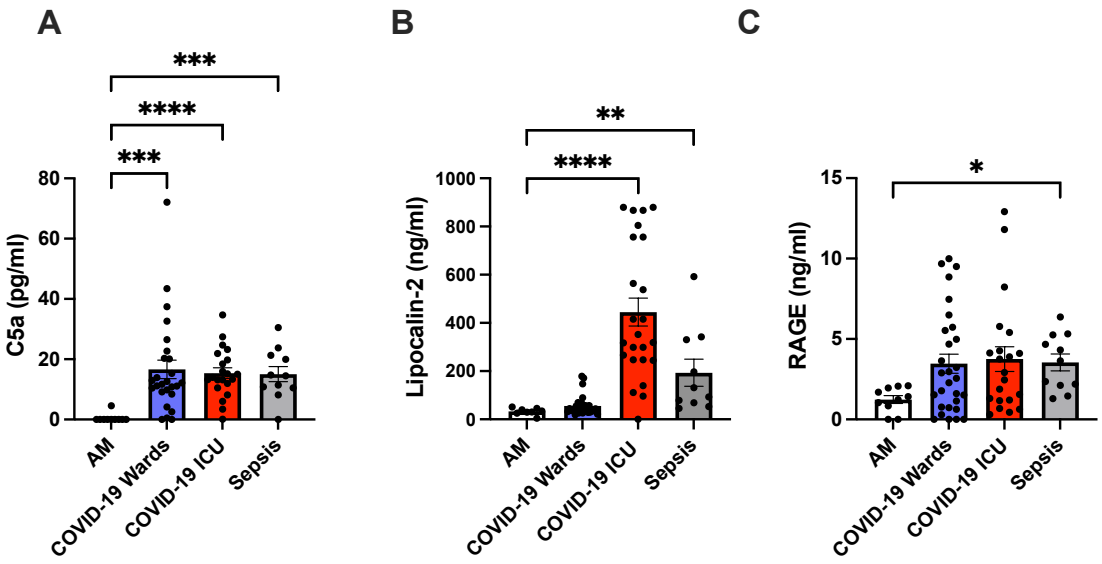


Figure 6. Increased complement activation and lung and kidney damage markers and in patients with sepsis and COVID-19

(A) complement C5a, (B), lipocalin-2 and (C) (RAGE) s were quantified using the Luminex assay in patients with COVID-19 hospitalized in wards and ICU and with sepsis. The statistical difference between multiple groups was analysed using a paired one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05 **p < 0.01 ***p < 0.001.

3.1.E NETosis-associated DAMPs are increased in patients with sepsis and COVID-19

Finally, we assessed the levels of markers released during NETosis. Patients with COVID-19 in ICU had increased Histone 4 but this was not seen in patients on wards or in patients with sepsis (Figure 7-A). The absence of histone H4 in patients with sepsis could be related to the anti-coagulant used. Blood from some patients with COVID-19 on wards were collected on heparin as anticoagulant and this was the case for all plasma from patients with sepsis whereas AM and COVID-19 ICU patients were collected on EDTA. The high affinity binding of heparin to histone might have compromised this assay. Plasma from

patients with COVID-19 (on ward and in ICU) and with sepsis had increased S100A8/A9 compared to age-matched controls (Figure 7-B). This data shows that patients with COVID-19 in ICU have significant levels of NET-associated DAMPS, which can contribute to EC activation and thrombotic complications.

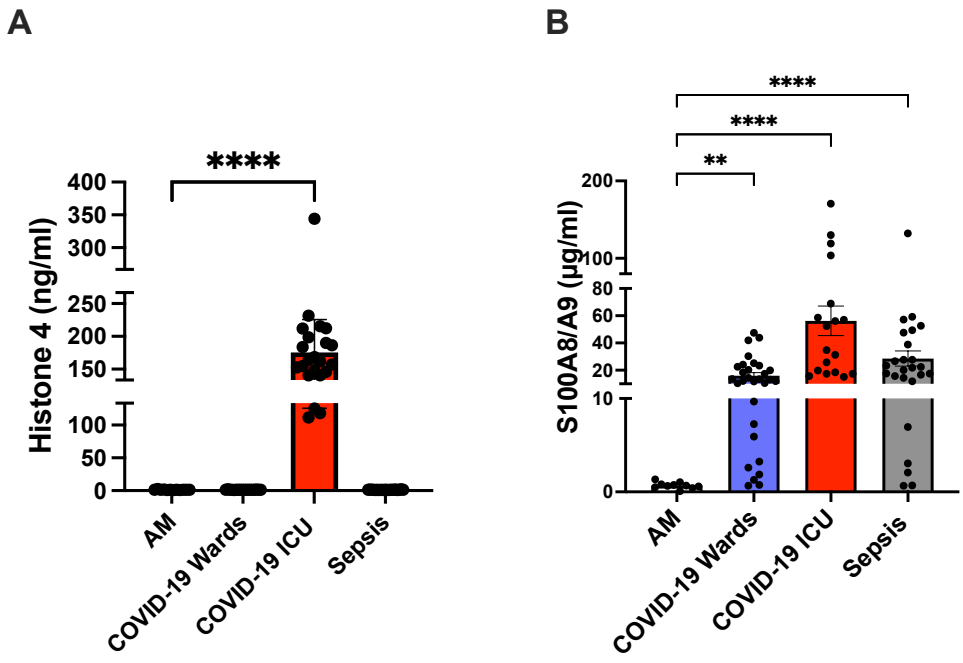


Figure 7. Increased levels of Histone 4 and S100A8/A9 in patients with severe COVID-19

Soluble NET-associated DAMPs (A) histone H4 and (B) S100A8/A9 were quantified using the Luminex assay in patients with COVID-19 hospitalized in wards and ICU and with sepsis. The statistical difference between AM and multiple groups was analysed using a paired one-way ANOVA with Tukey's multiple comparisons test, *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$.

In summary, measurement of different EC inflammatory and thrombotic markers shows evidence of EC activation associated with immune cell recruitment, thrombosis and increased junctional permeability in patients with COVID-19 and with sepsis. This was

greater in COVID-19 patients in ICU compared to COVID-19 patients on wards. Key DAMPs associated with NETosis (histone 4 and S100A8/A9) were both increased in COVID-19 patients in ICU whereas S100A8/A9 was increased in the COVID-19 wards and sepsis population.

3.2 Plasma from patients with sepsis and COVID-19 differentially alters heparan sulfate and VWF expression on HUVECs

To further explore the mechanisms of EC activation in patients with COVID-19 and sepsis, we assessed the effect of plasma from patients with COVID-19 in ICU and on wards and from patients with sepsis on HS and VWF expression using HUVECs. HUVEC monolayers were cultured with 10% patient plasma alongside healthy control and age-matched plasma for 24 hours. Heparan sulfate and VWF expression was assessed by immunofluorescence. We quantified the average median of fluorescence of HS as a marker of glycocalyx degradation/increase synthesis of the proteoglycans and the average median of fluorescence for VWF in WPB and on the surface of ECs as a marker of EC activation.

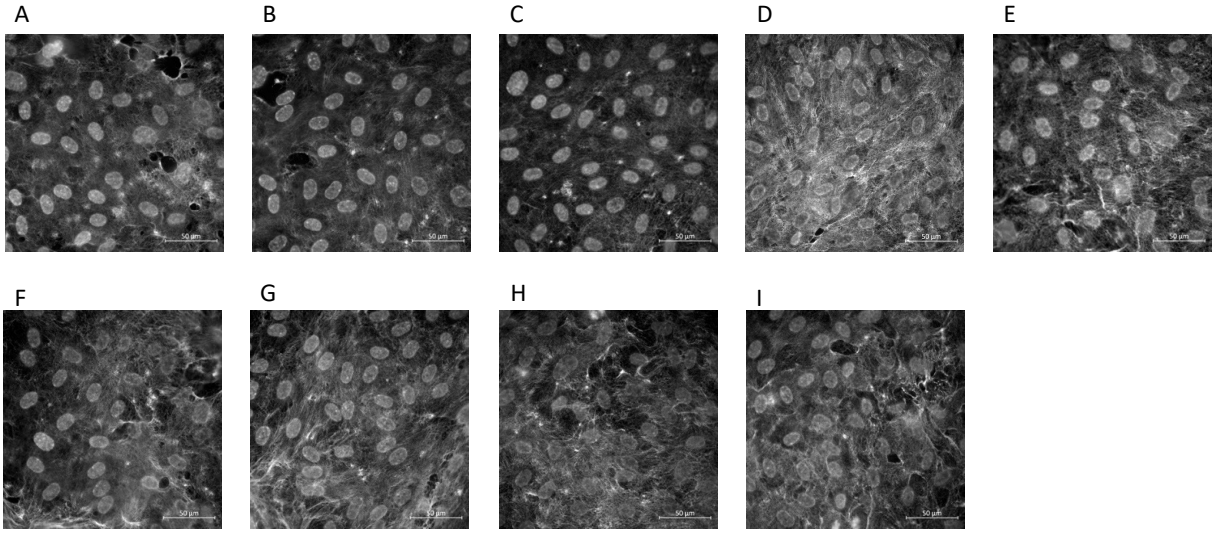
3.2.A Plasma from patients with sepsis and COVID-19 differentially alter heparan sulphate expression on HUVECs

We first assessed the levels of HS on HUVECs following addition of plasma from COVID-19 patients and sepsis and compared to AM controls. Our data showed a large variation in HS expression on HUVEC monolayers cultured with plasma from COVID-19 patients (figure 8 -

C-F). Whereas some plasma showed an increase in fluorescence compared to control (Figure 8 - D - COVID-19 ICU patient) suggesting an increase in HS synthesis, plasma from other patients showed a decrease in the fluorescence of HS suggesting HS degradation (Figure 8 - E and F - COVID-19 ICU patients). However, overall mean immunofluorescence did not differ from healthy and age-matched conditions (Figure 8 – J). When fluorescence was reduced, we observed a subjective loss of fine fibre structure with the appearance of thick fibers of HS (figure 8 – E-F) compared to healthy and age-matched plasma conditions. These results show a heterogeneous effect of plasma from patients with COVID-19 in ICU and that this could be regulated by different factors in the plasma.

On the other hand, plasma from the sepsis cohort had a more uniform effect on HS expression, although median immunofluorescence still did not differ from healthy and age-matched conditions (figure 8 – J). However, we again observed a loss of fine fibre structure and the appearance of thick fibres of HS (figure 8– G-I).

Therefore, whereas overall intensity was not altered, plasma from COVID-19 and sepsis patients increased the thickness of HS fibres on HUVECs, possibly regulating immune cell recruitment.



J

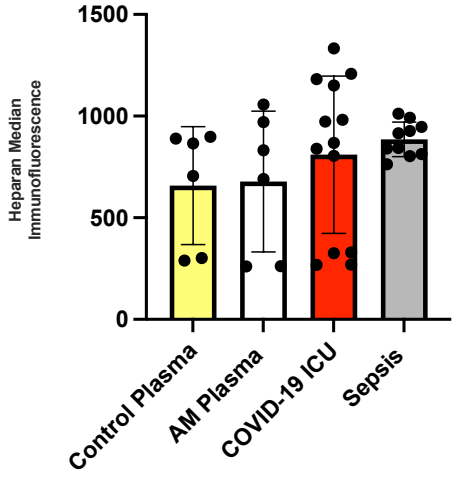


Figure 8. Expression of heparan sulphate on the surface of HUVEC monolayers

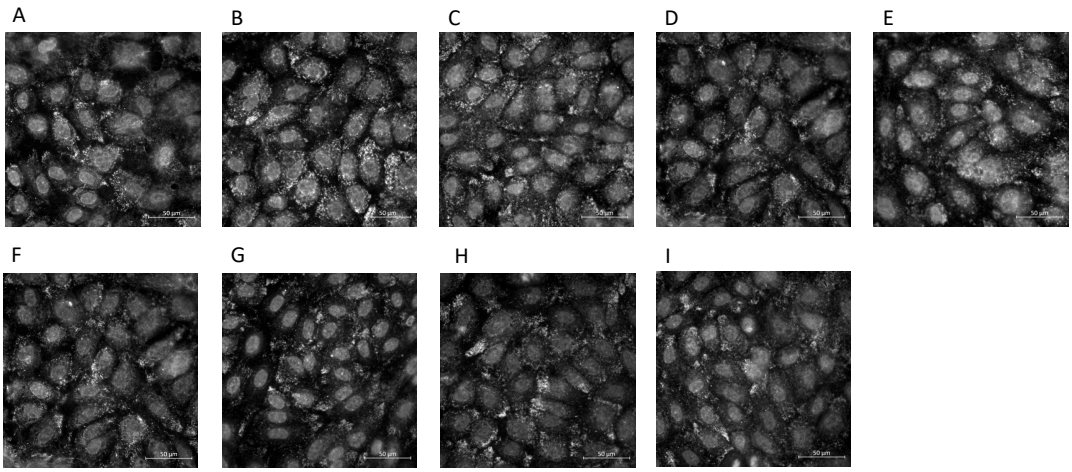
HUVECs were cultured until confluent then incubated with 10% plasma from control (A), age-matched (B), COVID-19 ICU patients (C-F) and sepsis patients (G-I) for 24 hours. Cells were then fixed, permeabilized, auto-fluorescence quenched, and non-specific binding sites blocked before staining for heparan sulfate. Images were captured with Epi

fluorescence microscope at 63x magnification and analysed using ImageJ software (J). Quantification of HS was determined based on 5 images taken across each monolayer and a macro used to calculate the median immunofluorescence for each condition. The statistical difference between control and multiple groups was analysed using a paired one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05 **p < 0.01 ***p < 0.001.

3.2.B Plasma from patients with sepsis and COVID-19 does not alter Von Willebrand expression on HUVECs

We next assessed the expression of VWF on HUVECs following addition of plasma. Our data showed a variable effect on overall VWF expression in HUVEC monolayers cultured with plasma from COVID-19 patients (figure 9 – C-F). Whereas some plasma showed an increase in fluorescence compared to control (Figure 9 - C - COVID-19 ICU patient), plasma from other patients showed a decrease in the fluorescence (Figure 9 - F- COVID-19 ICU patient). However, overall mean immunofluorescence did not differ from healthy and age-matched conditions (figure 9 – J).

Plasma from the sepsis cohort had a more uniform effect on VWF expression and median immunofluorescence was lower compared to the age-matched control (figure 9 – J), possibly suggesting VWF release from ECs (figure 9 -J).



J

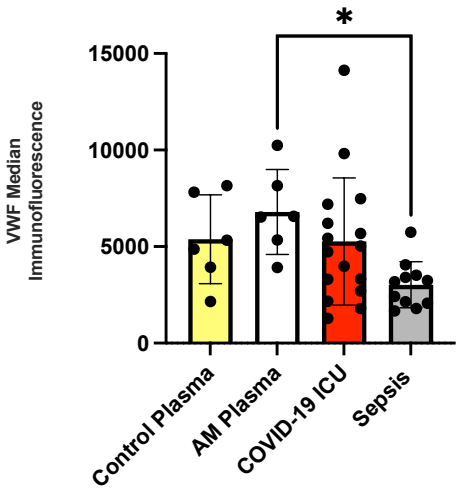


Figure 9. Expression of VWF on HUVEC monolayers

HUVECs were cultured until confluent then incubated with 10% plasma from control (A), age-matched (B), COVID-19 ICU patients (C-F) and sepsis patients (G-I) for 24 hours. Cells were then fixed, permeabilized, auto-fluorescence quenched, and non-specific binding sites blocked before staining for VWF. Images were captured with Epi fluorescence microscope at 63x magnification and analysed using ImageJ software (J). Quantification of

VWF was determined based on 5 images taken across each monolayer and a macro used to calculate the median immunofluorescence for each condition. The statistical difference between control, AM and multiple groups was analysed using a paired one- way ANOVA with Tukey's multiple comparisons test, *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$.

In summary, although overall intensity of HS was not altered, plasma from sepsis and COVID-19 patients increased the thickness of HS fibres on HUVECs, possibly regulating immune cell recruitment. Overall mean immunofluorescence of VWF was decreased in sepsis patients compared to age-matched controls but not in COVID-19 patients.

3.3 SARS-Cov-2 glycoproteins do not directly activate HUVECs or mediate indirect complement activation of HUVECs

Endothelial activation is reported in SARS-CoV-2 infection but controversy still exists as to whether this is due to direct viral infection of ECs or indirect effects of the innate immune system, such as complement pathway activation. Because the spike and nucleocapsid glycoprotein have been reported to directly infect EC (299-301) but also activate complement pathways for indirect activation (305, 381, 382), we compared the levels of EC bound complement and markers of EC activation following activation of HUVECs by spike and nucleocapsid glycoproteins in the presence of decomplemented and non-decomplemented serum. We then also used IL-1β as a positive control to see if priming the HUVECs increased complement deposition or EC activation. We assessed EC complement

deposition and EC activation by flow cytometry using an anti C3b antibody and antibodies against TF, P-selectin and ICAM-1.

3.3.A Culture of HUVECs with SARS-Cov-2 glycoproteins did not directly activate endothelial cells nor provide evidence for complement activation

We first assessed complement deposition and EC activation in the presence of decomplemented and non-decomplemented serum. This data showed that incubation of HUVECs with spike glycoprotein, with or without the addition of the nucleocapsid protein, did not result in direct EC activation as assessed by flow cytometry. This effect was not altered in the presence of non-decomplemented serum, showing that these proteins did not significantly induce complement deposition on EC or led to complement mediated indirect activation of ECs (Figure 10).

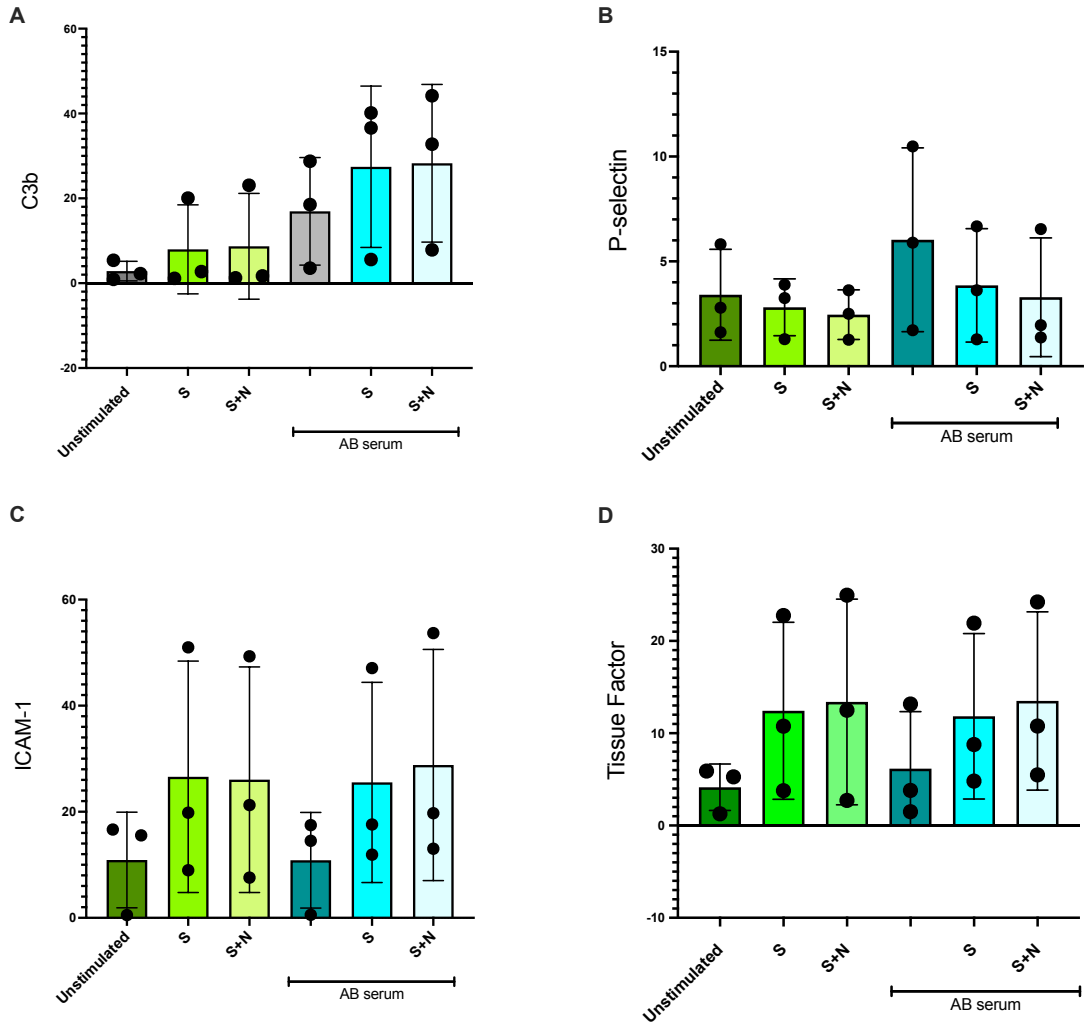


Figure 10. SARS-CoV-2 glycoproteins do not directly or indirectly activate HUVEC monolayers

HUVEC monolayers were cultured until confluent and then incubated with spike (S) protein (100nM) alone or with the addition of nucleocapsid (N) protein (100nM) or 24 hours. Wells were washed then healthy human AB serum containing complement was added 1:2 with ECGM2 for 1 hour at 37^{o}C while decomplemented serum was used in the remaining conditions as a negative control. Non-enzymatic detachment was performed using 5% EDTA and 5mg/ml Lidocaine. HUVEC surface expression of (A) C3b, (B) P-selectin, (C) ICAM_1 and (D) Tissue Factor was assessed by flow cytometry. The statistical significance

between unstimulated and multiple groups was tested using a one-way ANOVA with Tukey's multiple comparisons test.

3.3.B Culture of HUVECs with SARS-Cov-2 glycoproteins and IL-1β does not directly activate endothelial cells nor provide evidence for complement activation

We then assessed complement deposition and EC activation in the presence of decomplemented and non-decomplemented serum with the addition of IL-1β as a positive control. This data showed that incubation of HUVECs with the addition of IL-1β still did not lead to direct EC activation or complement deposition and indirect EC activation (Figure 11).

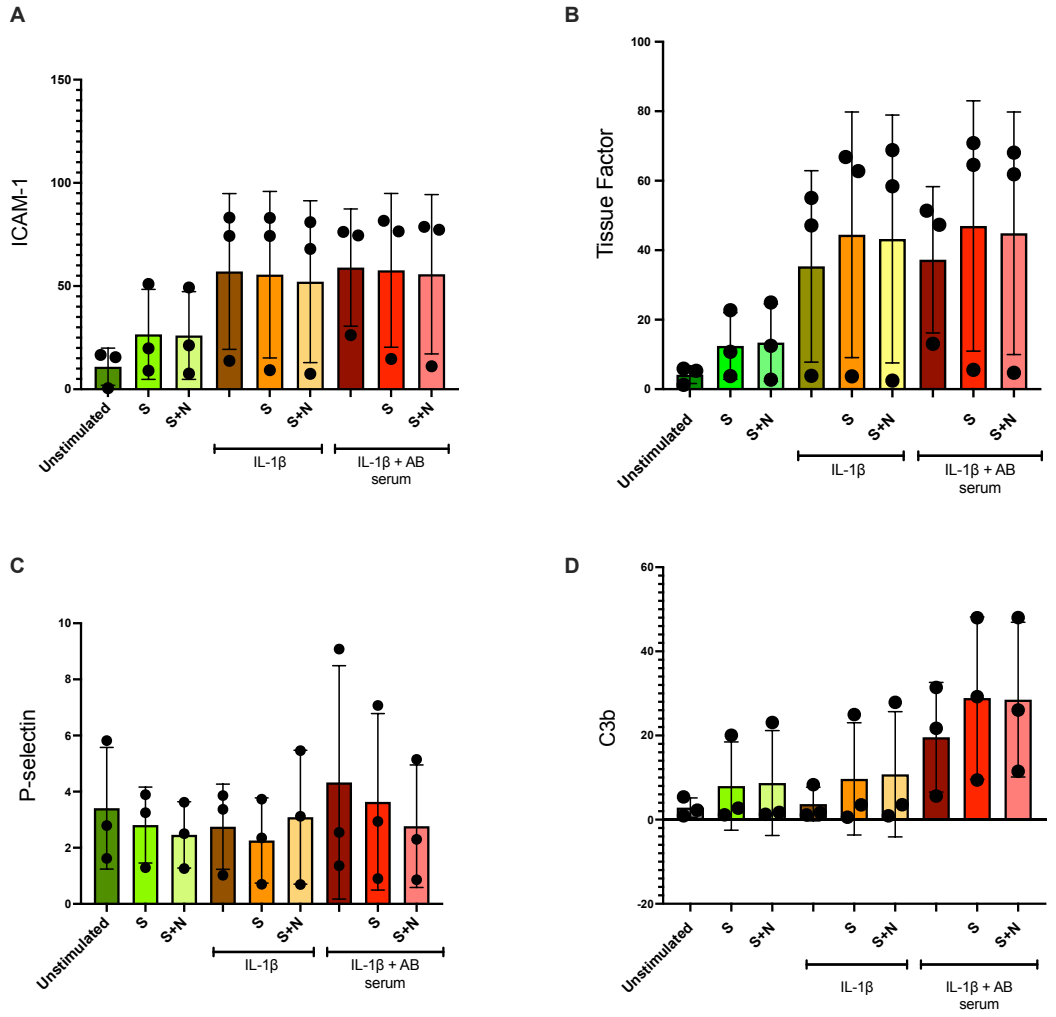


Figure 11. The addition of IL-1β to SARS-CoV-2 glycoproteins neither directly nor indirectly activates HUVEC monolayers

HUVEC monolayers were cultured until confluent and then incubated with spike (S) protein (100nM) alone or with the addition of the nucleocapsid (N) protein (100nM) and/or IL-1β (20ng/ml) for 24 hours. Wells were washed then healthy human AB serum containing complement added 1:2 with ECGM2 for 1 hour at 37°C while decomplemented serum used in the remaining conditions as a negative control. Non-enzymatic detachment was performed using 5% EDTA and 5mg/ml Lidocaine. HUVEC surface expression of (A) ICAM,

(B) TF, (C) P-selectin and (D) C3b was assessed by flow cytometry. The statistical significance between unstimulated and multiple groups was tested using a one-way ANOVA with Tukey's multiple comparisons test.

In summary, incubating HUVECs with spike and nucleocapsid glycoproteins resulted in no evidence of direct EC activation. There was also no evidence of complement activation and indirect activation of ECs. The addition of IL-1β did not change this.

3.4 Hyperglycaemia increases histone 4 binding to endothelial cells without additional amplification of endothelial activation

Given hyperglycaemia has been shown to induce endothelial dysfunction and diabetics who contract COVID-19 are at risk of progression and worse clinical outcome (383). in this study we investigated whether D-glucose modulation of HUVECs for 24 hours to mimic clinical hyperglycaemia resulted in independent EC activation or an increase in activation when HUVEC monolayers were subsequently stimulated by NETosis components Histone 4 and S100A8A9 to mimic activation of the innate immune system. We assessed EC binding of histone 4 and S100A8A9 and markers of EC activation or dysfunction using flow cytometry and antibodies against histone 4, S100A8A9, VWF, heparan, TF, P-selectin and ICAM-1. L-Glucose was used as an osmotic control for D-Glucose. Representative images for the gating strategy for unstimulated and histone 4 stimulated ECs is given in figure 12.

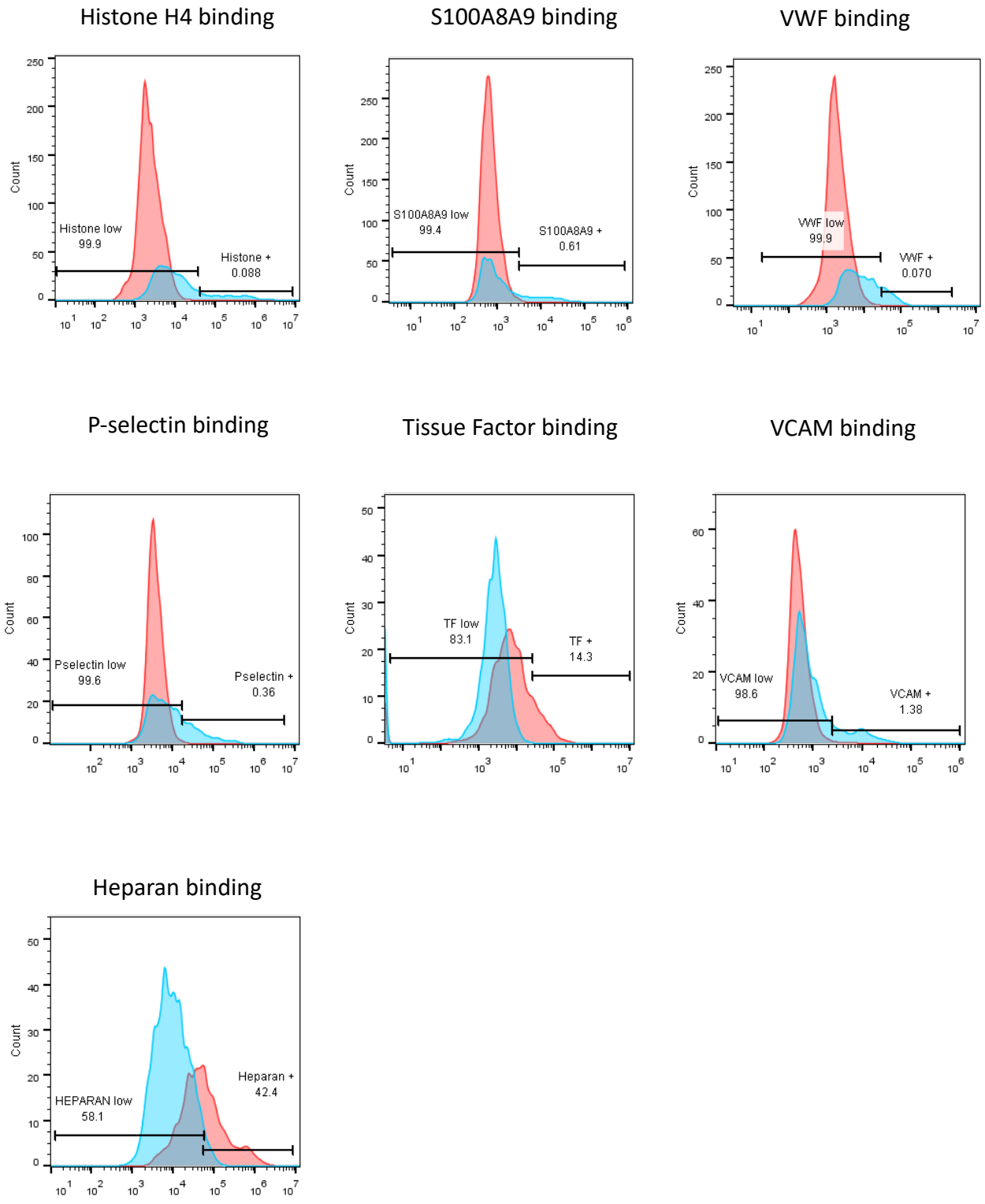
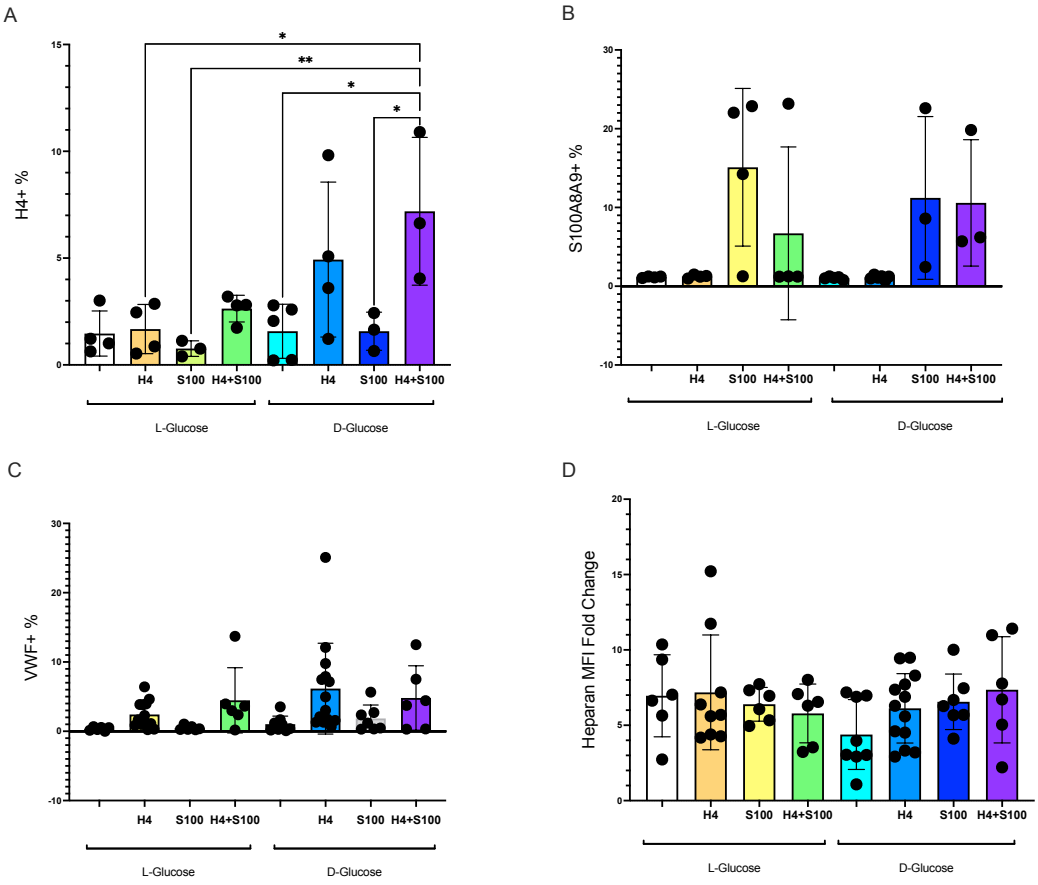


Figure 12. Representative images for the gating strategy for unstimulated and histone 4 stimulated ECs

3.4.A Hyperglycaemic modulation of HUVECs increases endothelial cell histone 4 binding but does not increase other markers of endothelial activation

This data showed that modulation of HUVECs with D-glucose for 24 hours, followed by stimulation with histone-4 with or without S100A8A9 for 24 hours, led to increased EC binding of histone 4 (Figure 13 - A). This increased binding was not seen following modulation with L-Glucose (Figure 13 – A).

There was no increase in S100A8A9 binding (Figure 13 - B) or increase in other markers of EC activation or dysfunction following 24 hours of hyperglycaemia or following 24 hours of hyperglycaemia and stimulation with histone 4 and S100A9A9 (Figure 13 - C-G).



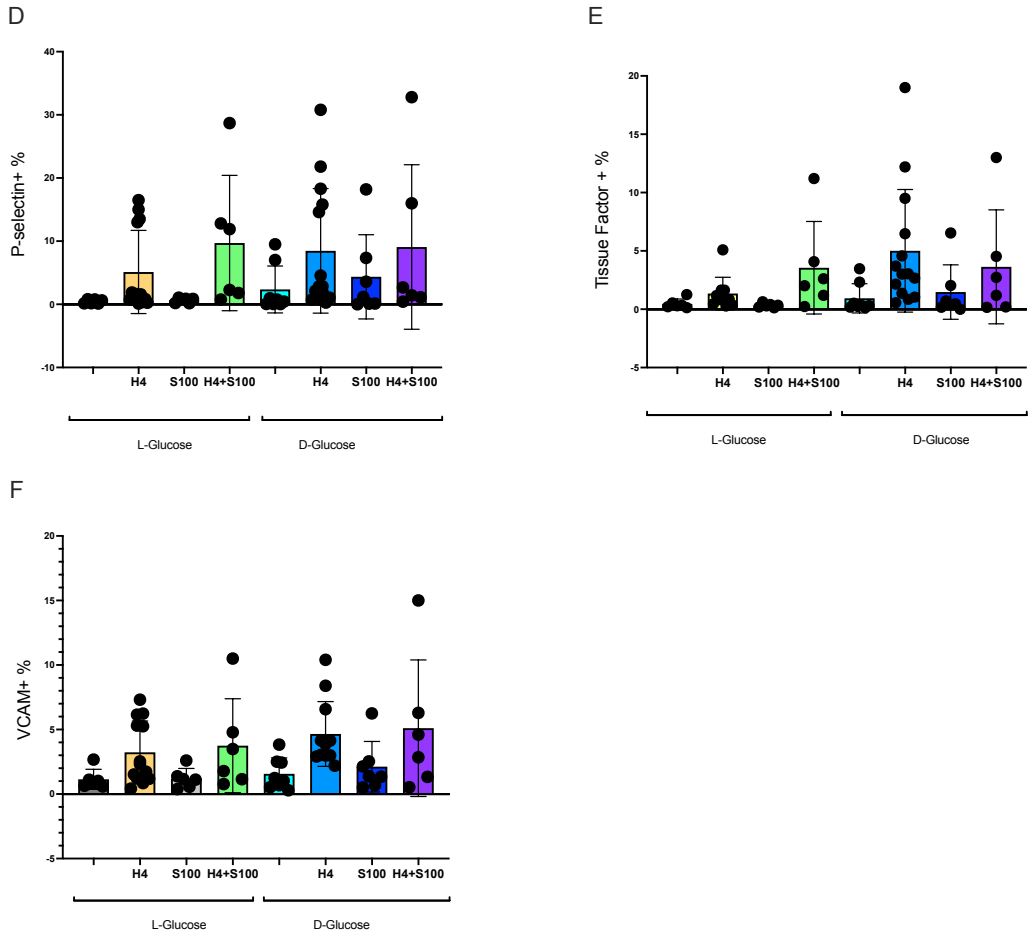


Figure 13. Hyperglycaemic modulation of HUVECs with D-Glucose increases histone binding

HUVEC monolayers were cultured until confluent and then incubated with 30mM D-Glucose or L-Glucose 30mM as control for 24 hours. Wells were washed then monolayers stimulated with Histone 4 (10µl/ml) and / or S100A8A9 20µl/ml for 24 hours. HUVEC surface binding / expression of (A) Histone 4, (B) S100 A8A9, (C) vWF, (D) Heparan, (E) P-selectin, (F) Tissue Factor and (G) VCAM was assessed by flow cytometry. The statistical significance between all conditions was tested using a one-way ANOVA with Tukey's multiple comparisons test, *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$.

In summary hyperglycaemia appears to increase EC histone binding when ECs are subsequently stimulated with histones, but this does not increase other markers of endothelial activation.

3.5 Modulation of HUVEC monolayers with heparanase and hyaluronidase does not increase histone 4 or S100A8A9 binding or result in increased markers of endothelial cell activation

Enzymatic degradation of the glycocalyx has been widely reported in both sepsis (332, 384) and COVID-19 (385, 386) with increased plasma markers of glycocalyx degradation being associated with increased leukocyte recruitment and pro-thrombotic potential in both conditions.

Here we investigated whether heparanase and hyaluronidase modulation of HUVECs for 1 hour resulted in altered histone 4 or S100A8/A9 binding, independent EC activation or an increase in histone 4 or S100A8A9 binding or EC activation when HUVEC monolayers were subsequently stimulated by histone 4 and S100A8A9. We assessed EC binding of histone 4 and S100A8A9 and markers of EC activation or dysfunction using flow cytometry and antibodies against histone 4, S100A8A9, VWF, P-selectin, TF, VCAM-1 and heparan sulphate.

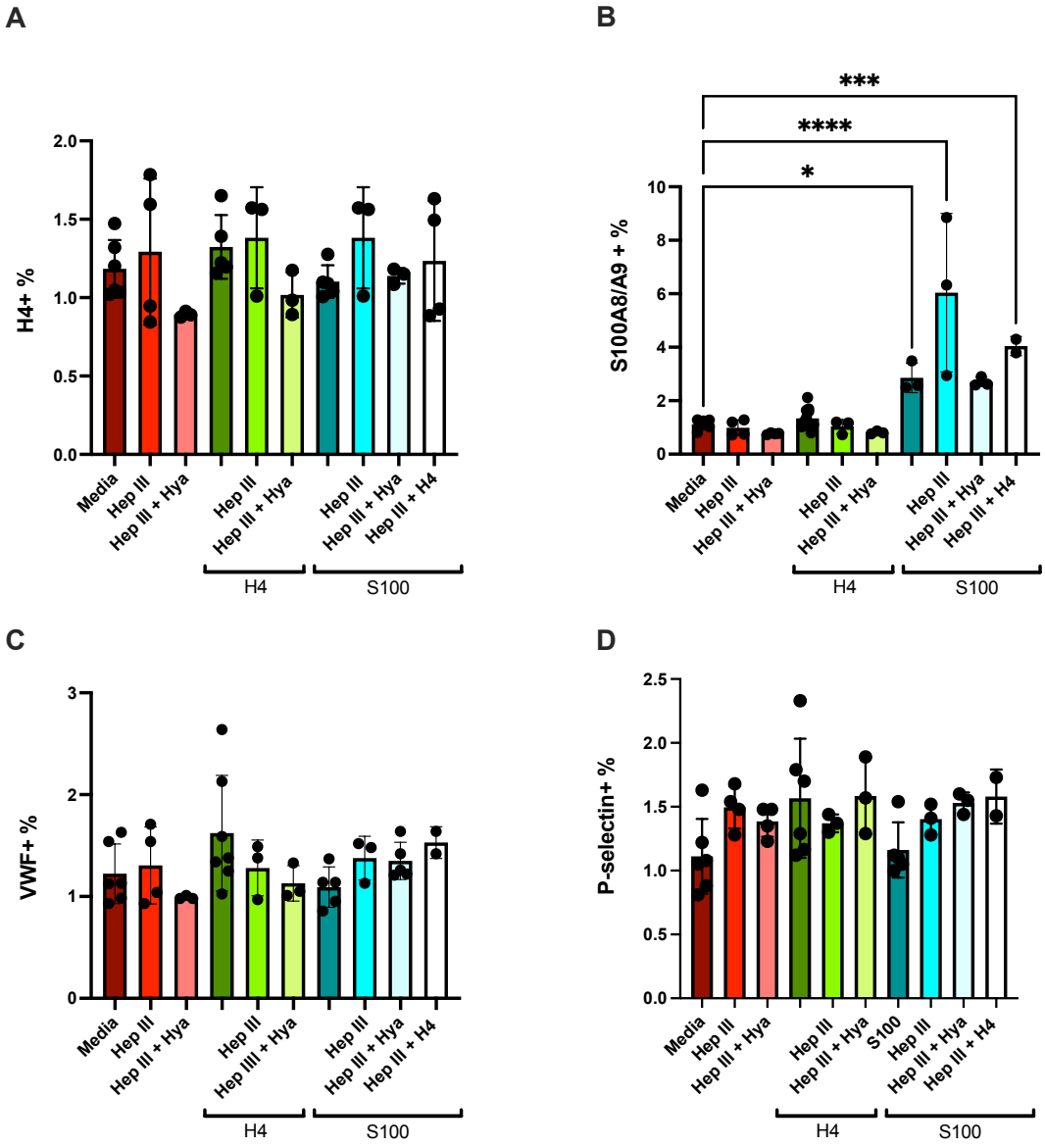
3.5.A Modulation of HUVEC monolayers with heparanase and hyaluronidase does not increase histone 4 or S100A8A9 binding or result in increased markers of EC activation

This data showed that, without heparanase and hyaluronidase treatment, activation of ECs with histone 4 did not increase histone 4 binding compared to control (figure 14-A). However, activation of ECs with S100A8A9 did increase S100A8/A9 binding (figure 14-B).

Following modulation of HUVECs with heparanase and hyaluronidase for 1 hour, histone 4 binding was still not increased and S100A8A9 binding was not significantly increased compared to activation with S100A8A9 without modulation (figure 14 – A and B).

Neither activation with histone 4 or S100A8A9, nor modulation with heparanase, with or without hyaluronidase, nor modulation with subsequent activation with histone 4 or S100A8A9 resulted in increased markers of EC activation (figure 14 – C-F).

Flow cytometry assessment of heparan sulphate on ECs also did not show any significant decrease following heparanase treatment (figure 14 – G).


A
H4+ %
2.0
1.5
1.0
0.5
0.0
Media
Hep III
Hep III + Hya
Hep III
Hep III + Hya
Hep III
Hep III + Hya
Hep III + H4
H4
S100
B
S100A8/A9 + %
***
****
*
10
8
6
4
2
0
Media
Hep III
Hep III + Hya
Hep III
Hep III + Hya
Hep III
Hep III + Hya
Hep III + H4
H4
S100
C
VWF+ %
3
2
1
0
Media
Hep III
Hep III + Hya
Hep III
Hep IIII + Hya
Hep III
Hep III + Hya
Hep III + H4
H4
S100
D
P-selectin+ %
2.5
2.0
1.5
1.0
0.5
0.0
Media
Hep III
Hep III + Hya
Hep III
Hep III + Hya
S100
Hep III
Hep III + Hya
Hep III + H4
H4
S100

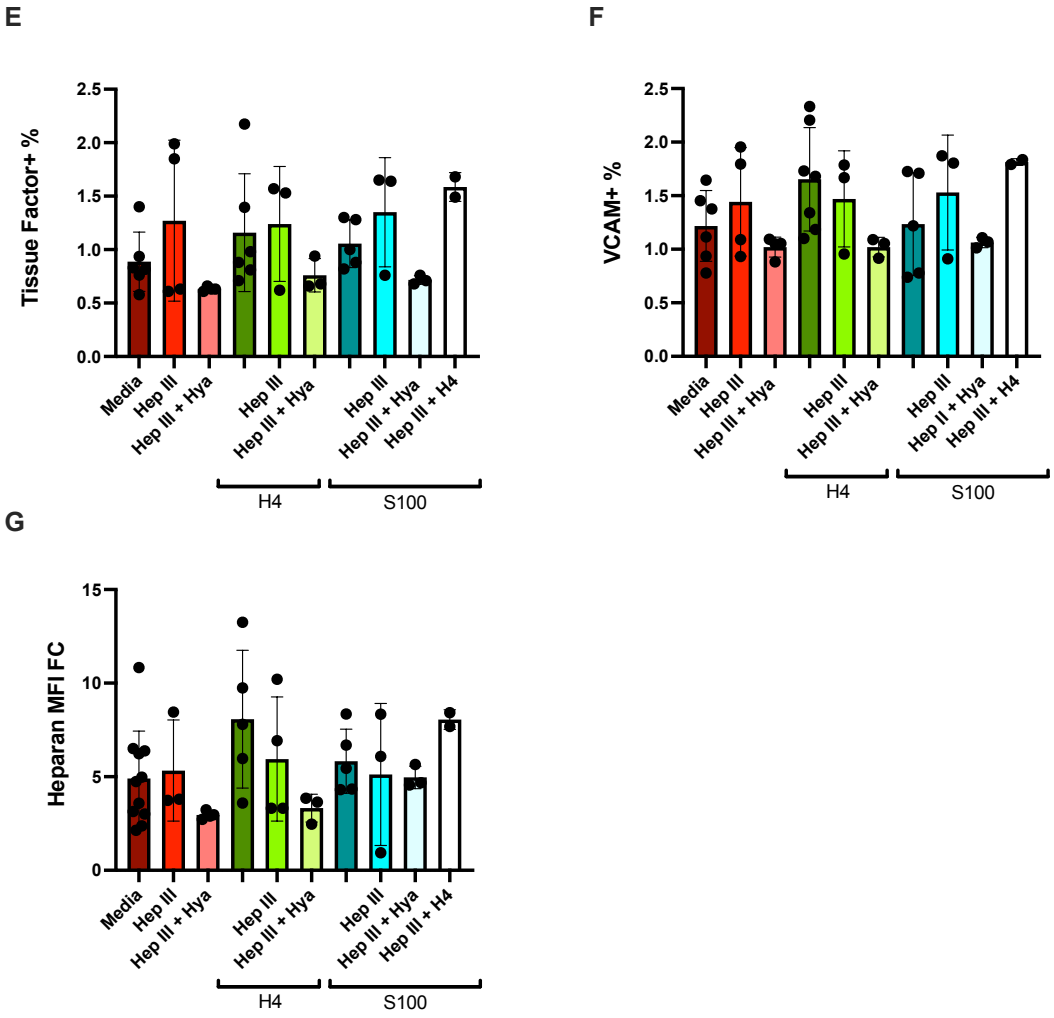


Figure 14. Modulation of HUVEC monolayers with heparanase and hyaluronidase does not increase histone 4 or S100A8A9 binding or result in increased markers of EC activation

HUVEC monolayers were cultured until confluent and then incubated with either heparanase III (1U/ml) alone or heparanase III (1U/ml) and hyaluronidase (10U/ml) for 1-hour. Wells were washed then monolayers stimulated with Histone 4 (10µl/ml) and / or S100A8A9 20µl/ml for 24 hours. HUVEC surface binding / expression of (A) Histone 4, (B) S100 A8A9, (C) vWF, (D) Heparan, (E) P-selectin, (F) Tissue Factor and (G) VCAM was

assessed by flow cytometry. The statistical significance between all conditions was tested using a one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05 **p < 0.01 ***p < 0.001.

In summary, heparanase and hyaluronidase showed no evidence of altering histone 4 or S100A8A9 binding and did not result in increased markers of EC activation following stimulation with histone 4 or S100A8A9.

3.6 Patients with ARDS have increased endothelial cell binding of histone 4 and S100A8A9 following stimulation with histone 4 and increased markers of EC activation

The pathogenesis of indirect ARDS involves diffuse endothelial dysfunction and is associated with increased risk of secondary bacterial infections and increased mortality (347-349). We therefore investigated whether the plasma from patients with sepsis, with and without ARDS, resulted in differing levels of histone 4 and S100A8A9 binding and EC activation. To do this, HUVEC monolayers were cultured in either low serum media or with 10% patient plasma alongside healthy control plasma. We also investigated whether, when HUVEC monolayers were pre-treated with plasma from patients with sepsis with and without ARDS for 24 hours, and subsequently stimulated with histone 4, whether there was an increase in histone 4 or S100A8A9 binding or increased EC activation. We also grouped patients as to whether they met the criteria for SIC to determine whether the clinical presence of SIC increased histone 4 or S100A8A9 binding or EC activation. We assessed EC binding of histone 4 and S100A8A9 and markers of EC activation or dysfunction using flow

cytometry and antibodies against histone 4, S100A8A9, VWF, P-selectin, TF, VCAM-1 and heparan sulphate.

3.6.A Addition of histone 4 to plasma from septic patients with ARDS induces endothelial cell activation

This data showed that when untreated ECs were stimulated with histone 4, there was no increase in H4 or S100A8A9 binding and no increase in markers of EC activation or dysfunction (Figure 15 A-G).

When ECs were treated with control plasma, histone 4 and S100A8A9 binding was not increased compared to untreated ECs, even after stimulation with histone 4 (Figure 15- A-B). There was also no increase in markers of EC activation or dysfunction (Figure 15- C-G).

Similarly, when ECs were treated with plasma from patients with sepsis but without ARDS, histone 4 and S100A8A9 binding was also not increased, even after subsequent stimulation with histone 4 (Figure 15- A-B). There was no increase in markers of EC activation or dysfunction (Figure 15- C-G).

However, when ECs were treated with plasma from patients with sepsis and ARDS and subsequently stimulated with histone 4, histone 4 and S100A8A9 binding was increased compared to treated but non-stimulated ECs (Figure 15 - A-B).

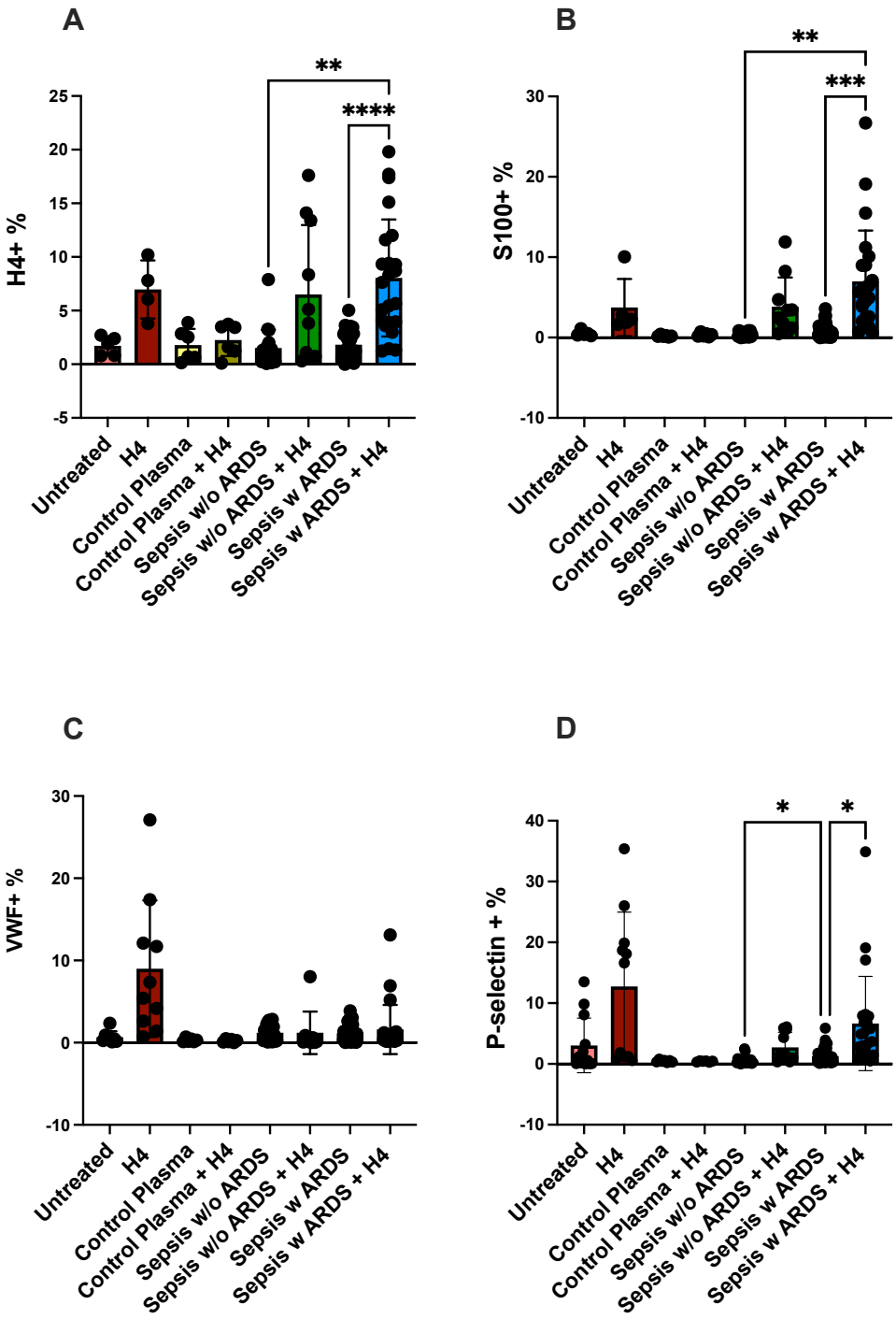
P-selectin expression was increased in ECs treated with plasma from patients with sepsis and ARDS compared to patients with sepsis without ARDS. Subsequent stimulation of ECs with histone 4 further increased P-selectin expression in the sepsis with ARDS group but not the sepsis without ARDS group (Figure 15- D).

Similarly, TF expression was increased in ECs treated with plasma from patients with sepsis and ARDS compared to patients with sepsis but without ARDS. Subsequent stimulation of ECs with histone 4 further increased P-selectin expression in the sepsis with ARDS group but not the sepsis without ARDS group (Figure 15- E).

VCAM expression was increased in ECs treated with plasma from patients with sepsis with ARDS and stimulated with histone 4 compared to treatment with plasma from patients with sepsis but without ARDS. Subsequent stimulation of ECs with histone 4 further increased VCAM expression in the sepsis with ARDS group but not the sepsis without ARDS group (Figure 15- F).

VWF expression on ECs was not increased under any conditions (Figure 15- C). Heparan expression was also not altered under any condition (Figure 15- G).

In summary, EC treated with plasma from patients with sepsis and ARDS and subsequently stimulated with histone 4 had increased histone 4 and S100A8A9 binding. The plasma from patients with sepsis with ARDS also led to increased expression of EC activation markers after the ECs were subsequently stimulated with histone 4.


A
H4+ %
25
20
15
10
5
0
-5
**
****
Untreated
H4
Control Plasma
Control Plasma + H4
Sepsis w/o ARDS
Sepsis w/o ARDS + H4
Sepsis w ARDS
Sepsis w ARDS + H4
B
S100+ %
30
20
10
0
-10
**
***
Untreated
H4
Control Plasma
Control Plasma + H4
Sepsis w/o ARDS
Sepsis w/o ARDS + H4
Sepsis w ARDS
Sepsis w ARDS + H4
C
VWF+ %
30
20
10
0
-10
Untreated
H4
Control Plasma
Control Plasma + H4
Sepsis w/o ARDS
Sepsis w/o ARDS + H4
Sepsis w ARDS
Sepsis w ARDS + H4
D
P-selectin + %
40
30
20
10
0
-10
*
*
Untreated
H4
Control Plasma
Control Plasma + H4
Sepsis w/o ARDS
Sepsis w/o ARDS + H4
Sepsis w ARDS
Sepsis w ARDS + H4

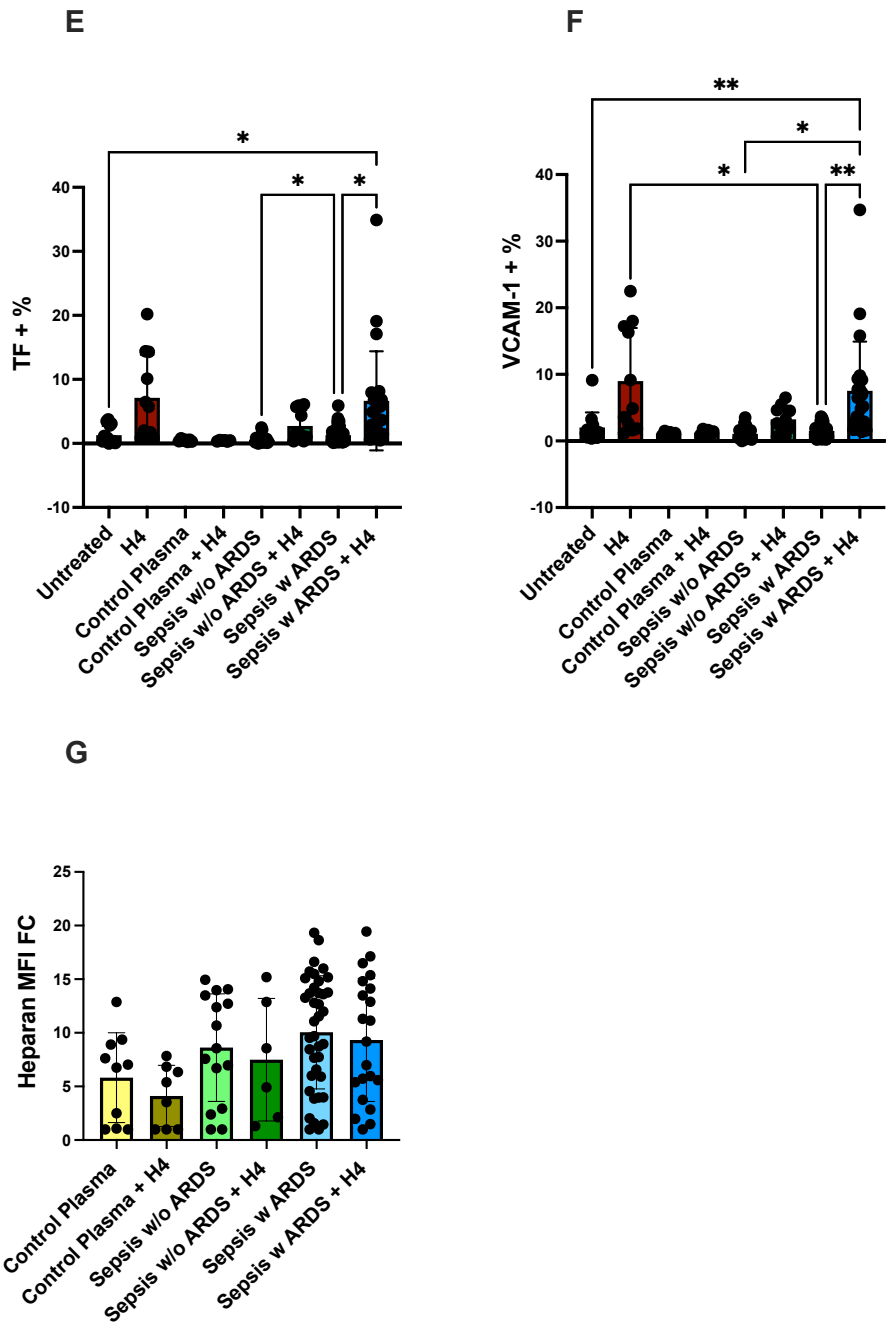


Figure 15. Treatment of HUVECs with plasma from patients with sepsis and ARDS subsequently stimulated with histone 4 have increased histone 4 and S100A8A9 binding and increased markers of EC activation

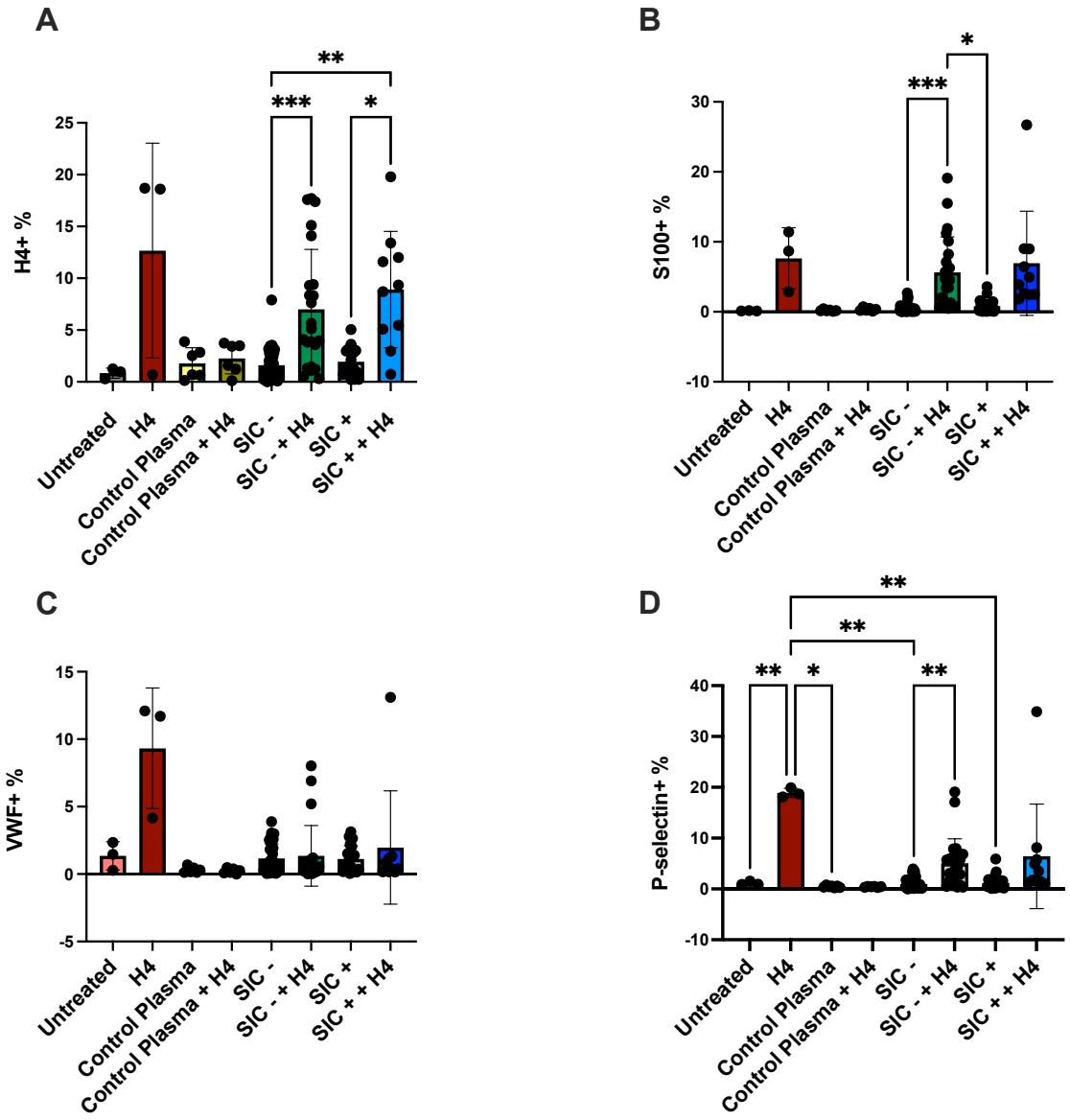
HUVEC monolayers were cultured until confluent and then incubated either in low serum media (LSM) or with 10% plasma in duplicate from either healthy donors or patients with sepsis with or without ARDS for 24 hours. Histone 4 (10µl/ml) was added to one duplicate such that each patient had its own control, also for 24 hours. HUVEC surface binding / expression of (A) Histone 4, (B) S100 A8A9, (C) vWF, (D) P-selectin, (E) TF, (F) VCAM-1 and (G) Heparan was then assessed by flow cytometry. The statistical significance between conditions was tested using a one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05 **p < 0.01 ***p < 0.001.

3.6.B Presence of sepsis induced coagulopathy did not affect histone 4 or S100A8A9 binding to endothelial cells or expression of activation or dysfunction markers

This data shows that when patients were re-classified based on the presence or absence of SIC, stimulating ECs with histone 4 increased histone 4 binding irrespective of SIC status (Figure 16-A). Only the ECs treated with plasma from patients without SIC had increased S100A8A9 binding following stimulation with histone 4 (Figure 16- B). Similarly, P-selectin and VCAM expression was only increased in ECs treated with plasma from patients without SIC when stimulated with histone 4 (Figure 16 - D and F).

Heparan expression was increased compared to treatment with control plasma in patients without SIC; patients without SIC stimulated with histone 4 and in patients with SIC but not in patients with SIC stimulated with histone 4 (Figure 16- G).

Von Willebrand's Factor and TF expression on ECs did not differ between patients with or without SIC (Figure 16 - C and E). This data shows that the presence of ARDS rather than SIC is a key determinant in the effect of histone 4 on ECs.


A
H4+ %
25
20
15
10
5
0
**
***
*
Untreated
H4
Control Plasma
Control Plasma + H4
SIC -
SIC - + H4
SIC +
SIC + + H4
B
S100+ %
30
20
10
0
-10
*
***
Untreated
H4
Control Plasma
Control Plasma + H4
SIC -
SIC - + H4
SIC +
SIC + + H4
C
VWF+ %
15
10
5
0
-5
Untreated
H4
Control Plasma
Control Plasma + H4
SIC -
SIC - + H4
SIC +
SIC + + H4
D
P-selectin+ %
40
30
20
10
0
-10
**
**
**
*
**
Untreated
H4
Control Plasma
Control Plasma + H4
SIC -
SIC - + H4
SIC +
SIC + + H4

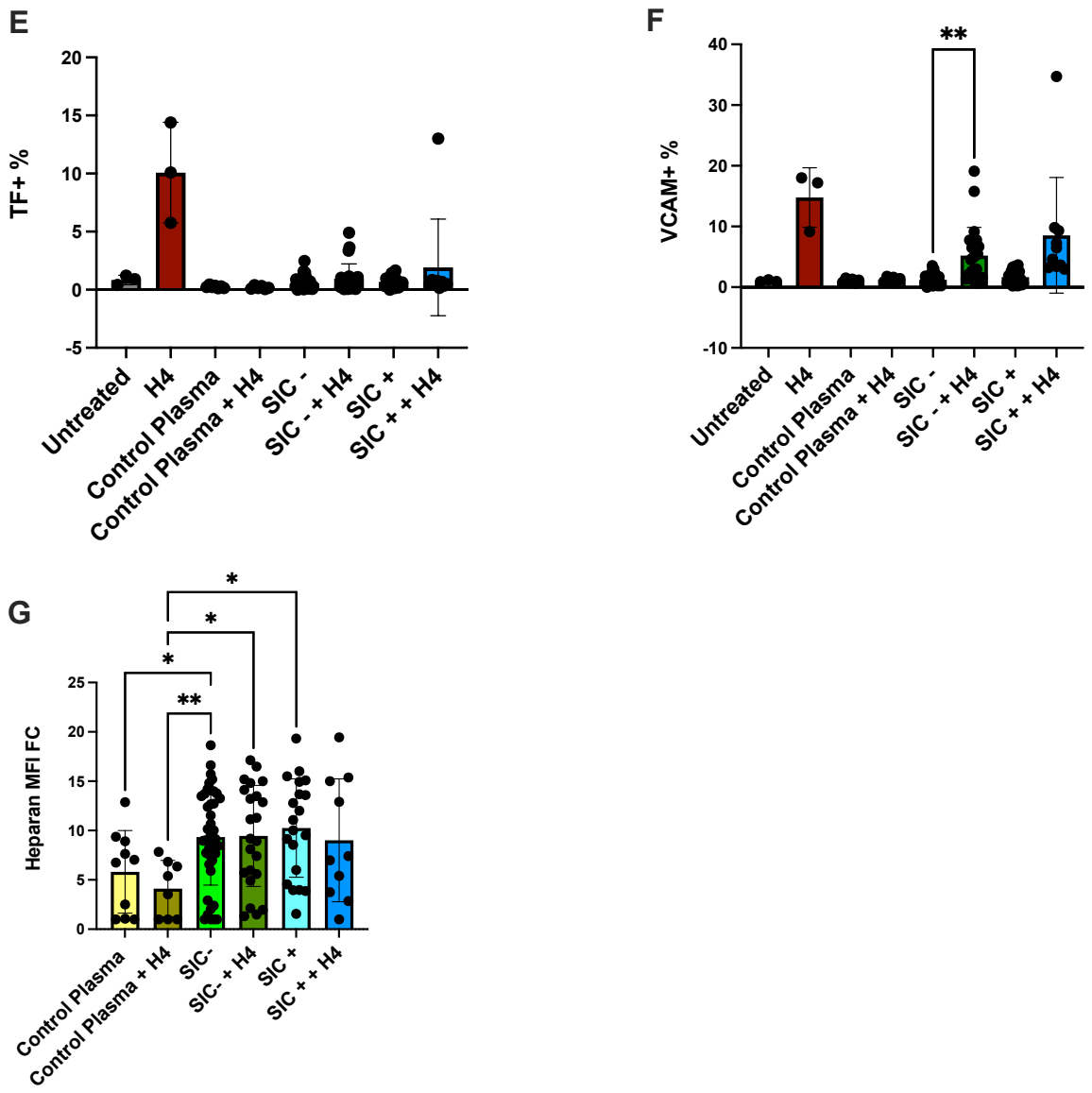


Figure 16. Presence of sepsis induced coagulopathy did not affect histone or S100A8A9 binding to ECs or expression of activation or dysfunction markers

HUVEC monolayers were cultured until confluent and then incubated either in low serum media (LSM) or with 10% plasma in duplicate from either healthy donors or patients with sepsis with or without ARDS for 24 hours. Histone 4 (10µl/ml) was added to one duplicate such that each patient had its own control, also for 24 hours. HUVEC surface binding / expression of (A) Histone 4, (B) S100 A8A9, (C) vWF, (D) Heparan, (E) P-selectin, (F) Tissue

Factor and (G) VCAM was then assessed by flow cytometry. The statistical significance between all conditions was tested using a one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05 **p < 0.01 ***p < 0.001.

3.6.C Endothelial cells expressing more heparan sulphate had reduced histone 4 binding. This data shows that higher levels of EC HS expression correlated with a reduction in H4 binding (Figure 17- A). There was no correlation between heparan expression and VCAM expression (Figure 17- B).

R	-0.50
R squared	0.25
95% Confidence interval	-0.68 to 0.27
P value	0.0001

r	0.18
R squared	0.03
95% Confidence interval	-0.09 to 0.42
P value	0.19

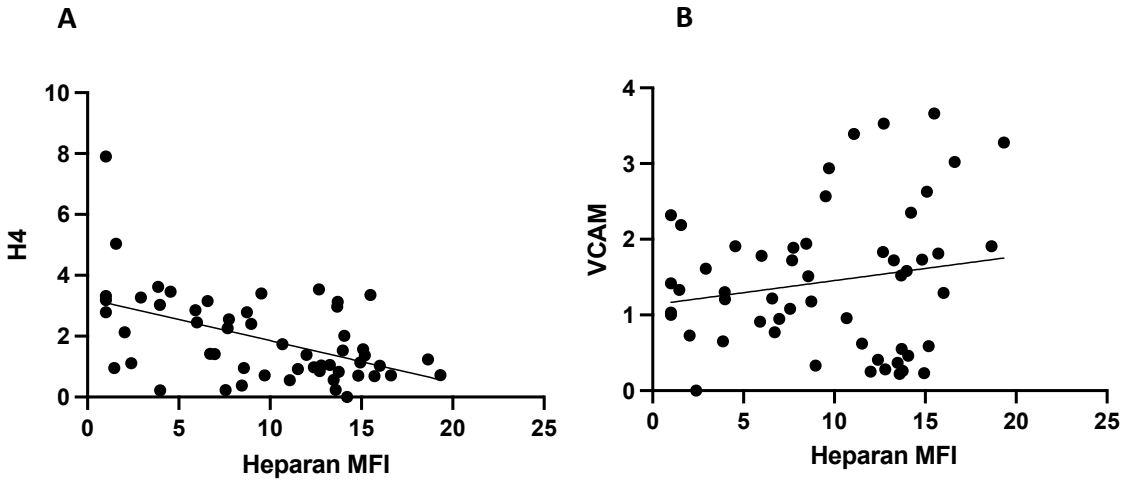


Figure 17. Endothelial cell heparan expression is negatively correlated with histone 4 binding.

HUVEC monolayers were cultured until confluent and then incubated either in low serum media (LSM) or with 10% plasma from either healthy donors or patients with sepsis with or without ARDS for 24 hours. HUVEC surface binding / expression of (A) Histone 4, (B) VCAM was assessed by flow cytometry. Correlation binding heparan expression and H4 and VCAM expression was assessed using Pearson correlation coefficient.

Chapter 4. Discussion

In this project we investigated mechanisms of endothelial activation in patients with sepsis and COVID-19 of different severity. We found:

1. Plasma from patients with sepsis and COVID-19 results in EC activation associated with immune cell recruitment, thrombosis and increased junctional permeability.
2. Plasmas from patients with sepsis and COVID-19 altered the structure of heparan sulfate on HUVEC monolayers. Plasma from patients with sepsis but not COVID-19 resulted in a decrease in VWF expression on HUVEC monolayers compared to healthy control plasma.
3. COVID-19 S and N proteins did not induce direct EC activation or support indirect activation through the activation of the complement system.
4. Hyperglycaemic modulation of HUVEC monolayers increases histone 4 binding but does not alter EC activation.
5. Plasma from septic patients with ARDS results in increased histone 4 and S100A8A9 binding to ECs compared to healthy control and patients with sepsis without ARDS and subsequent activation following histone 4 stimulation.

In summary our data shows evidence of endothelial cell activation markers in patients with sepsis and COVID-19 and identifies a possible role of NET-associated DAMPs, in particular histone 4, as a factor contributing to endothelial cell activation in patients.

4.1 Are endothelial cell activation markers in the plasma from patients with sepsis and COVID-19 associated with disease severity?

Infections, both bacterial and viral, can lead to endothelial cell activation and support inflammation and thrombosis. Our findings support the presence of systemic activation of the endothelium in sepsis and COVID-19. As well as contributing to our understanding of the pathophysiology of these conditions, clinical utility may be further provided if these biomarkers relate to severity and outcome.

4.1.1 Activated endothelial cells drive immune cell recruitment

In our cohort, E-selectin, P-selectin, ICAM-1, VCAM-1 and endoglin were all increased in septic and COVID-19 patients in ICU. However, only VCAM-1 and endoglin were increased in COVID-19 patients on wards. An increase in CAMs has previously been reported in patients with sepsis (387-389) however, the degree of CAM elevation is inconsistently predictive of severity and outcome (390, 391). This could be related to the concomitant mechanisms of cell activation, injury and death. For example, although EC apoptosis contributes to shedding of CAM, proteolytic enzymes also actively regulate leukocyte-endothelial interactions. This can both reduce receptor density and soluble CAM can result in competitive inhibition of granulocyte receptors (392-394). In fact, increased soluble CAM and deletion of CAM genes have been associated with survival in multiple sepsis studies (395-397) and are also impacted by age (398). This currently limits the utility of CAMs as biomarkers for specific mechanisms of endothelial cell activation and/or injury in sepsis.

Regarding COVID-19, although many studies do report an increase in E-selectin, P-selectin, VCAM-1 and ICAM-1 (322, 399-402), other studies have only identified increases in VCAM-1 (403, 404). This is potentially in line with our findings where we observed a selective increase in CAMs. The difference among studies could be related to the time of sampling of patients, comorbidities, age or other factors yet to be determined. Further studies with larger populations and a focus on assay variation and COVID-19 severity scoring are required.

4.1.2 Endothelial cell activation at the interface between inflammation and thrombosis

In this cohort, soluble TF and VWF were increased in patients with sepsis and in patients with COVID-19 in ICU and on wards. Patients with sepsis had the greatest increases in TF and VWF whereas the increase in TF and VWF was similar between COVID-19 patients in ICU and wards. The high levels of TF in septic patients supports the concept of systemic activation of coagulation, although tissue factor has only previously been reported to be increased in septic patients with ARDS (405). Furthermore, patients with COVID-19 have also previously been reported to have higher levels of EV-TF compared to patients with sepsis, alongside higher levels of PAI-1 (406). The differences seen in our cohort may be due to increased representation of the earlier, lung restricted stage of SARS-CoV-19 infection, with more local EC upregulation of TF in the lungs and local thrombosis associated with systemic hypofibrinolysis (41, 407-409).

Previous studies have also reported an association between TF levels and mortality in COVID-19 patients but this was only in patients with moderate or severe disease (410, 411). In this study, increases in TF were similar between COVID-19 ICU and wards patients,

suggesting that systemic hypercoagulability may not be associated with clinical severity. However, again this may reflect a more localized stage of disease as ICU COVID-19 patients in this study only had lung injury compared to systemic disease as in the sepsis group. Another explanation could be related to the time of sample collection as plasma from patients in ICU was collected 24 hours post admission, whereas plasma from patients on wards were not adjusted to admission.

In this cohort, VWF was increased in both septic and COVID-19 patients consistent with systemic EC activation (213, 412, 413). However, similarly to CAM levels, increased VWF is inconsistently associated with severity and prognosis in sepsis (314, 412, 413). In patients with COVID-19, VWF has been associated with severity and prognosis (414-419) although this also wasn't the case in our cohort. Imbalance of VWF to ADAMTS-13 and complement consumption due to the VWF-A2, via complement factor B, binding C3b, may also be relevant (420, 421) but ADAMTS-13 was also not significantly reduced compared to healthy controls.

4.1.3 Sepsis and COVID-19 patients have markers of increased endothelial cell permeability

In this studied cohort, patients with sepsis but not COVID-19 had decreased soluble angiopoietin-1 levels compared to age-matched controls. However, patients with sepsis and COVID-19 in ICU had increased angiopoietin-2. This was not seen in COVID-19 patients on wards. Both angiopoietin-1 and angiopoietin-2 levels have been widely associated with the presence and severity of sepsis, although serial trends and the Angiopoietin1:2 ratio may provide the best predictor of mortality (422-426).

In contrast to our findings, angiopoietin-1 levels have been reported to be decreased in COVID-19, although only in severe disease (427). However, like our findings, angiopoietin-2 levels are reported to increase over time in ICU patients but remain stable in wards patients and increases have been associated with mortality (49, 428, 429). In spite of this, angiopoietin-2 in vitro has been reported to increase endothelial permeability to a lesser extent and shorter duration compared to plasma from COVID-19 patients and the main plasma factor for permeability is still considered to be unidentified (430). Pulmonary ECs exposed to SARS-CoV-2 have also been shown to suffer a greater loss and more prolonged time to recovery of barrier function when compared to glomerular ECs, reflecting differences in capillary beds (429). The increase in angiopoietin-2 without increase in angiopoietin-1 in ICU COVID-19 patients could therefore reflect more localized alterations in permeability compared to sepsis.

4.1.4 Complement activation in patients: a trigger for EC activation or a hallmark of infection and inflammation

In our cohort, plasma from patients with sepsis and COVID-19 in ICU and on wards had increased levels of complement C5a compared to age-matched controls.

C5a levels are reported to predict sepsis (431) and are associated with disease severity and mortality in patients with septic shock (432). Complement inhibition during severe sepsis has also been shown to reduce immune cell recruitment and mitigate the procoagulant phenotype of sepsis (433). Several studies have shown increased complement in COVID-19 patients and this has also been associated with disease severity (305, 434-436), although there was no difference in C5a between ICU and wards COVID-19 patients in our

population. Complement activation and the release of C5a is a strong inflammatory and chemoattractant stimulus for innate immune cell recruitment. However, it remains unknown whether complement activation results from the opsonization of the pathogen and PAMPs or whether it is secondary to the inflammatory response.

4.1.5 Are NET-associated DAMPs responsible for EC activation?

In this cohort, patients with COVID-19 in ICU had increased histone 4 levels but this was not seen in patients on wards or in patients with sepsis. Plasma from patients with sepsis and COVID-19 in ICU and on wards had increased S100A8/A9.

The current literature suggests that histone 4 levels are increased in patients with sepsis and that this is associated with increased mortality (437-439). The absence of histone H4 is sepsis patients is surprising and this data should be interpreted with caution. It is important to note that plasma from COVID-19 patients in ICU was collected on EDTA whereas plasma from patients with sepsis was collected on heparin. As heparin is known to bind to histone 4, it is possible that its detection by ELISA in patients with sepsis was compromised.

Histone 4 has also previously been shown to be increased in COVID-19 and to be associated with severity (440-442). This is consistent with the findings in this study, although heparin was also used to anticoagulate some COVID-19 wards patients whereas EDTA was used for COVID-19 ICU patients.

Prior studies also suggest that S100A8/A9 levels are increased in COVID-19 and have been suggested to discriminate between mild and severe disease (443-445). However, S100A8/A9 levels did not differ between COVID-19 patients in ICU or on wards in this population. This could be related to patients' comorbidities and inclusion time.

Furthermore, neutrophils are not the only source of S100A8/A9 as it is released by other cells, such as adipocytes. Moreover, S100A8/A9 binds to heparin and as all ICU patients (COVID-19 and sepsis) received prophylactic treatment of low molecular weight heparin, it is possible that the detection of S100A8/A9 was also altered. In summary, it is evident that histone 4 and S100A8/A9 are increased in some patients with COVID-19 but the interaction between heparin and histone 4 in patients with sepsis requires further investigation.

Overall, this data suggests that endothelial dysfunction is clearly present in patients with sepsis and COVID-19 but only some parameters reflect disease severity. It is possible that stratification of patients on wards based on their admission could reduce the spread of the data across groups.

4.2.1 Does sepsis and COVID-19 alter glycocalyx structure? What would be the consequences on EC activation?

Patients with sepsis are widely reported to have increased plasma HS and other glycocalyx components with levels being associated with severity (446-449). Shedding of the glycocalyx in patients with COVID-19 was also described early during the pandemic, with increased plasma GAGs and syndecan-1 reported, even compared to COVID-19 negative sepsis patients (450, 451). Syndecan-1 levels have also been shown to predict non-survival; development of moderate-to-severe ARDS and requirement for ventilation (335, 452, 453). Plasma HS has also been associated with disease severity (454). By testing the effect of the plasma from patients on HS expression and structure on HUVEC monolayers, our data showed that overall intensity of HS was not altered but plasma from COVID-19 and sepsis

patients subjectively increased the thickness of HS fibres. Such changes may have functional effects on immune cell recruitment as thick fibres of HS may alter cytokine and chemokine presentation (455, 456).

Although we only assessed HS intensity via immunofluorescence in this experiment, endotoxin, heparanase and hyaluronidase have all been shown to reduce glycocalyx thickness or result in loss of the fine hair-like structure (457-460). To further explore these findings, measurement of glycocalyx degradation products in the supernatant of cultured HUVECs by ELISA and subtraction of this value from the plasma supernatant could be performed. This would help to establish any relationship between shed glycocalyx components and qualitative appearance, as intensity alone seems unable to quantify glycocalyx changes. In vivo, sepsis has also been shown to be associated with the absence of reparative pulmonary endothelial FGFR1 induction (461).

However, an additional limitation in this experiment was that HUVECs were not grown under flow conditions. This has been reported to reduce the thickness of the glycocalyx and increase neutrophil adhesion (462). Measuring the glycocalyx itself is challenging in vitro and does not necessarily correspond to the known dimensions of individual proteoglycan components (463).

4.2.2 Is Weibel-palade body release of VWF regulated by disease severity?

In contrast to HS intensity, treatment of ECs with plasma from patients decreased the overall mean immunofluorescence of VWF in sepsis patients, although this alteration was not seen in response to plasma from COVID-19 patients. This is interesting as VWF levels were increased in the plasma of both sepsis and COVID-19 patients in the first experiment.

In this experiment, HUVECs were permeabilized, such that both intracellular and EC bound VWF were identified. VWF is not only released in the supernatant but also can remain bound on the EC surface. It is possible that the VWF is released from WPB but a good fraction of it remains attached to the surface. Future studies could therefore include measurement of VWF in the HUVEC supernatant to better characterize this finding, and these experiments need to take into consideration the basal level of VWF in the plasma of patients. Cleavage of VWF also typically requires flow conditions to expose the ADAMTS-13 A2 binding site so this also needs to be considered (44).

4.3 Do COVID-19 glycoproteins directly activate ECs or show evidence of activating complement in order to indirectly activate ECs?

Although there is some evidence to suggest that the COVID-19 spike glycoprotein mediates direct viral cellular entry (299-301, 464) , indirect EC activation is also supported by studies showing plasma from critically ill and convalescent patients with COVID-19 causes EC cytotoxicity and NET release (306, 465, 466). Our data showed that, neither the addition of the spike or nucleocapsid proteins, nor the addition of serum as a source of complement, induced EC activation. This suggests that complement activation may not result from direct contact with the viral proteins. This is in contrast to other reports in the literature which suggest the spike protein causes exuberant C3 and C5b-9 deposition on ECs (467, 468) and the N protein can interact and potentiate the mannose-binding lectin pathway (469). However, it is still possible that direct actions of the S and N proteins indirectly induce complement activation by other pathways, such as the thrombo-inflammatory response.

Complement activation has also been suggested to be more specific to severe, late COVID-19 (>30 days) compared to the innate hyperinflammatory response of the early disease (<14 days) which may account for the lack of complement activation in this experiment (470).

4.4 Does hyperglycaemic alter endothelial cell activation by regulating DAMP binding?

Although diabetes itself has not been shown to be associated with outcome during sepsis (471, 472), interestingly, hyperglycaemia has been associated with higher mortality in nondiabetic populations with sepsis (259). Our findings in this experiment show that hyperglycaemia appears to increase EC histone 4 binding when ECs are subsequently stimulated with histones, as might be seen during secondary infections in hospitalised patients. Histones are thought to bind to HS binding sites within the glycocalyx (273), although the anionic GAGs are also thought to protect the cells against histone-mediated damage (473, 474).

Direct extracellular cytotoxicity due to circulating histones, particularly in the lungs, is thought to contribute to organ failure and death during sepsis (475, 476). Alternatively, histones are thought to induce complement activation and EC injury (477-479).

Unexpectedly, there was no significant increase in other markers of endothelial activation in conjunction with this finding. This contrasts with the literature which reports histones increase EC CAM expression and WPB release of VWF, as well as angiopoietin-2 (225, 480). This may be due to overwhelming cytotoxicity, an inability to generate complement for indirect EC activation or effective neutralization of histones by EC GAGs or thrombomodulin

in the absence of other inflammatory mediators (481, 482). Finally, multiple comparison statistical testing may have masked any significance.

4.5 Is the EC response to histone 4 in the presence of patients' plasma regulated by disease severity?

As histone 4 was not detected in the plasma of patients with sepsis by ELISA, we wanted to explore whether this could be related to increased histone 4 binding to ECs or heparin. We also wanted to assess the relationship between the response of ECs to histone 4 and patients' disease severity. To do this, we assessed the binding of histone 4 and the activation status of cells in the presence of plasma of control and septic patients' plasma. HUVECs cultured with plasma from patients with sepsis and ARDS and subsequently stimulated with histone 4, resulted in increased EC binding of histone 4 and S100A8A9 compared to control plasma and plasma from septic patient without ARDS. Like histone 4, S100A8/A9 can also bind to GAGs and activate ECs to increase CAM expression (245), although this too can be blocked via endogenous and exogenous heparin (246). The increased histone 4 and S100A8A9 binding was seen in spite of the samples being anti-coagulated with heparin, and that patients received prophylactic doses of low molecular weight heparin which would normally neutralize these DAMPs, suggesting that heparin is not effective in neutralizing histone 4 in patients with sepsis and ARDS. This finding is highly relevant as this cohort of patients had the highest mortality (around 40% versus 10% for patients without ARDS).

In addition, P-selectin, TF and VCAM-1 expression were also increased in ECs exposed to plasma from patients with sepsis and ARDS. Subsequent stimulation with histone 4 further increased their expression. This could suggest that the ability of EC GAGs and thrombomodulin to neutralize the effects of histone 4 are overwhelmed with the plasma from sepsis patients with ARDS.

When the studied population was retrospectively grouped based on presence or absence of SIC, the effects of histone 4 and S100A8A9 binding and EC activation were lost. This suggests that it is systemic inflammation rather than systemic hypercoagulability that is most relevant.

Further assessment of the impact of DAMPs on EC activation could focus on adding inhibitors of the EC TLR 2 and 4 receptors for histone 4 and S100A8A9 or adding exogenous GAGS and thrombomodulin to understand if this would mitigate the EC activation seen. TLR2 and TLR4 null mice have already been reported to be protected in an in vivo histone release model (234) and inhibition of TLR-4 has been shown to reduce CAM expression in a ROS- NF-kB dependent manner (483).

4.6 Limitations:

The main limitation from these experiments relate to challenges associated with patient recruitment. This includes both the number of patients recruited and the variation in clinical severity when grouping patients by ICU or wards. For example, patients with sepsis had systemic disease but COVID-19 patients admitted to ICU may only have had lung injury. These COVID-19 ICU patients may therefore not truly represent a 'severely affected'

population. Both sepsis and COVID-19 are clearly heterogenous diseases and increased sample size and better standardization of severity scoring may have helped to reduce the spread of data. Furthermore, we were also limited by plasma sample size for some patients. Ideally all patients would have had 3 replicates but this wasn't always possible. The use of different anticoagulants during sample collection also impacted our ability to draw conclusions about the levels of histone 4 in the sepsis population and about the impact of DAMPs on EC activation.

Regarding the exploration of glycocalyx degradation on EC activation using heparanase and hyaluronidase, the expression of EC HS did not differ significantly from untreated samples. This may be due to failure to effectively cleave the HS, despite using similar doses and times as that reported in other studies. However, a recent study found that cultured HUVECs retain only 10% of the proteoglycans on the cell surface and secrete the rest into the culture medium (10). This may have contributed to an inability to detect a difference between treated and untreated conditions. It is possible that culturing HUVECs under flow conditions may also impact their response to histone 4 and S100A8/A9.

A final limitation is our use of different lots of histone 4 during serial replicates. These may have had differing abilities to activate EC.

4.7: Future directions:

Although we have shown that sepsis and COVID-19 induce EC activation, future work could include measurement of glycocalyx components and VWF in the supernatant of cultured

HUVECs after treatment with patient plasma. These levels would then be compared to the levels already present in the plasma.

Secondly, the functional relevance of alterations in HS structure and EC activation could be explored using neutrophil transmigration to assess immune recruitment and platelet adhesion to assess their contribution to immunothrombosis. The use of TLR inhibitors could then specially assess the contributions of histone 4 and S100A8/A9 to these effects.

4.8: Conclusion:

Both sepsis and COVID-19 result in EC activation. This seems primarily immune driven with DAMPs such as histone 4 and S100A8/A9 potentially playing a major role. Heparin is not effective in neutralizing the effects of histone 4 on EC activation in patients with sepsis and ARDS which may contribute to the increased mortality seen in this population.

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