Endothelial glycocalyx biomarkers in sepsis and trauma: associations with inflammation, organ failure and bolus fluid therapy

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This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

Division of Emergency Medicine
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THESIS DECLARATION

I, Lisa Smart, certify that:

This thesis has been accomplished during enrolment in the degree and supervised by Professor Daniel Fatovich, Dr Erika Bosio, Associate Professor Glenn Arendts and Professor Giselle Hosgood. My candidature was supported by an Australian Postgraduate Award, a University of Western Australia Safety Net top-up Scholarship, a University of Western Australia Emergency Medicine top-up scholarship and the Jean Kahan Memorial Scholarship (Harry Perkins Institute of Medical Research).

The novel research presented in this thesis was my own work, except where stated. Work presented was primarily carried out in the Centre for Clinical Research in Emergency Medicine, Harry Perkins Institute of Medical Research, Perth, Australia. Animal experimental work was conducted at the School of Veterinary Medicine, Murdoch University, Perth, Australia.

Research involving human subjects reported in this thesis followed the National Statement on Ethical Conduct in Human Research, and was approved by the Human Research Ethics Committee at The University of Western Australia (approval numbers: 2009-080, 2011-091). Research involving animal subjects reported in this thesis followed the University of Western Australia and National Standards for the Care and Use of Laboratory Animals, and was approved by the Murdoch University Animal Ethics Committee (approval number: R2666/14).

This thesis contains work prepared for publication, all of which has been co-authored. This thesis does not contain material that has been submitted for the award of any other degree or diploma in my name, in any university or other tertiary institution. In the future, no part of this thesis will be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia and where applicable, any partner institution responsible for
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Blood vessels are lined with endothelial cells that have a protective layer of proteoglycans and glycosaminoglycans on their apical surface. This superficial layer of the endothelium is called the glycocalyx. Widespread inflammation resulting from conditions such as sepsis or severe traumatic injury can lead to shedding of the endothelial glycocalyx, causing endothelial dysfunction. This dysfunction is thought to contribute to impaired microvascular perfusion and organ dysfunction in patients with critical illness.

Endothelial glycocalyx shedding can be detected by measurement of circulating glycocalyx components. Several studies conducted in the intensive care unit (ICU) setting have shown an association between elevated concentrations of these glycocalyx biomarkers and worse clinical outcome, such as higher risk of organ failure or death. Importantly, standard interventions delivered in the emergency department (ED) before ICU admission, such as intravenous bolus fluid therapy, may exacerbate glycocalyx shedding. The first key finding of this thesis is that glycocalyx biomarker concentrations increased over time during ED treatment of sepsis and were positively associated with the total volume of intravenous fluid delivered, concentrations of inflammatory biomarkers and organ failure. The second key finding was that glycocalyx biomarker concentrations did not increase over time during ED treatment of patients with major trauma. Based on other work, it was postulated that pre-hospital fluid administration in trauma patients might have caused glycocalyx shedding before measurement was made, which led to investigation of an animal model of haemorrhagic shock to explore glycocalyx shedding and inflammation in the time surrounding fluid resuscitation.

A canine atraumatic haemorrhagic shock model was used to compare fluid resuscitation strategies: large-volume crystalloid fluid, two different synthetic colloid fluids and autologous whole blood. Crystalloid fluid was associated with rapid glycocalyx shedding and higher inflammatory biomarker concentrations than other fluid groups. Given the concern for large-volume crystalloid
resuscitation causing glycocalyx shedding and inflammation, biomarker comparisons were made between ED patients with suspected infection randomised to receive either a single bolus of hypertonic saline or isotonic saline for fluid resuscitation. No relevant differences were identified between the treatment groups in glycocalyx or inflammatory biomarker concentrations, however there were also no significant differences between groups in the volume of intravenous fluid delivered subsequent to the intervention, which may have negated any beneficial effects of hypertonic saline.

An important observation that has emerged from the studies conducted in this thesis is the varied temporal patterns of shedding between three different glycocalyx biomarkers. These studies have also contributed to the body of evidence that there exists a signal of harm associated with large volumes of crystalloid fluid, including exacerbation of glycocalyx shedding and inflammation. Further work is needed to understand the varied sources of these biomarkers during critical illness before a higher level of interpretation can be applied to the impact of interventions on glycocalyx shedding, and any benefit that glycocalyx protection may confer to patients with critical illness.
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This research was supported by an Australian Government Research Training Program (RTP) Scholarship. This research was funded by the Jean Kahan Memorial Scholarship, the Canine Research Foundation, the American College of Veterinary Emergency and Critical Care and departmental funds from the Centre for Clinical Research in Emergency Medicine. I am honoured to be the first recipient of the Jean Kahan Memorial Scholarship; she was one of the first women in Western Australia to graduate with a Science degree and was described as a woman that taught us to ‘pursue our dreams in spite of the obstacles we face’. These words resonated with me throughout my candidature.

I thank my colleagues for making this journey possible, including Peter Irwin who pushed me out the door while facilitating retention of my position at Murdoch University so that I could maintain my veterinary academic career, Simon Brown for embracing the idea of including a veterinarian as a clinical researcher on his team and Shelley Stone for providing the support I needed to embark on laboratory work.

I thank my supervisory team at the Centre for Clinical Research in Emergency Medicine (CCREM). Daniel Fatovich provided his research expertise and sage advice for timely progress. Erika Bosio facilitated the day-to-day operative needs of my candidature and dedicated much of her time for long, supportive conversations; scientific or otherwise. Glenn Arendts has never failed to be generous with his time and provided a much-needed chock when my wheels were spinning. Giselle Hosgood has provided invaluable guidance on biostatistical methodology and precision in scientific writing.

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Finally, I would like to thank an intelligent, kind and generous ex-ICU nurse who understood the challenges I faced and was always willing to listen; my mother, Helen Smart. Without her, I may not have survived the juggling act. I would also like to thank my companion, Frodo Baggins, who never failed to make me laugh each and every day, and is always by my side. Life is never dull with a Gordon Setter.


**AUTHORSHIP DECLARATION – Co-authored publications**

This thesis contains work that has been published or prepared for publication.

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I, Erika Bosio, certify that the student's statements regarding their contribution to each of the works listed above are correct.

As all co-authors' signatures could not be obtained, I hereby authorise inclusion of the co-authored work in the thesis.

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PRESENTATIONS ARISING FROM THIS RESEARCH

Conference abstracts


Invited presentations

1. The glycocalyx: the gatekeeper to the endothelium. Australian College of Veterinary Scientists, Annual Scientific Meeting, Gold Coast, QLD, Australia, July 2016.


6. Surviving Sepsis Campaign guidelines: where should we depart? Australian and NZ College of Veterinary Scientists, Annual Scientific Meeting, Gold Coast, QLD, Australia, July 2017.

7. The adverse effects of rapid fluid administration. World Small Animal Veterinary Association Congress, Singapore, October 2018.


CHAPTER 1 - Introduction

Critical illness typically includes a systemic inflammatory response, whereby an insult such as infection or massive tissue injury incites a cascade of inflammatory mediators that can damage organ systems distal to the source of the insult. This systemic inflammatory response can be associated with circulatory shock, multiple organ failure and death. Optimisation of macrohaemodynamic variables to ensure adequate tissue perfusion, and therefore prevent organ failure, is an important component of initial stabilisation for patients with shock. However, despite measures taken to ensure adequate tissue perfusion in critical illness, such as normalisation of cardiac output and blood pressure, microcirculatory dysfunction and tissue hypoxia may persist in some forms of critical illness, such as sepsis. In patients with sepsis, early goal directed therapy aimed at optimising macrohaemodynamic variables has failed to improve survival and the case fatality rate for sepsis remains high. Further, the presence of microcirculatory perfusion deficits in septic patients, either during or following macrohaemodynamic resuscitation, has been associated with an increased risk of organ failure and death.

Activation or damage to the surface of the endothelium is implicated as the main cause of microcirculatory dysfunction and subsequent multiple organ failure in critical illness. Given that early microcirculatory dysfunction may be a key protagonist of organ dysfunction, recent focus has been placed on identifying circulating biomarkers that characterise endothelial damage, predict clinical outcome and gauge effect of treatments aimed at reversing endothelial dysfunction. The endothelial glycocalyx is the most superficial layer of the endothelium. Studies exploring biomarkers of endothelial glycocalyx shedding have become prevalent in critical illness research in the last decade. Characterising glycocalyx shedding in the clinical setting is important to not only improve understanding of the pathophysiology of multiple organ failure but also to investigate therapies to reduce this damage.
Optimisation of tissue perfusion in the treatment of many types of shock includes expansion of blood volume, with rapid intravenous administration of crystalloid fluid strongly recommended as the first choice in patients with sepsis.\textsuperscript{12} Bolus crystalloid fluid therapy improves macrohaemodynamic variables but may also promote endothelial glycocalyx shedding, exacerbating endothelial activation and furthering inflammation. Published data on shedding of the endothelial glycocalyx in human sepsis, via measurement of circulating glycocalyx biomarker concentrations, focuses on intensive care unit (ICU) patients. However, crystalloid fluid loading typically starts in the emergency department (ED). Therefore, elevations in glycocalyx biomarker concentrations on admission to the ICU may reflect the impact of prior interventions in the ED, in addition to the effects of sepsis itself.

The literature review provided with this thesis (Chapter 2) amalgamates evidence from \textit{in vitro} and animal model studies on the nature and function of components of the glycocalyx, in order to deepen understanding of the various sources of circulating glycocalyx biomarkers and their possible downstream effects. This section also reviews mechanisms of glycocalyx shedding pertaining to systemic inflammation and bolus fluid therapy, as relevant to sepsis. The first two papers (Chapters 3 and 4) characterise the temporal patterns of endothelial glycocalyx shedding during ED treatment of sepsis, with a particular focus on associations with inflammatory biomarkers and fluid therapy intervention. Given that a positive association was identified between glycocalyx shedding and cumulative fluid volumes administered in septic patients, a further two papers explored glycocalyx biomarker concentrations, fluid volumes and inflammation in patients with trauma (Chapter 5) and in a canine haemorrhagic shock model (Chapter 6). Finally, given the evidence that large volume crystalloid fluid was associated with glycocalyx shedding and inflammation, the final paper of this thesis (Chapter 7) compared concentrations of similar biomarkers in ED patients with suspected infection that were randomised to receive either hypertonic saline or isotonic saline. This thesis has been prepared as a series of manuscripts; however, a short literature review relevant to each objective is provided at the start of each chapter. Given that Chapter 1 focuses on laboratory
research, these short literature reviews accompanying Chapters 3 through to 7 provide a review of clinical studies pertaining to either sepsis or trauma. Chapter 6 also reviews haemorrhagic shock models.

This thesis has enabled me to acquire skills in diagnostic laboratory techniques, animal model design and implementation, clinical data acquisition, clinical trial methodology and statistical methods. This submission is consistent with the University of Western Australia Doctor of Philosophy rules for the content and format of a thesis and is presented as a series of papers that are either published or formatted for publication.

**Thesis Aims**

The overarching aim of this thesis is to explore the temporal patterns of endothelial glycocalyx biomarkers, and associations between change in these biomarkers over time, and inflammation, organ failure and intervention. Fluid resuscitation has been focused on as the intervention of choice. I hypothesised that endothelial glycocalyx biomarkers would increase over time during Emergency Department treatment, and that the magnitude of change would be positively associated with biomarkers of inflammation, organ failure scores and fluid volumes delivered.

**Thesis Objectives**

- To understand the structure and function of the endothelial glycocalyx and mechanisms of glycocalyx shedding, as relevant to critical illness (Chapter 2 – Literature Review)
- To review clinical studies describing measurement of glycocalyx biomarker concentrations in patients with sepsis (Chapter 3 – Literature Review)
- To describe the temporal pattern of circulating concentrations of three glycocalyx biomarkers during ED treatment of sepsis, and assess
associations between changes over time, cumulative fluid volumes and organ failure scores (Chapter 3 – Paper)

- To review studies characterising the endothelial activation biomarker, endocan, and its relationship with inflammation and critical illness severity, especially respiratory failure (Chapter 4 – Literature Review)

- To describe the temporal patterns of circulating concentrations of two glycocalyx biomarkers with endocan in patients with sepsis secondary to pneumonia, and test associations between these biomarkers and inflammatory biomarker concentrations, respiratory failure, need for mechanical ventilation and mortality (Chapter 4 – Paper).

- To review clinical studies describing measurement of glycocalyx biomarker concentrations in patients with trauma (Chapter 5 – Literature Review).

- To describe the temporal pattern of circulating concentrations of three glycocalyx biomarkers and inflammatory biomarkers during ED treatment of major trauma, compare these changes over time with that of patients with sepsis, and assess associations between glycocalyx biomarkers and cumulative fluid volumes (Chapter 5 – Paper).

- To review studies reporting circulating glycocalyx biomarker concentrations in haemorrhagic shock models and comparison of different fluid resuscitation strategies (Chapter 6 – Literature Review).

- To measure the effect of four different fluid strategies on glycocalyx and inflammatory biomarker concentrations in a canine haemorrhagic shock model (Chapter 6 – Paper).

- To review laboratory and clinical studies examining the effect of hypertonic saline on inflammation, and reduction of subsequent fluid administration in the clinical setting (Chapter 7 – Literature Review).

- To compare the effect of hypertonic saline with isotonic saline on glycocalyx and inflammatory biomarker concentrations when used for fluid resuscitation in ED patients with suspected infection (Chapter 7 – Paper).
References


CHAPTER 2 – Literature Review

Activation or damage to the surface of blood vessels, the endothelium, is implicated as one of the key steps to multiple organ failure in both sepsis and trauma.\(^2\) The endothelial glycocalyx is the most superficial layer of the endothelium, therefore studies exploring biomarkers of endothelial glycocalyx shedding have become prevalent in critical illness research in the last decade. The importance of characterising this shedding, or damage, is not only to improve understanding of the pathophysiology of multiple organ failure but also to investigate therapies to reduce this damage. The focus of this thesis is detection of endothelial glycocalyx shedding early in the treatment of sepsis and trauma, with a particular focus on associations with inflammation and fluid therapy interventions.

This chapter provides a broad overview of the structure, function and alteration of the endothelial glycocalyx (Section 2.1), and then detail on the location and function of individual glycocalyx components (Section 2.2). Focus has been placed on the soluble components that are often measured as clinical biomarkers of glycocalyx shedding, and the relationship between these components and inflammation. This review explores alternative sources of these soluble biomarkers, other than the endothelium, as pertinent to critical illness. Further, conditions that may shed the endothelial glycocalyx are also reviewed, with particular emphasis on those relevant to sepsis and trauma (Section 2.3), as well as the potential pro-inflammatory downstream effects of glycocalyx shedding (Section 2.4).

2.1 The endothelial glycocalyx - general structure and function

Many cells in the body are covered with a mesh-like protective layer, called the glycocalyx, which consists of a carbohydrate scaffold housing many resident molecules. The general structure of the endothelial glycocalyx is composed of proteoglycans, glycosaminoglycans (GAGs), glycoproteins and
soluble components, such as albumin (Figure 1). Proteoglycans, and their associated GAG chains, are ubiquitous molecules that assist with cell to cell, or cell to matrix, interaction. An additional GAG, hyaluronan, is not usually associated with a proteoglycan; instead it is attached to the endothelium via receptors such as CD44 or other GAG molecules. Glycoproteins contribute to the structural scaffold between the glycocalyx and endothelial cells, and include adhesion molecules such as integrins and selectins. Two of these adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), play an important role in leucocyte trafficking during states of inflammation. Soluble components of the endothelial glycocalyx include proteins such as albumin and anticoagulants. All of these components on the endothelial surface can change in composition according to various stimuli, with up- or down-regulation as appropriate. This delicate glycocalyx layer can be prompted to shed in states of inflammation, releasing many of these components into circulation and exposing adhesion receptors on the surface of the denuded endothelium. Measurement of shed proteoglycans and GAGs are among the most commonly used biomarkers for assessment of glycocalyx shedding in vivo.

Figure 1. General structure of the endothelial glycocalyx. The shaded area represents the immobile, relatively protein-poor plasma layer of the glycocalyx. Blood flow occurs above this layer, in a central column within the blood vessel.
The dominant proteoglycans on the endothelial surface are the glypicans (subtype 1) and syndecans (subtypes 1, 2, and 4), each with their own configuration, distribution and unique functions.\textsuperscript{11-13} The glypicans are attached to the endothelial surface via a glycosylphosphatidylinositol anchor whereas the syndecans are transmembrane proteins (Figure 1). It has been suggested that glypican-1 is mainly present on the apical surface whereas syndecans predominate on the basolateral surface,\textsuperscript{12} though most representations of the endothelial glycocalyx feature both proteoglycans across the cell surface. Syndecan-1, in particular, may be more apical than syndecan-2 and -4,\textsuperscript{14,15} though it is unknown if location is important when considering which proteoglycan ectodomains may be shed during critical illness. Glypican-1 also has the additional property of changing its distribution across the surface in response to extracellular conditions.\textsuperscript{15,16} Another glycocalyx proteoglycan, perlecan, is located on the basement membrane\textsuperscript{17} so is perhaps less relevant in regards to shedding at the endothelial-blood interface. Proteoglycans provide a scaffold for the attached sulfated GAG side-chains, which includes heparan, chondroitin and dermatan sulfate. Heparan sulfate is the most abundant GAG on syndecans and glypican-1. This has influenced the nomenclature of these proteoglycans; they are often referred to as heparan sulfate proteoglycans.

The endothelial glycocalyx serves a range of functions, many of which are still under investigation. This includes maintenance of a surface barrier that repels circulating leucocytes, inhibits coagulation, regulates fluid flux and communicates changes in vascular wall shear forces. Negative charge maintained by the sulfated GAGs repels similarly charged cells and other molecules from the endothelial surface.\textsuperscript{18} One caveat to this general rule is albumin: due to its amphoteric properties, positively charged groups within the structure allow for incorporation of albumin into the glycocalyx. Loss of negative charge on the surface of the endothelium is related to extravasation of albumin, therefore maintenance of an electrostatic barrier is important for maintaining normal permeability.\textsuperscript{19-21} The glycocalyx houses anticoagulants, such as antithrombin and tissue factor pathway inhibitor.\textsuperscript{22} This highlights its role in preventing intravascular thrombosis. It also provides a barrier to fluid filtration by
maintaining a protein-poor sub-glycocalyx layer, therefore maintaining an oncotic pressure gradient favouring fluid retention within the vasculature.\textsuperscript{18}

When flow is applied to the vascular wall, the glycocalyx plays an important role in transmembrane signalling, leading to morphological changes in endothelial cells.\textsuperscript{23,24} Heparan sulfate proteoglycans, especially syndecans, play a vital role in this response to shear stress or pressure.\textsuperscript{11,25,26} Transmission of shear stress forces via glypican-1 also increases nitric oxide production, a potent vasodilatory mediator.\textsuperscript{14,17} Changes to the distribution of glycocalyx components can also occur under shear stress. For example, glypican-1, along with its associated heparan sulfate side-chains, moves to the ‘downstream’ edge of the cell under flow conditions.\textsuperscript{15} Components can also be upregulated, including glypican-1 and syndecan-1, whose cell surface expression increases over 24 hours of shear stress conditions.\textsuperscript{27,28} These changes to the distribution of proteoglycans might be important when considering the dynamic changes in shear forces that occur in states of microcirculatory dysfunction, which in turn may influence shedding of individual components in these altered states of blood flow.

Shedding of the endothelial glycocalyx is a vital step after tissue injury in order to facilitate leucocyte and platelet adherence (see Section 1.3 for more detail on shedding).\textsuperscript{29} Shed components of the glycocalyx can also propagate the inflammatory response. Shedding alters capillary perfusion, causing a decrease in functional capillary density,\textsuperscript{30} in addition to increasing endothelial permeability, as mentioned above.\textsuperscript{31} Further details on the downstream effects of glycocalyx shedding are reviewed at the end of this chapter (see Section 1.4). Evidence for the time period over which restoration of the endothelial glycocalyx occurs after shedding is mostly based on \textit{in vivo} cell culture or \textit{ex vivo} vascular models. In cell culture models, structural glycocalyx recovery is delayed up to 7 days,\textsuperscript{23,32} even in systems employing shear stress flow. This is in contrast to the evidence in \textit{in vitro} studies that individual components of the glycocalyx can be regenerated within 24 hours, such as heparan sulfate proteoglycans and hyaluronan.\textsuperscript{27,33} Endothelial damage and downstream inflammatory processes
that may occur after glycocalyx shedding could delay structural repair of the layer.\textsuperscript{32}

These variations in the time taken for glycocalyx regeneration highlight the difficulty in comparing endothelial cell responses between \textit{in vitro} and \textit{ex vivo} models. As mentioned by Becker et al,\textsuperscript{18} there are large differences between the responses of cultured endothelial cells and those \textit{in situ}, not only in the structure of the glycocalyx but also in their permeability, such that ‘cultured cells are at best, a model for endothelium in a chronically inflamed state’. Other authors echo this sentiment. For example, Savery et al stated that ‘...HUVECs (human umbilical vein endothelial cells) do not possess a glycocalyx that is structurally and functionally equivalent to that found \textit{in vivo}.’\textsuperscript{34} These findings have been corroborated by the direct comparison of the \textit{in vitro} endothelial glycocalyx to that observed \textit{ex vivo}.\textsuperscript{35} These limitations of \textit{in vitro} studies hamper progress in understanding the structure and function of the endothelial glycocalyx in any kind of model other than \textit{in vivo} live models using real-time microscopy methods, which faces its own methodologic challenges.

\subsection*{2.2 Key endothelial glycocalyx biomarkers}

Most clinical studies assessing endothelial glycocalyx shedding in critical illness rely on measurement of soluble glycocalyx components, with the most frequent choice being syndecan-1, with or without an additional GAG, such as heparan sulfate or hyaluronan. Other syndecans, such as syndecan-4, have been rarely explored in clinical studies. This section reviews the most frequently reported endothelial glycocalyx biomarkers (syndecan-1, heparan sulfate and hyaluronan) in further detail, including their distribution, individual function and conditions affecting their up- or downregulation. A subsection is dedicated to syndecan-4, given its wide distribution on the endothelial glycocalyx. Glypican-1, though prevalent within the endothelial glycocalyx, has not been historically included in critical illness studies and will not be discussed further.
Syndecans are a family of transmembrane proteoglycans that serve a wide variety of cell signalling functions, enabling key intracellular responses to changes in the extracellular matrix and the circulation (Figure 1). Four syndecan sub-types (-1, -2, -3 and -4) share a basic conserved structure of a cytoplasmic domain, a transmembrane domain and an extracellular domain. It is the extracellular domain, or ectodomain, that differs in structure and length between subtypes, which likely relates to their differences in function. Side-chains of GAGs, mostly heparan sulfate but also chondroitin sulfate, are attached to the ectodomain at multiple attachment sites. The distribution of syndecan subtypes varies with cell type. For instance, syndecan-1 is predominantly expressed on epithelial and mesenchymal cells, whereas syndecan-2 is present predominantly on fibroblasts but has constitutive expression on endothelial cells.

In descriptions of the proteoglycan makeup of the endothelial glycocalyx, syndecan has been presented as a relatively minor component of endothelial cells, compared to perlecan and glypican. However, all syndecan subtypes are present in varying degrees on the endothelium in its resting state, with syndecan-2 and -4 predominating, and syndecan-1 being less present. Many different factors can upregulate or downregulate syndecan expression, such as tissue injury or inflammation, therefore this distribution of syndecans may vary in disease states. For example, in a study assessing HUVEC expression of all four syndecans using a quantitative reverse transcription polymerase chain reaction technique, syndecan-1 and -2 were down-regulated after application of lipopolysaccharide or interleukin-1β, an accepted technique for replicating conditions of sepsis. This is in contrast to syndecan-4, which was upregulated and showed increased surface expression. A different study showed that syndecan-2 was up-regulated after application of tumour necrosis factor-α (TNF-α) to HUVECs. It is interesting to note that syndecan-1 has become the most frequently used biomarker to represent glycocalyx shedding when previous work suggests that syndecan-2 and -4 may also hold value.
When discussing states of inflammation, it is also relevant to consider the cell surface expression of syndecans on neighbouring cells to the endothelium, such as those in circulation. In particular, the surface of leucocytes also bears proteoglycan ectodomains and GAGs. Human macrophages express syndecan-1, -2 and -4, with apparent inter-individual variations in expression, whereas glypican-1 expression is minimal. Syndecan-2 and -4 are also present on human T lymphocytes. Syndecan-1 has also been identified on the surface of neutrophils and its expression may increase in patients with diabetes mellitus.

**Syndecan-1**

Syndecan-1 is one of the most frequently measured endothelial glycocalyx biomarkers yet documentation of its presence on the surface of the endothelium is less consistent compared to other proteoglycans. Most studies have shown low to no constitutive expression of syndecan-1 though one study did show that syndecan-1 on rat endothelium had a similar distribution of expression as glypican-1. The presence of syndecan-1 may not even be essential to the creation of an endothelial glycocalyx, as syndecan-1 deficient mice still displayed a ‘hydrodynamically relevant’ glycocalyx, albeit slightly thinner than wild-type mice. The cell-surface composition of the GAG, heparan sulfate, also appears unaffected by lack of syndecan-1 in mice. Despite this, syndecan-1 plays an important role in some key processes in the body, the majority of which have been characterised for epithelial cells, and its role in cell proliferation and wound healing. Lack of syndecan-1, as demonstrated in knockout mouse models, has important ramifications in response to disease, including enhanced inflammation, increased organ injury after an insult, and delayed wound healing. Syndecan-1 has been shown to play a role in neutrophil chemotaxis, and shedding of syndecan-1 can interfere with transendothelial neutrophil migration.

The distribution of syndecan-1 can be altered during cellular injury or repair. Although syndecan-1 was not detected on the endothelia of normal mouse skin, syndecan-1 expression was increased during dermal wound repair,
as was syndecan-4.42 Similarly, pro-inflammatory cytokines have been shown to increase expression of both syndecan-1 and -4 on mouse glomerular endothelial cells57 and myocardial cells.58 In contrast, another study has shown down-regulation of syndecan-1 mRNA in HUVECs after application of lipopolysaccharide.44 The difference in response to pro-inflammatory stimuli in these studies may be explained by timing of measurements. Upregulation of syndecan-1 on endothelial cells can vary over a matter of hours, such as those induced by shear stress conditions,28 therefore peak changes may be missed. Thus, although syndecan-1 does not feature as a large component of endothelial cell surface proteoglycans, states of illness creating pro-inflammatory conditions may change the cell surface landscape.

The syndecan-1 ectodomain undergoes constitutive shedding, and is shed by various mediators in addition to those mentioned above. One study demonstrated shedding after application of phorbol 12-myristate 13-acetate (PMA), thrombin, plasmin and endothelial growth factors.49 In this study, shedding stimulated by plasmin or thrombin could be inhibited by the presence of serum, which is an important consideration when addressing the effects of haemodilution on glycocalyx shedding (see Section 1.3.3). In regards to accelerated shedding of syndecan-1, although concentrations increased in the cell culture medium, the concentration of syndecan-1 mRNA was not increased and cell surface syndecan-1 was unaffected, or increased. This suggested that stimulation increased the turnover of syndecan-1 at the cell surface, maintaining cell surface concentrations. Hence, states of inflammation in vivo may prove to increase proteoglycan shedding but not necessarily decrease surface expression. Furthermore, measurement of circulating syndecan-1 may not just be a biomarker of loss of the endothelial glycocalyx; it may also indicate increased cell surface turnover.

Shed syndecan-1 ectodomain may also come from other cells in circulation. As mentioned above, studies have demonstrated the presence of syndecan-1 on the surface of some leucocytes, such as neutrophils and lymphocytes.48,59 Interestingly, for B-lymphocytes, it seems that inflammatory
states do not cause shedding of syndecan-1, and actually downregulate its presence on the cell surface, as demonstrated in murine myeloma cells exposed to interleukin-6. Furthermore, it appears that syndecan-1 expression is lost through the maturation stage and is not present on circulating B lymphocytes, though specific circumstances may induce re-expression. Syndecan-1 expression is also low in circulating murine monocytes, but, in contrast to B-lymphocytes, is upregulated in activated murine macrophages. Perplexingly, the up-regulation demonstrated was not accompanied by increased cell surface expression or shedding of syndecan-1, unless accompanied by specific experimental conditions that increase intracellular cyclic AMP. It is unknown if shedding of syndecan-1 ectodomains from leucocytes occurs in vivo during states of inflammation but it is possible that this process may contribute to circulating biomarker concentrations.

**Syndecan 4**

Syndecan-4 has not been typically used as a biomarker of endothelial glycocalyx shedding in previous publications, even though its expression has been documented on endothelial cells. Its absence in clinical biomarker research is curious given that it is, along with syndecan-3, the most abundant constitutive syndecan expressed on the surface of the endothelium. Syndecan-4 is also the most widely distributed proteoglycan in the body, with constitutive expression on most cell types, including endothelial cells, epithelial cells, fibroblasts, macrophages, lymphocytes and neutrophils. The function of syndecan-4 is varied and includes facilitating cell binding to the extracellular matrix and the endothelium, assisting neutrophil migration and playing an anti-inflammatory role during tissue injury. Shedding of the syndecan-4 ectodomain appears to be stimulated in a similar way to syndecan-1, such as in response to PMA, thrombin, plasmin and endothelial growth factors. Similarly, this shedding can be inhibited by the presence of serum under certain conditions.

Upregulation of syndecan-4 on endothelial cells occurs in conditions relevant to critical illness, including response to acute tissue injury.
hypoxia, inflammatory cytokines, shear stress, cardiac pressure overload, procoagulants or exposure to bacterial elements. Upregulation also occurs on other cell types in response to inflammatory cytokines and bacterial elements. Increased expression of syndecan-4 can occur as early as one hour after stimulus, and may peak 24 to 72 hours. One study in HUVECs showed upregulation and increased expression of syndecan-4 within 4 hours of application of both lipopolysaccharide and interleukin-1β, whereas syndecan-1 was downregulated. Results indicated that up-regulation of syndecan-4 is important for modulating inflammation and wound healing. Studies in mice have shown upregulation occurring in multiple organ types in response to bacterial components, including the lung, liver, intestine and kidneys. In particular, one study modelling pneumonia showed that only syndecan-4 was upregulated in response to intra-tracheal lipopolysaccharide, compared to other syndecans. Upregulation of syndecan-4 was also demonstrated in human alveolar epithelial cells, where syndecan-4 expression was increased in response to mycobacteria, whereas syndecans-1, -2 and -3 were not. Therefore, given the propensity for syndecan-4 to be upregulated under these conditions, it is possible that measurement of shed syndecan-4 ectodomains as a biomarker of glycocalyx shedding may be informative in patients with critical illness.

Like syndecan-1, syndecan-4 is also present on the surface of some leucocytes, including monocytes, macrophages, lymphocytes and neutrophils. Its presence serves a range of functions, including intercellular adhesion, response to migration signals and activation of intracellular signalling pathways. Investigation using cell culture has identified upregulation of syndecan-4 mRNA in mouse macrophages in response to lipopolysaccharide as well as after exposure to mycobacteria. The latter study also demonstrated increased macrophage surface expression in murine lung tissue, as well as in human lung tissue from patients with active tuberculosis. Other studies have confirmed expression of syndecan-4 on monocytes or macrophages but not necessarily upregulation in response to stimulation.
Studies assessing the presence of syndecan-4 on the surface of neutrophils have provided mixed results. One study measuring murine syndecan-4 expression after lipopolysaccharide administration used flow cytometry to assess cells of different organs for the expression of two markers; Mac-1 and Ly-6G. Whereas Mac-1 is expressed on both monocytes and neutrophils, Ly-6G is only present on neutrophils. This study found only Mac-1 expression on cells expressing syndecan-4, concluding that this was consistent with monocytes or macrophages. In contrast, an ovine burn and smoke inhalation model detected expression of syndecan-4 on the surface of neutrophils via flow cytometry and observed an increase from 8% to ~50% after injury, consistent with upregulation. Finally, in a study using isolated human neutrophils, a weak signal was found for syndecan-4 on the surface of neutrophils, as detected by flow cytometry, along with some detection of syndecan-4 mRNA via reverse transcriptase polymerase chain reaction, though much less evident compared to HUVECs. Despite the known presence of syndecan-4 on leucocytes, similar to syndecan-1, it is unknown if shedding of syndecan-4 ectodomains from leucocytes, as opposed to endothelium, contributes to circulating biomarker concentrations in vivo.

**Glycosaminoglycans**

The endothelial glycocalyx is rich with carbohydrate chains of GAGs, including sulfated GAGs associated with proteoglycans and hyaluronan. Amongst these molecules, heparan sulfate and hyaluronan are the two GAG biomarkers used to represent endothelial glycocalyx shedding in vivo, though both of these molecules undergo constant constitutive turnover from the endothelium. The functions of the sulfated GAGs overlap with those of proteoglycans, as it is the GAG side-chains that provide the ligand binding capacity for most interactions with circulating cells or proteins. Therefore, there is much overlap between the functions of the syndecans, and those of heparan sulfate.

**Heparan sulfate**

Heparan sulfate, like other sulfated GAGs, helps to maintain the electrostatic charge on the endothelial surface that repels similarly charged cells,
while maintaining incorporation of plasma proteins that are vital to the glycocalyx structure. Heparan sulfate also provides an anticoagulant layer via its interactions with antithrombin. One key function for heparan sulfate is maintenance of a chemokine gradient along the endothelium in order to facilitate leucocyte trafficking. Other immune functions include assisting with rolling and margination of neutrophils during acute inflammation. For example, heparan sulfates associated with syndecan-1 bind chemokines, including interleukin-8 and monocyte chemoattractant protein-1, encouraging a chemotactic gradient for leucocyte migration. Interestingly, soluble heparan sulfate also inhibits elastase, serving a dual function in limiting the cytotoxic effects of the neutrophil at the local site of inflammation. These functions overlap with those of their associated proteoglycans, as mentioned above, and are not necessarily separated in the literature.

Heparan sulfate varies in its structure or function according to location or stimulus. For example, its charge was less negative in areas where leucocyte trafficking is essential, such as venules, compared to areas of minimal leucocyte margination, such as arteries. In another example, exposure to inflammatory cytokines not only increased heparan sulfate expression but also changed the sulfation pattern present on glomerular endothelial cells. Heparan sulfate was also released from activated endothelium after cytokine stimulation, and the increase in free heparan sulfate was important for modulating further leucocyte adhesion. A further study showed that heparan sulfate was not only shed from endothelium after exposure to cytokines but there was evidence of increased turnover on the cell surface. Soluble heparan sulfate also induces cytokine release from various cells and this is discussed in further detail below (Section 1.4). The presence of heparan sulfate on the surface of leucocytes is also important for a range of immune cell functions, in line with those described for leucocyte-associated syndecans.

Hyaluronan

Hyaluronan is a unique GAG molecule within the endothelial glycocalyx; it is a long polysaccharide chain synthesised in the plasma membrane and is not
always associated with a proteoglycan scaffold. It is a ubiquitous molecule throughout the connective tissues of the body, with probably only a small proportion of total body hyaluronan residing on the surface of the endothelium. Linkage of hyaluronan chains within the extracellular matrix, either as free molecules or via binding with proteins such as aggrecan, contributes considerably to the hydrosopic structure and viscoelastic properties of tissue. These properties of hyaluronan has led to its therapeutic use in many domains, including cosmetic dermal augmentation and joint lubrication. The importance of hyaluronan to tissue structure is evident when considering the destructive effects of hyaluronidases, also called ‘spreading factors’, such as those found in venoms or microbial secretions. The hydrosopic properties of hyaluronan likely assist in providing structure to the endothelial glycocalyx.

Unlike sulfated GAGs that are associated with a proteoglycan scaffold, incorporation of hyaluronan into the endothelial glycocalyx is mostly achieved by binding to the CD44 receptor on the endothelial cell. An important role of both hyaluronan and CD44 is facilitating rolling and adhesion of leucocytes in preparation to marginate. For example, hyaluronan on endothelial cells interacts with CD44 on the surface of lymphocytes to aid rolling. In a reciprocal fashion, hyaluronan present on neutrophils interacts with CD44 on endothelial cells. Inflammatory cytokines can upregulate cell surface expression of hyaluronan, including in pulmonary fibroblasts and endothelial cells. Similar to heparan sulfate, the upregulation may be location-dependent. Inflammatory cytokines or ischaemic injury also increases CD44 expression on glomerular endothelial cells. Therefore, hyaluronan appears to play an important role in inflammation as a ligand for CD44.

Hyaluronan present in healthy tissue or fluid mostly exists as a high molecular weight molecule, greater than 1000 kDa. However, inflammation and tissue injury can cause fragmentation of hyaluronan. Reactive oxygen species can also degrade hyaluronan in a non-enzymatic fashion, a process that may be relevant during critical illness. As low molecular weight
hyaluronan can act as a pro-inflammatory molecule, fragmentation of hyaluronan at high concentrations may contribute to the ‘inflammatory storm’ of critical illness. This is covered in more detail in Section 1.4. The half-life of hyaluronan ranges from hours to 5 days for most tissues. Breakdown of hyaluronan via endogenous hyaluronidases can occur in situ or within lymph nodes, or hyaluronan can be transported back into circulation via the lymphatic ducts. The CD44 receptor plays an important role in clearance of low molecular weight hyaluronan from sites of inflammation, with macrophages involved in the post-injury or infection clean-up. Once in circulation, removal of hyaluronan is rapid via hepatic metabolism, with only a small amount metabolised and excreted by the kidneys. Rapid clearance of hyaluronan should be considered during investigation of temporal patterns of serum hyaluronan concentration as a biomarker of glycocalyx shedding.

2.3 Mechanisms of proteoglycan and glycosaminoglycan shedding

The previous section discussed the general structure and function of the endothelial glycocalyx and its individual components, including the unique structure, function and interaction with other molecules. Mechanisms of upregulation and shedding of individual glycocalyx components have been briefly covered. This section reviews general mechanisms of shedding of the endothelial glycocalyx structure as a whole, bringing focus on mechanisms that are pertinent to critical illness, including effects of inflammation, natriuretic peptides, endothelial sheer stress and haemodilution. This section summarises literature from mostly in vitro studies that provides mechanistic evidence for these effects. Further detail from animal models or clinical studies is provided in the Background sections of each subsequent chapter.

Inflammation

Shedding of the endothelial glycocalyx facilitates exposure of endothelial cells and their adhesion molecules to circulating leucocytes and platelets. It is one of the first steps in acute inflammation that is necessary for leucocytes to
adhere and marginate into the extravascular space, towards the site of injury or infection. Studies that explore the interaction between inflammation and glyocalyx shedding usually examine three different aspects: removal of glyocalyx components and subsequent stimulation of inflammation; use of genetically-modified rodents that lack certain components; or imaging of the glyocalyx to determine effects of inflammatory mediators on glyocalyx thickness.

An inflammatory mediator that has been extensively explored is TNF-α. It is a potent pro-inflammatory cytokine released from activated macrophages and T-lymphocytes as a part of the innate immune response, whose main function is to activate endothelial cells and neutrophils. Modulation of the endothelial glyocalyx by TNF-α has been demonstrated in both in vitro and in vivo studies. Endothelial cells in culture exposed to TNF-α have shown shedding of both syndecan-4 and sulfated GAGs, as well as upregulation of syndecan-4 mRNA and increased GAG turnover at the cell surface. Furthermore, a reduction in thickness of the endothelial glyocalyx following TNF-α treatment has been demonstrated using a dye exclusion technique in hamster cremaster muscle. Shedding has also been visualised, via electron microscopy, in guinea pig coronary arteries exposed to TNF-α, which was accompanied by a spike of heparan sulfate release, as well as sustained release of syndecan-1. In contrast, some studies have shown opposing effects of TNF-α on the endothelial glyocalyx. Tumour necrosis factor-α applied to HUVECs reduced syndecan-1 mRNA expression in a dose dependent fashion; detectable at 6 hours after application, with maximal effect at 10 hours. This was accompanied by downregulation of syndecan-1 surface expression, which was maximal at 24 hours. The contrasting results of these studies highlight the complexity of proteoglycan expression and shedding at the endothelial surface.

Other inflammatory cytokines also induce shedding of endothelial glyocalyx components, including interleukin-1 and C-reactive protein, whereas other cytokines, such as interukin-6 and interferon-γ, have not demonstrated shedding properties. In one study, shedding of sulfated GAGs was
accompanying loss of anionic charge across the endothelium. Co-culturing endothelial cells with neutrophils also resulted in increased shedding of sulfated GAGs, which was not repeatable in isolated neutrophil culture without endothelial cells present. Similarly, studies have examined the effects of tissue injury, which inevitably involves a component of inflammation, on the endothelial glycocalyx. For example, conditions of ischaemia-reperfusion injury in rodents causes glycocalyx shedding, which can be prevented by provision of antioxidants. These shedding effects in rodent models appear to occur early, before a measurable inflammatory response develops. Reactive oxygen species in endothelial cell culture can also overwhelm the embedded antioxidants in the glycocalyx and cause shedding of GAGs from endothelial cells. Reactive oxygen species can also cause fragmentation of soluble hyaluronan, generating a pro-inflammatory low molecular weight hyaluronan.

In addition to inflammatory cytokines, activated neutrophils and platelets release polycationic proteins, which disrupt the endothelial barrier via binding of anionic sites on sulfated GAGs. Polycations cause increased vascular permeability, loss of circulating albumin and non-inflammatory-type tissue oedema. These effects can be mitigated by provision of albumin or heparin, which presumably compete for anionic binding sites on sulfated GAGs. In one in vitro study, the protective effect of foetal bovine serum was timing-dependent; the prevention of albumin ‘leakage’ in cell culture was lost when serum was added after exposure to the polycation. Although these studies did not assess glycocalyx thickness specifically, other studies did demonstrate vasoconstriction and morphological changes to the endothelium after exposure to polycations. It is possible that binding of polycations to sulfated GAGs might alter the structure of the glycocalyx, however this requires further investigation. Leucocyte proteases also degrade the endothelial glycocalyx, including matrix metalloproteinases and neutrophil elastase.

Other mediators that tie in closely with the acute inflammatory cascade include activated components of the coagulation system. Two of these, plasmin and thrombin, cleave syndecan ectodomains. Thrombin production is an
important component of normal clot formation but also has pro-inflammatory and platelet activation properties. These studies show that there is a close link between coagulation and glycocalyx shedding, as there is with inflammation and glycocalyx shedding.

A pro-inflammatory and hypercoagulable state is created by the syndrome of sepsis due to the host’s response to microbial invasion of tissues. Therefore, the above mechanisms for both inflammation and products of coagulation causing glycocalyx shedding are highly relevant to sepsis. Bacterial components or secretions, such as lipopolysaccharide and chemotactic peptides, also directly affect the endothelial glycocalyx. Lipopolysaccharide upregulates the expression of syndecan-1 and -4 on the endothelium of murine myocardium, as well as accelerate shedding of these ectodomains. Shedding and increased synthesis of sulfated GAGs also occurs with the addition of lipopolysaccharide to endothelial cell culture, either alone or in combination with inflammatory cytokines. The effects of lipopolysaccharide have also been demonstrated in vivo, with Okada and others visualising shedding of the endothelial glycocalyx in mice using transmission electron microscopy, 48 hours after lipopolysaccharide infusion. This was accompanied by a gradual increase in serum syndecan-1 concentration, peaking at 24 hours. The bacterial peptide, formylmethionyl-leucyl-phenylalanine (fMLP), is often used experimentally to induce an inflammatory response and can also shed GAGs from the endothelial glycocalyx. Release of these types of molecules during sepsis may accelerate the systemic inflammatory response by their direct effects on endothelial glycocalyx shedding. Although models utilising components of infection, such as lipopolysaccharide, to induce inflammatory states do not mirror the clinical syndrome of sepsis, it stands to reason that these elements may contribute directly to the assault on the endothelial glycocalyx.

**Natriuretic peptides**

Natriuretic peptides include a family of peptide hormones that are mainly synthesised in the cardiac myocytes, though other sources exist such as endothelial, nerve and kidney cells. Atrial and brain natriuretic peptide
(ANP and BNP, respectively) are secreted by both the atrial and ventricular myocardium in response to myocyte stretch, via pressure or volume overload. These molecules promote natriuresis in response to hypervolaemia, though many other paracrine and autocrine functions have been identified. C-type natriuretic peptide (CNP) is expressed by in the nervous system as well as by endothelial cells, where it has vasoactive and cell growth effects.

Conditions eliciting atrial and ventricular stretch are frequently encountered in critically ill patients due to the use of fluid bolus therapy. Rapid infusion of crystalloid fluids, which may cause cardiac chamber stretch, is standard of care for improving circulation in patients with either sepsis or trauma. Given that natriuretic peptides can increase vascular permeability, interest has arisen in their role in causing glycocalyx shedding in states of critical illness. All three major natriuretic peptides (ANP, BNP and CNP) can shed the endothelial glycocalyx, as measured by increased syndecan-1 and heparan sulfate concentrations in the effluent of guinea pig coronary arteries, accompanied by increased vascular permeability. These studies also visualised glycocalyx destruction via electron microscopy.

Several human studies have demonstrated that bolus fluid therapy causes an increase in ANP, as well as shedding of the glycocalyx. In a pilot study of surgical patients subject to volume loading under general anaesthesia, both ANP and glycocalyx biomarkers (hylauronan and syndecan-1) increased immediately after a bolus of hydroxyethyl starch solution, whereas the same effect was not seen in patients undergoing normovolaemic haemodilution. Another study in periparturient women examined ANP concentration and glycocalyx shedding after fluid loading to prevent hypotension associated with spinal anaesthesia. This scenario has been previously shown to rapidly increase plasma ANP concentration. However, although an increase in plasma heparan sulfate and syndecan-1 concentration was observed after the crystalloid fluid bolus, there was no significant increase in serum ANP concentration. Finally, a small study in on-pump coronary artery bypass patients identified an early rise in both ANP and heparan sulfate at the onset of cardiopulmonary bypass, followed by
increases in syndecan-1, hyaluronan and inflammatory cytokines (interleukin-6, -8 and -10) after removal of the aortic cross-clamp. A limitation of the above studies mentioned was indexation of glycocalyx biomarkers to serum albumin concentration, which may not accurately reflect the degree of haemodilution in states of increased vascular permeability. The challenges of accounting for haemodilution in resuscitation research are discussed further in Chapter 5.

**Endothelial shear stress and haemodilution**

In addition to causing release of natriuretic peptides, fluid bolus therapy also dilutes plasma components. *In vitro* research examining glycocalyx shedding has provided evidence that dilution of plasma, or lack of provision of plasma, either increases shedding of glycocalyx components or reduces glycocalyx thickness. Pries and others used mathematical modelling of vascular resistance, derived from a meta-analysis of studies, to show that dilution of blood with artificial media reduced vascular resistance independent of haematocrit and colloid osmotic pressure. There was less change in resistance after haemodilution with prior heparinase infusion, which would remove the glycocalyx prior to the experiment. The authors inferred that the loss of resistance was due to removal of the endothelial glycocalyx, secondary to haemodilution. Prior studies showed perfusion of blood vessels with crystalloid solution increased capillary permeability, compared to albumin solution, supporting the conclusion that crystalloid solutions change the surface of endothelium.

Perfusion with plasma appears to restore endothelial permeability after crystalloid infusion to a greater degree than albumin alone. *In vitro* studies have explored the differences between plasma and crystalloid solution on changes to the endothelium. One study, using human pulmonary endothelial cells in culture, found that lactated Ringer's solution increased both endothelial permeability and leukocyte binding compared to incubation with fresh frozen plasma. Other studies exploring mechanisms of endothelial glycocalyx
sheding have found that incubation of cell cultures with serum can prevent glycocalyx shedding.\textsuperscript{49,122} It is likely that there are constituents within serum or plasma, beyond albumin, which bind to the endothelial surface and help rebuild its structure.

Experimental \textit{in vivo} studies, both in healthy human subjects and rodent haemorrhagic shock models, have compared the effects of crystalloid solutions and plasma (or albumin) administered rapidly. One study compared 7.1 mL/kg of 4\% albumin solution, 7.1 mL/kg of dextrans 40 and 21.4 mL/kg of Ringer’s acetate solution administered to euvolaemic healthy male volunteers in a crossover design.\textsuperscript{146} Those given crystalloid fluid had a rapid increase in plasma hyaluronan concentration, whereas the other fluid types did not elicit this response. It is unclear if this effect was due to more marked hypervolaemia, and therefore greater release of natriuretic peptides, or greater dilution of plasma constituents causing glycocalyx shedding. Several rodent models of haemorrhagic shock have compared large volume crystalloid fluid resuscitation with smaller-volume blood products, such as plasma or whole blood.\textsuperscript{145,147-151} Large-volume crystalloids were associated with decreased glycocalyx thickness, as well as increased plasma syndecan-1 and heparan sulfate concentration. Several studies also demonstrated other effects of crystalloid fluid such as increased microvascular permeability, pro-inflammatory effects and organ injury in the crystalloid groups, compared to the mice that received blood products.\textsuperscript{145,150,151} Review of the literature in further detail concerning glycocalyx shedding after fluid resuscitation in haemorrhagic shock models is found in Chapter 6.

One of the major limitations of studies comparing different methods of fluid resuscitation, such as those mentioned above, is difficulty in separating effects of variability in blood volume expansion with different types of fluid products and the effects of haemodilution. Some products may cause transient hypervolaemia and natriuretic peptide release. However, two studies, have examined the effect of haemodilution separately to the degree of blood volume expansion. The first study used a rodent haemorrhagic shock model to examine
the effects of haemodilution while keeping the degree of blood volume expansion constant. They compared the administration of 15 mL/kg of three different preparations of blood products; fresh whole blood, a standard preparation of packed red blood cells and a washed preparation of packed red blood cells. Glycocalyx thickness was reduced, and plasma heparan sulfate concentration increased, after the washed preparation but not after fresh whole blood or standard packed red blood cells, implying that the lack of plasma proteins in the washed preparation contributed to glycocalyx shedding. The second study examined the effects of degree of blood volume expansion while keeping haemodilution constant. They compared two different states of haemodilution in cardiac surgical patients: one group with normovolaemic haemodilution (target haematocrit; 24%) and one with hypervolaemic haemodilution (20 mL/kg) using a balanced low molecular weight hydroxyethyl starch (Volulyte®). The hypervolaemic group showed a significant increase in serum ANP, syndecan-1 and hyaluronan concentration directly after fluid administration, whereas the normovolaemic group did not. This study does have the limitation of indexation to albumin concentration however the authors also measured urine concentration of syndecan-1, which replicated the results in serum. The results of both of these studies suggest that both hypervolaemia and haemodilution play a role in endothelial glycocalyx shedding.

Fluid bolus therapy may also create some increase in shear stress on endothelial cells. Cell culture studies focusing on the endothelial glycocalyx have found that shear stress, compared to static conditions, increases incorporation of GAGs into the glycocalyx and increases GAG shedding into the supernatant. These studies support that shear stress conditions promote reinforcement, rather than destruction, of the glycocalyx, however an increase in shear stress may increase circulating concentrations of glycocalyx components, such as hyaluronan, due to increased protein synthesis. This effect should also be considered when interpreting increased circulating glycocalyx biomarkers after rapid fluid administration.

**Other factors that cause glycocalyx shedding**
There are other factors that cause endothelial glycocalyx shedding that may be relevant to critical illness, such as hyperglycaemia,\textsuperscript{154} oxidised low-density lipoproteins,\textsuperscript{125} cancer-derived sheddases,\textsuperscript{155} smoking,\textsuperscript{156} or even simply ageing.\textsuperscript{157} These factors will not be covered further.

### 2.4 Shedding of the glycocalyx may lead to inflammation

One premise for the importance of glycocalyx shedding in critical illness is that it may amplify inflammation, and thus contribute to development of multiple organ failure and subsequent death. This literature review has, so far, discussed the effects of inflammatory mediators on glycocalyx shedding. However, studies have also explored whether shedding the glycocalyx, as the inciting event, leads to an inflammatory response. Cell culture models that explore mechanisms of glycocalyx shedding often use agents that shed GAGs from the cell surface, such as heparanase or hyaluronidase. Such models have found that enzymatic removal of GAGs from endothelial cells \textit{in vitro} leads to increased ICAM-1 expression, leucocyte adhesion, upregulation of nuclear factor-\kappa B and increased inflammatory cytokine production.\textsuperscript{23,158-160} Glycocalyx shedding induced by fMLP, a chemotactic peptide, also leads to increased leucocyte adhesion in rat mesenteric venules.\textsuperscript{120} Shedding the glycocalyx by oxidised low-density lipoproteins has also shown to transiently increase platelet adhesion to the endothelium.\textsuperscript{125}

Shedding of the glycocalyx is thought to unveil binding sites for circulating cells. The relationship between shedding and subsequent inflammation has been implicated as an important pathophysiologic component of many syndromes, including reperfusion injury, diabetes and congestive heart failure.\textsuperscript{161} Conversely, other studies have shown conflicting results, with selective removal of GAGs decreasing monocyte interactions with TNF\alpha-activated endothelium,\textsuperscript{162} and with epithelial cells.\textsuperscript{91} This highlights the complexity of glycocalyx shedding and the role GAGs play in cell adhesion. The type and magnitude of alteration of the endothelial cell surface, for example
selective GAG removal or destruction of the entire layer, may determine the ultimate effects on the immune system.

Shedding of the glycocalyx releases many different components into circulation that have a variety of biological actions. One of these downstream effects is the stimulation of further inflammation, by these components acting as danger-associated molecular patterns (DAMPs). This seems mostly relevant to heparan sulfate and fragmented hyaluronan, both heavily distributed across the endothelial glycocalyx. Soluble heparan sulfate molecules play a key role in modulating inflammation, including leukocyte activation, increasing production of cytokines and endothelial activation. Soluble heparan sulfate may also limit the cytotoxic effects of the neutrophil at the local site of inflammation. A similar function of binding neutrophil end-products has been identified for syndecan-1 and -4 ectodomains; however, this may be related to the presence of GAG side-chains. Shed heparan sulfate can also bind to Toll-like receptor-4, inducing a pro-inflammatory response. One study showed infusion of heparan sulfate into murine pancreas induced neutrophil infiltration and increased myeloperoxidase activity, likely via Toll-like receptor-4 activation. In this way, heparan sulfate behaves as a DAMP, similar to high mobility group B1 and heat shock proteins. In addition, heparan sulfate can induce inflammatory cytokine release from macrophages and B lymphocytes, as well as increase expression of adhesion molecules on macrophages and cardiac myocytes.

Similar to heparan sulfate, hyaluronan can also be pro-inflammatory, though this effect is restricted to fragmented or low molecular weight hyaluronan. The role of hyaluronan acting as a DAMP is important in tissue injury when hyaluronan is abundant, but also likely important for responding to certain microbes coated in hyaluronan, such as certain Streptococcus and Pasteurella species. When low molecular weight hyaluronan (<200kD) was added to macrophages, increased mRNA production of a range of inflammatory mediators via the Toll-like receptor-2 pathway was noted. Another study used hyaluronan fragments (<500kD) to induce chemokine mRNA production from mouse alveolar macrophages, evident within 3 hours of incubation. This study
also found that other GAGs, such as chondroitin sulfate, heparan sulfate or dermatan sulfate, did not upregulate these chemokines. Similarly, hyaluronan fragments upregulated interleukin-8 mRNA from human alveolar macrophages after a 6-hour stimulation. A study using a different human cell line found that low molecular weight hyaluronan from human umbilical cord stimulated interleukin-10 production from human monocytes after an 18-hour incubation.\textsuperscript{169} Low molecular weight hyaluronan has also been shown to activate dendritic cells via the Toll-like receptor-4 pathway, leading to secretion of pro-inflammatory cytokines over 4 to 24 hours of co-incubation in cell culture.\textsuperscript{170} Therefore, it is possible that shedding of the endothelial glycocalyx may promote inflammation by release of hyaluronan fragments. Further to this, as discussed above, inflammation and reperfusion injury may perpetuate the response by releasing mediators that fragment hyaluronan in circulation.

2.5 References


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CHAPTER 3 - Endothelial glycocalyx biomarkers in clinical sepsis

3.1 Gap in knowledge

Sepsis is associated with endothelial glycocalyx shedding and dysfunction, which may form an important part of the pathophysiology of multiple organ failure. Studies have shown that biomarkers of endothelial glycocalyx shedding are increased in patients with sepsis. However, these studies have all been conducted in the intensive care unit (ICU), usually well after interventions have commenced. The few studies that have measured these biomarkers over time during hospitalisation have shown a decreasing pattern, consistent with an improving clinical course. However, the pattern of endothelial glycocalyx shedding has not been examined early in the treatment of sepsis and it is unknown if these biomarkers may increase during the time of initial intravenous fluid loading, especially given rapid fluid administration has been associated with inducing endothelial glycocalyx shedding.

The background information provided below reviews clinical studies on endothelial glycocalyx shedding that were published up to and including 2017, providing supporting literature to preface the observational study that measured glycocalyx biomarkers over time in patients with simple infection and sepsis. The results of this study were published in *Journal of Critical Care* in 2017\(^1\) and the manuscript is presented here as published.

3.2 Background information

According to the latest international consensus definitions, sepsis is defined as ‘life-threatening organ dysfunction caused by a dysregulated host response to infection’.\(^2\) Previous definitions had included those with systemic response to infection but without organ dysfunction,\(^3\) however the new definition published in 2016 seeks to refine the syndrome to those with more
severe features. This dysregulated response to infection includes a massive release of pro-inflammatory and anti-inflammatory cytokines, as well as other cell signalling molecules including complement, danger-associated molecular patterns, reactive oxygen species and neutrophil extracellular traps. Characterisation of the early phase of this innate immune response has led researchers to focus on the endothelium as a key player in propagating inflammation and contributing to organ dysfunction. Endothelial activation and ‘dysfunction’ leads to structural changes in the cells, such as changes in the expression of adhesion molecules, cell swelling and increased permeability. Shedding of the endothelial glycocalyx is likely one of the first steps in this process.

Endothelial glycocalyx shedding in clinical sepsis has been inferred by observing increased circulating concentrations of glycocalyx biomarkers, predominantly syndecan-1, heparan sulfate and hyaluronan. The first documentation of an elevation in these biomarkers in sepsis was a study published by Berg and authors in 1998, who measured plasma hyaluronan concentration in 44 patients with ‘severe infection’. The highest concentrations were found in patients with septic shock, compared to patients without septic shock. Proposed mechanisms for elevated hyaluronan in sepsis at the time included increased tissue hyaluronan synthesis, increased ‘washout’ of hyaluronan from the interstitium and decrease hepatic uptake. This was further explored by the same author in 2002, when human male volunteers given an infusion of isotonic crystalloid fluid showed a large increase in plasma hyaluronan concentration, whereas the same effect was not seen with albumin or dextran infusion. Again, this was attributed to tissue washout of hyaluronan.

It was not until 2008 that increased circulating glycosaminoglycan (GAG) concentrations in patients with sepsis, such as hyaluronan, were attributed to endothelial glycocalyx shedding. In this particular study, GAGs were measured by an Alcian blue slot binding assay in 18 patients with septic shock; a technique that does not differentiate between types of GAGs. Plasma GAG concentrations
were significantly higher in non-survivors than survivors. Plasma syndecan-1 concentration was also elevated in patients with septic shock, compared with patients scheduled for craniotomy, and was moderately correlated ($r=0.69$, $P<0.01$) with cardiovascular Sequential Organ Failure Assessment (SOFA) score. The authors also found that GAGs inhibited the antibacterial properties of plasma, possibly by binding antimicrobial peptides, creating a plausible link between increased circulating GAGs and poorer outcomes in sepsis. Several more studies on increased hyaluronan or GAG concentrations in patients with sepsis soon followed, showing positive associations with illness severity and inflammatory biomarker concentrations.

Multiple studies have also emerged showing increased concentrations of syndecan-1, predominantly, and heparan sulfate in patients with sepsis. Associations between syndecan-1, at a single point in time, and sepsis severity or SOFA score have also been demonstrated in other cohorts of patients with sepsis, in addition to those mentioned above. Several ICU studies have assessed change in syndecan-1 concentration over time in sepsis, with some of these also investigating GAG concentration. One of the earliest studies compared these biomarkers between patients with sepsis ($n=104$) and a control population of patients that had undergone major abdominal surgery ($n=28$) without a systemic inflammatory response. Plasma concentrations of biomarkers were measured at either the time of sepsis diagnosis or at the end of surgery, and then 6, 24 and 48 hours later. In addition to endothelial glycocalyx shedding, the authors also assessed endothelial activation (intercellular adhesion molecule-1, vascular adhesion molecule-1) and inflammation (interleukin-6). They found that patients with sepsis had significantly higher syndecan-1, compared to healthy controls and patients with abdominal surgery, and that this concentration remained elevated over time. In contrast, heparan sulfate was higher in the abdominal surgery patients, compared to sepsis, throughout the study, though both groups had higher concentrations than healthy controls. A significant correlation was found between syndecan-1 and interleukin-6, however no correlation coefficient was reported.
Another study assessing plasma syndecan-1 concentration over time in patients with septic shock (n=20) found that syndecan-1 was higher than healthy control concentrations, and was not significantly different between measurement on day 1 and day 4 of ICU hospitalisation.\textsuperscript{17} There was also a significant positive correlation between syndecan-1 and SOFA score (\(\rho=0.67\)). Finally, a more recent study measured both syndecan-1 and hyaluronan on days 1, 3, 5 and 7 of ICU hospitalisation.\textsuperscript{18} Overall, peak concentrations were identified on day 1 with a falling trend over time. However, this pattern differed for non-survivors, whereby both biomarkers trended upwards or stayed static over time. Like other studies, they found a positive association between both biomarkers and scores indicating the severity of sepsis.

Syndecan-1 has generally been the only glycocalyx proteoglycan used in clinical sepsis studies to reflect endothelial glycocalyx shedding. However, syndecan-4 is possibly better distributed across the endothelium and is upregulated during inflammation (see Section 2.2). Despite limited studies measuring circulating syndecan-4 in patients with sepsis, there are several studies that measured this biomarker in patients with pneumonia, due to there being evidence for upregulation of syndecan-4 in the inflamed lung. One study compared syndecan-4 concentration between children with viral infection and children with bacterial infection (n=110), and found no difference between the groups.\textsuperscript{19} They also found no difference between the first sample and a sample taken 5 days later. A second study measured serum syndecan-4 concentration in adults with pneumonia (n=30) on admission, then 8 and 15 days later.\textsuperscript{20} They found higher syndecan-4 on admission, compared to healthy controls (n=11), though the healthy control group was skewed towards a younger age. When dichotomised into mild and moderate/severe pneumonia, they found that syndecan-4 in the mild group remained higher than control whereas the moderate/severe group was no different. There was also a weak negative correlation with pneumonia severity score (\(\rho=-0.391\)). Patients that responded to a short course of antibiotics had syndecan-4 concentrations that generally increased over the 15-day period, whereas patients that did not respond had static concentrations. Another study in adults with idiopathic
interstitial pneumonia measured syndecan-4 concentration during stable disease and then during an acute exacerbation. Syndecan-4 was lower during acute exacerbation, compared to stable disease, and was significantly higher during stable disease compared to healthy volunteers. During acute exacerbation, it was weakly positively correlated with white blood cell count (Spearman’s correlation coefficient 0.426, P=0.001). An interesting pattern emerging from these studies is that low concentrations of circulating syndecan-4 may be present in acute infection, in contrast to the elevated concentrations observed with syndecan-1. However, this biomarker has not been measured in a septic population specifically, and patterns of shedding over time in the acute phase have not been characterised. Therefore, this biomarker was added to the investigation that was performed.
3.3 Endothelial glycocalyx biomarkers increase in patients with infection during Emergency Department treatment

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Abstract

Purpose: Endothelial glycocalyx (EG) shedding may promote organ failure in sepsis. This study describes temporal changes in EG biomarkers from Emergency Department (ED) arrival, and associations with clinical characteristics.

Materials and Methods: This prospective observational study included 23 patients with simple infection, 86 with sepsis and 29 healthy controls. Serum EG biomarkers included syndecan-1, syndecan-4 and hyaluronan. Samples were taken on enrolment in the ED (T0), 1 hour (T1), 3 hours (T3) and 12-24 hours (T24) later.

Results:
Syndecan-1 concentration increased incrementally over time (T0 to T24, both patient groups, \( P<0.001 \)) whereas hyaluronan concentration peaked at T3 (T0 to T3, sepsis group, \( P<0.001 \)). Hyaluronan was positively associated with cumulative fluid volumes (\( P<0.001 \)) at T0, T1 and T3, independent of illness severity. Both syndecan-1 (OR 1.04, 95% CI 1.01-1.07, \( P=0.017 \)) and hyaluronan (OR 1.83, 95% CI 1.46-2.30, \( P<0.001 \)) were associated with organ failure, independent of age and comorbidity. Syndecan-4 concentration was not different between groups or over time.

**Conclusions:** In contrast to previous ICU studies, EG biomarkers increased during the first 24 hours of sepsis treatment and were associated with fluid volumes and organ failure. Further investigation is required to determine if interventions delivered in the ED contribute to EG shedding.

**Key words**
Glycocalyx, endothelium, sepsis, infection, fluids

**Abbreviations**
- CCS  Charlson Comorbidity Score
- CISS  Critical Illness and Shock Study
- ED  Emergency department
- EG  Endothelial glycocalyx
- ELISA  Enzyme-linked immunosorbent assay
- ICU  Intensive care unit
- NGAL  Neutrophil gelatinase-associated lipocalin
- SOFA  Sequential organ failure assessment
Introduction

Multiple organ failure is central to the morbidity and mortality associated with sepsis. A key player in the pathogenesis of organ failure is endothelial dysfunction, featuring increased fluid extravasation, microthrombosis and loss of vasomotor tone. Increased endothelial activation early in the treatment of sepsis is associated with organ failure severity and mortality. More recently, it has been discovered that endothelial activation is preceded by shedding of its luminal surface layer, the endothelial glycocalyx (EG). Shedding of the EG can be initiated by inflammatory cytokines and pathogen-associated molecules, such as lipopolysaccharide, and is associated with endothelial-leukocyte adhesion and increased vascular permeability. Therefore, it has been postulated that EG shedding is an important part of the pathogenesis of multiple organ failure in sepsis.

Biomarkers of circulating EG components, such as syndecan-1 and hyaluronan, are increased in patients with sepsis and are associated with illness severity, sequential organ failure assessment (SOFA) score, and mortality. Studies in the intensive care unit (ICU) assessing EG biomarker concentrations over time have reported the highest concentrations at ICU admission, with a decreasing trend during the ICU stay. Patients admitted to the ICU from the emergency department (ED) represent the greatest proportion of ICU patients with sepsis. Interventions given to patients in the ED may increase EG biomarkers in the first 24 hours of treatment, partly explaining high concentrations found at ICU admission.

A frequent intervention given early in the treatment of sepsis is intravenous fluid boluses, with most volume loading occurring in the first 6 hours of treatment. Crystalloid fluid boluses have been associated with EG shedding in multiple studies. Therefore, it is plausible that the initial resuscitation period of treating sepsis may be associated with increasing EG biomarker concentrations. Understanding the time course of EG shedding in sepsis, and any relationships with therapeutic intervention, is important for...
devising strategies aimed at mitigating this injury and improving clinical outcomes.

The main objective of this study was to compare the pattern of EG shedding in patients with either simple infection or sepsis over the first 24 hours from ED arrival, as determined by three different EG biomarkers. We hypothesized that EG biomarker concentrations would increase during this time period. A secondary objective was to investigate associations of each EG biomarker with fluid volumes administered, independent of infection severity and degree of inflammation, as well as clinical outcomes.

**Materials and methods**

*Study design*

Patients meeting criteria for sepsis in the ED were identified from the Critical Illness and Shock Study (CISS) (HREC permit number 2009-080), which is an observational database of patients meeting physiologic criteria consistent with critical illness (Figure 1). As previously described, CISS enrolment criteria focused on patients with evidence of shock or organ failure. Formal written consent was obtained from patients or next-of-kin. Patients underwent real-time data collection and research blood sampling during the initial 24 hours from enrolment, and were then followed for clinical outcomes. Recruitment into CISS occurred during rostered research nurse hours 0700-2100, up to 7 days of the week. This study included sequential CISS enrolments meeting sepsis criteria between January 2012 and July 2013 that were a subset of a larger sepsis biomarker study. Sepsis was defined as meeting at least 2 of 4 SIRS criteria [27]; temperature >38°C or <36°C, heart rate >90 bpm, respiratory rate >20bpm or white cell count >12x10⁹/L or <4x10⁹/L, as well as clinical suspicion of infection and administration of intravenous antibiotics. Patients were excluded if there was a clear alternative diagnosis not consistent with infection, based on review of medical record and discharge summary. Healthy control samples were selected based on age and sex-stratification to approximate the distribution of the infection group.
**Patient data collection and categorization**

Patients were classified into two infection severity categories, according to 2012 Surviving Sepsis Campaign definitions.\(^{39}\) To correspond with recently updated sepsis definitions,\(^{2}\) simple infection was defined as having a SOFA score <2,\(^{40}\) with no requirement for subsequent organ support, and sepsis was defined as a SOFA score ≥2, thus combining severe sepsis and septic shock into a single group. The SOFA score was calculated from parameters collected on the first day of hospitalization. Source of infection was decided based on clinical judgement, informed by review of microbiology results and subsequent clinical course. Charlson Comorbidity Score (CCS)\(^{41}\) was retrieved from the CISS database. Cumulative fluid volume at each blood sampling time point was calculated for each patient, based on patient records, and included isotonic or hypertonic crystalloid and gelatine artificial colloid fluid. The calculated volume at each time point included all fluids given up until that time, including pre-hospital fluids.

**Biomarker measurement**

Blood samples were collected soon after enrolment in the ED (T0) and then 1-2 hours (T1), 3-5 hours (T3) and 12-24 hours later (T24). Samples were collected into a serum clot tube then centrifuged at 3000rpm at 4°C for 10 minutes. Serum was then aliquoted into cryogenic tubes and stored at -80°C. Biomarkers syndecan-1, syndecan-4 and hyaluronan were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Inflammatory cytokines interleukin-6, interleukin-10 and neutrophil gelatinase-associated lipocalin (NGAL) were also measured at each time point using either a commercial multiplex cytometric bead array kit (interleukin-6, interleukin-10), as previously described,\(^{42}\) or commercial ELISA kit (neutrophil gelatinase-associated lipocalin) (R&D Systems, Minneapolis, MN, USA). Samples were repeated if the intra-assay or inter-assay CV was >15% or if the result was outside the standard curve range, with an adjustment made to dilution factor.

**Statistical analysis**
Normality of data was assessed by visual inspection of histograms and Q-Q plots. Participant characteristics were summarized using number (proportion) for categorical variables, and median [Q1-Q3] or mean (95% confidence interval) for continuous variables depending on normality. Differences between patient groups for participant characteristics were tested using student’s t-test for normally distributed continuous variables, chi square test for dichotomous variables, negative binomial or truncated negative binomial regression for count variables and Kruskall Wallis for ordinal or continuous variables not normally distributed. Biomarker concentrations were log-transformed to produce normal or approximately normal distributions and summarized using geometric mean (95% confidence interval). Comparisons between groups for biomarker concentrations at T0 were tested using analysis of variance and, where significant, pair-wise comparisons were made.

Comparisons of biomarker concentration over time between simple infection and sepsis were made using the interaction of time and group in linear mixed regression models. These models use maximum likelihood estimation, which retains patients with incomplete outcome data in the regression analysis, and is known to produce unbiased estimates under the assumption of missing at random. Models were bootstrapped to obtain robust \( P \) values for biomarker concentrations that remained slightly skewed following log transformation. Regression models were also assessed with an adjustment made for age, sex and CCS to ensure that these variables did not significantly change the result. Due to a small number of patients with missing data due to death before T24, a sensitivity analysis was performed excluding these patients from the sample to assess their influence on the results.

Association between biomarker concentration over time and cumulative fluid volume was tested using linear mixed models. If a significant association was detected between biomarker concentration and cumulative fluid volume over time, a three-way interaction was tested to assess whether fluid type (crystalloid versus colloid) modified this association. The model was also repeated with adjustment made for infection severity category (simple infection
versus sepsis) as well as inflammatory cytokine concentration (interleukin-6, interleukin-10 and NGAL) at each time point, in order to test persistence of associations independent of these confounders. Associations between biomarker concentrations at T0 (as a covariate) and clinical outcomes were tested using logistic regression for 30-day mortality and ICU admission, ordinal logistic regression for SOFA score, and truncated negative binomial regression for length of stay. Linearity of associations was checked using multivariable spline regressions or fractional polynomials. For ordinal logistic regression, if the assumption for proportional odds was not satisfied, analysis was performed on log-transformed data.

All analyses were performed using Stata 14 (College Station, TX, USA) with significance set at \( P<0.05 \).

Results

Participant characteristics

A summary of participant characteristics for the control (n=29), simple infection (n=23) and sepsis group (n=86) are reported in Table 1. Patients with sepsis had significantly increased inflammatory cytokine and lactate concentrations, number admitted to ICU and 30-day mortality, compared to patients with simple infection. Positive microbiological results included 60 patients with a positive blood culture, 57 with a positive urine culture, 16 with a positive sputum culture, and 16 with a positive soft tissue/skin culture. The median time from ED arrival to the first research blood sample (T0) was 62 minutes (Q1-Q3, 42-90) in the simple infection group and 47 minutes (Q1-Q3, 30-108) in the sepsis group (P=0.74).

Comparison of biomarker concentrations

At T0, syndecan-1 concentration was significantly increased in both the simple infection \( (P=0.020) \) and sepsis \( (P<0.001) \) group, compared to the control group, and also in the sepsis group compared to simple infection \( (P<0.001) \)(Figure 2). Syndecan-1 concentration significantly increased between
T0 and T24 in both patient groups (both $P<0.001$), with no significant difference in the pattern over time between the groups ($P=0.15$). Syndecan-4 concentration at T0 was not significantly different between any of the groups and did not significantly change over time in either patient group.

At T0, hyaluronan concentration was significantly increased in the sepsis group, compared to the control group ($P=0.029$) and simple infection group ($P=0.002$) (Figure 2). In both the simple infection and sepsis groups, hyaluronan concentration significantly increased from T0 to T1 ($P=0.047$, $P=0.031$, respectively), T0 to T3 ($P=0.001$, $P<0.001$) and T0 to T24 (both $P<0.001$). When comparing the change over time between the two groups, the simple infection group had a greater increase in hyaluronan concentration between T0 and T24 ($P=0.020$), compared to the sepsis group.

Summarized biomarker concentrations and $P$ values for all comparisons are provided as supplementary data in Supplemental Digital Content 1.

**Associations with fluid administration**

Cumulative fluid volumes administered to participants, up until each sampling time point, are shown in Table 2. Fluid administration consisted of crystalloid (n=105) and gelatine colloid (n=33) fluid products. There was a significant positive association between cumulative fluid volume and hyaluronan concentration at T0 ($P=0.001$), independent of infection severity and inflammatory cytokine concentrations. For every 1000mL administered, hyaluronan concentration increased by 22%. This association was maintained at T1 and T3 but was no longer significant at T24. There was no evidence that type of fluid (crystalloid versus colloid) modified this association.

There were no significant associations between cumulative fluid volume and syndecan-1, or syndecan-4, concentration either, before or after adjustment for infection severity and inflammatory cytokine concentrations.

**Associations with clinical outcomes**
Increased syndecan-1 (OR 1.04, 95% CI 1.01-1.07, P=0.017) and hyaluronan concentration (OR 1.83, 95% CI 1.46-2.30, P<0.001) at T0 significantly increased the odds of a higher SOFA score, independent of age, sex and CCS (Table 3). There were no significant associations found between any of the three biomarkers at T0 and ICU admission or 30-day mortality, after adjustment for age, sex and CCS. Length of hospital stay was also not significantly associated with syndecan-1 (P=0.73), syndecan-4 (P=0.50) or hyaluronan (P=0.82) at T0.

Discussion

This study conducted soon after ED arrival found an increase in syndecan-1 and hyaluronan over time in patients with infection, whereas previous studies in ICU populations found a decrease over time.12,17,18 This study also found a significant association between hyaluronan concentration in the first 3 hours and volume of IV fluids delivered, in both infection severity groups. This association persisted after adjustment for biomarkers of systemic inflammation. Similar to studies in ICU settings, we also found an association with both syndecan-1 and hyaluronan, and organ failure.

The major finding of this study was an increase in both syndecan-1 and hyaluronan concentration during initial treatment of infection. This may reflect persistence of inflammation during the initial stages of treatment or be associated with interventions delivered during this time period. The association found between hyaluronan and cumulative fluid volume in the first three hours of this study may explain the result. Studies in both animal models and healthy volunteers have demonstrated an increase in EG biomarkers after bolus crystalloid fluid administration.8,34,35 In one of these studies, the increase in EG biomarkers was associated with a decrease in EG thickness and an increase in endothelial permeability.35 Shedding of the EG may partly explain the associations previously found between positive fluid balance and mortality.43 There is growing concern that liberal fluid administration early in the treatment of sepsis may be harmful44 and recent Surviving Sepsis Campaign guidelines
state that, ‘this is an area in which research is urgently needed’.

Although glycocalyx shedding may simply reflect disease severity to explain the fluid association identified in this study, our findings support the need for a prospective randomized trial to determine the relationship between fluid therapy and EG shedding in sepsis.

In this study, syndecan-4 concentration showed very different behaviour to that of syndecan-1. Evidence from bench-top studies show that syndecan-4 is widely distributed on endothelium and shows strong upregulation and shedding in response to bacterial elements or inflammatory cytokines. There are few studies in patients with critical illness that include syndecan-4 concentration. However, one study in patients with pneumonia found a negative association between syndecan-4 concentration and pneumonia severity, suggesting that circulating syndecan-4 may be decreased in critical illness. Published studies comparing syndecan-1 and syndecan-4 concentration in patients with infection are lacking, however, our results suggest that shedding of these EG components are discordant.

One explanation for the differences seen in syndecan shedding may be variation in the source of the biomarker. While both are expressed on different cell types, syndecan-1 is highly expressed on the surface of neutrophils, compared to syndecan-4, and shows increased expression in response to inflammatory stimuli. The contribution of neutrophil-derived syndecan-1 may also explain why syndecan-1 showed a slightly different pattern to hyaluronan during the first 3 hours of this study, and why a significant association was not found with cumulative fluid volume. Further work is needed to explore associations between shed syndecan concentrations and syndecan expression on circulating leukocytes in patients with infection.

Similar to several ICU studies, this study found an association between SOFA score and both syndecan-1 and hyaluronan. Some studies have also found associations with mortality. However, we found that any associations identified with 30-day mortality in this study became non-
significant after adjustment for age, sex and CCS. This was also true for ICU admission. Both syndecan-1 and hyaluronan concentrations can be increased by a range of chronic diseases, such as diabetes, renal disease and congestive heart failure. Given that cumulative comorbidities have also been associated with organ failure in sepsis, it is possible that comorbid disease burden is a significant confounder of EG biomarker concentrations. This should be taken into consideration when including EG biomarkers in studies concerning sepsis.

A limitation of this study was the lack of EG thickness estimation, for example, by techniques such as sidestream darkfield microscopy or tracer dilution. This would have complimented the interpretation of biomarker concentrations. Also, degree of inflammation was based on only three different inflammatory cytokines. Although all three biomarkers have established associations with severity of sepsis, the approach may be overly simplistic. Other limitations of this study include the observational design with convenience sampling, and some variability of sample timing in relation to both admission and interventions. In addition, we calculated only cumulative fluid volume, which did not account for type of fluid, rate of administration or timing in relation to the blood sampling time point.

Conclusions

In summary, this study found evidence of increasing EG shedding in patients with infection during early resuscitation and treatment in the ED. These increases were associated with cumulative fluid volume given in the first 3 hours and severity of organ failure. The association found with fluid volume, independent of infection severity and level of inflammation, supports the need for a prospective randomized trial to investigate causation. Factors influencing the heterogeneity seen between individual EG biomarkers also requires further exploration, including characterizing the contribution of leukocyte syndecan shedding and real-time relationships with EG thickness.

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Figures and Tables

Figure 1. Flow-chart describing selection of Emergency Department patients with simple infection or sepsis.

Enrolment into the Critical Illness and Shock Study at Emergency Department admission (March 2010 to July 2013)*
(n=948)
- Shock (hypotension OR shock index>1 OR lactate>4mmol/l)
- Hypoxemia
- Post-cardiac arrest
- Acute anaphylaxis
- Sepsis
  1. Likely infection,
  2. AND at least two out of four SIRS criteria,
  3. AND shock OR hypoxemia OR organ dysfunction

Participants screened for sepsis biomarker study
(n=621)**
- Met above criteria for sepsis

Included in sepsis biomarker study**
(n=186)
- Simple infection (n=28)
- Sepsis (n=158)

Consecutive participants between January 2012 and July 2013 (n=109)
- Simple infection (n=23)
- Sepsis (n=86)

Excluded (n=435)
- Diagnosis not consistent with infection
- Did not receive intravenous antibiotics
- Missing or inadequate blood sampling

Figure 2. Geometric mean (95% confidence interval) of syndecan-1, syndecan-4 and hyaluronan concentration over time in Emergency Department patients with simple infection or sepsis.
Table 1. Participant characteristics of Emergency Department patients with simple infection or sepsis. A healthy control group is provided for reference.

<table>
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<th>Characteristic</th>
<th>Control (n=29)</th>
<th>Simple Infection (n=23)</th>
<th>Sepsis (n=86)</th>
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<td>28 (33)</td>
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<td>Renal disease</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>13 (15)</td>
<td>0.010</td>
</tr>
<tr>
<td>Prior MI</td>
<td>2 (7)</td>
<td>2 (9)</td>
<td>13 (15)</td>
<td>0.41</td>
</tr>
<tr>
<td>CHF</td>
<td>2 (7)</td>
<td>2 (9)</td>
<td>10 (12)</td>
<td>0.68</td>
</tr>
<tr>
<td>Source, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary</td>
<td>-</td>
<td>7 (30)</td>
<td>36 (42)</td>
<td>0.31</td>
</tr>
<tr>
<td>Urinary</td>
<td>-</td>
<td>6 (26)</td>
<td>17 (20)</td>
<td>0.52</td>
</tr>
<tr>
<td>Skin/Soft Tissue</td>
<td>-</td>
<td>2 (9)</td>
<td>11 (18)</td>
<td>0.58</td>
</tr>
<tr>
<td>Abdominal</td>
<td>-</td>
<td>0 (0)</td>
<td>7 (8)</td>
<td>0.063</td>
</tr>
<tr>
<td>Other</td>
<td>-</td>
<td>2 (9)</td>
<td>14 (16)</td>
<td>0.83</td>
</tr>
<tr>
<td>Viral infection</td>
<td>-</td>
<td>3 (13)</td>
<td>1 (1)</td>
<td>0.007</td>
</tr>
<tr>
<td>SOFA score</td>
<td>-</td>
<td>1 [0-1]</td>
<td>4 [3-6]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C-reactive protein (mg/mL)</td>
<td>-</td>
<td>58 [27-310]</td>
<td>135 [51-225]</td>
<td>0.87</td>
</tr>
<tr>
<td>Interleukin-6 (pg/mL)</td>
<td>-</td>
<td>327 [122-1378]</td>
<td>989 [243-10396]</td>
<td>0.047</td>
</tr>
<tr>
<td>Interleukin-10 (pg/mL)</td>
<td>-</td>
<td>19.8 [12.7-30.5]</td>
<td>39 [12.7-30.5]</td>
<td>0.037</td>
</tr>
<tr>
<td>NGAL (ng/mL)</td>
<td>-</td>
<td>147 [106-252]</td>
<td>342 [187-646]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>-</td>
<td>1.5 [1.1-2.0]</td>
<td>2.6 [1.5-4.5]</td>
<td>0.003</td>
</tr>
<tr>
<td>ICU Admission, n (%)</td>
<td>-</td>
<td>1 (4)</td>
<td>38 (44)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Length of stay (days)</td>
<td>-</td>
<td>6 [3-16]</td>
<td>7 [4-13]</td>
<td>0.46</td>
</tr>
<tr>
<td>30-day mortality, n (%)</td>
<td>-</td>
<td>0 (0)</td>
<td>16 (19)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Data is presented as either median [Q1-Q3], mean (95% confidence interval) or number (percentage).

Abbreviations: CCS, Charlson Comorbidity Score; MI, myocardial infarction; CHF, congestive heart failure; SOFA, sequential organ failure assessment; NGAL, neutrophil gelatinase-association lipocalin; ICU, intensive care unit.
Table 2. Cumulative fluid volumes (median [Q1-Q3]) for patients with simple infection soon after Emergency Department admission (T0) and then 1 hour (T1), 3 hours (T3) and 12-24 hours later (T24).

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Cumulative fluid volume (mL)</th>
<th>T0</th>
<th>T1</th>
<th>T3</th>
<th>T24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T0</td>
<td>T1</td>
<td>T3</td>
<td>T24</td>
</tr>
<tr>
<td>Simple Infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colloid</td>
<td></td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
</tr>
<tr>
<td>Sepsis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colloid</td>
<td></td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
</tr>
</tbody>
</table>

Each time category includes all crystalloids and/or gelatine colloids given to the patient up until the respective research blood sample was drawn.
Table 3. Associations (odds ratio (95% confidence interval)) between endothelial glycocalyx biomarkers and clinical outcomes at Emergency Department admission in patients with infection.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Univariate Odds Ratios (95% CI)</th>
<th>Univariate P value</th>
<th>Multivariablea Odds Ratio (95% CI)</th>
<th>Multivariable P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Syndecan-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOFA scoreb</td>
<td>1.03 (1.01-1.07)</td>
<td>0.023</td>
<td>1.04 (1.01-1.07)</td>
<td>0.017</td>
</tr>
<tr>
<td>ICU admission</td>
<td>1.05 (0.99-1.12)</td>
<td>0.084</td>
<td>1.05 (0.87-1.26)</td>
<td>0.62</td>
</tr>
<tr>
<td>30-day mortality</td>
<td>1.02 (0.98-1.05)</td>
<td>0.31</td>
<td>1.02 (0.87-1.18)</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>Syndecan-4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOFA scoreb</td>
<td>1.05 (0.79-1.41)</td>
<td>0.73</td>
<td>1.08 (0.81-1.45)</td>
<td>0.60</td>
</tr>
<tr>
<td>ICU admission</td>
<td>0.91 (0.58-1.43)</td>
<td>0.68</td>
<td>0.82 (0.41-1.67)</td>
<td>0.59</td>
</tr>
<tr>
<td>30-day mortality</td>
<td>1.15 (0.74-1.78)</td>
<td>0.53</td>
<td>1.26 (0.41-3.86)</td>
<td>0.69</td>
</tr>
<tr>
<td><strong>Hyaluronan</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOFA scoreb</td>
<td>1.87 (1.49-2.33)</td>
<td>&lt;0.001</td>
<td>1.83 (1.46-2.30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ICU admission</td>
<td>6.68 (1.52-29.29)</td>
<td>0.012</td>
<td>5.53 (0.07-425)</td>
<td>0.44</td>
</tr>
<tr>
<td>30-day mortality</td>
<td>1.85 (1.09-3.14)</td>
<td>0.022</td>
<td>1.80 (0.10-32.20)</td>
<td>0.69</td>
</tr>
</tbody>
</table>

aThe multivariable model was adjusted for age, sex and Charlson Comorbidity Score

bOrdinal logistic regression performed for SOFA score estimates. Hyaluronan was log transformed to satisfy proportional odds assumption.

Odds ratios are for an increase of 1ng/mL for syndecan-1 and syndecan-4, and 1µg/mL for hyaluronan. For hyaluronan concentration that has been log transformed, the odds ratio is for an increase in 2.6µg/mL.
Supplemental Digital Content 1. Table. Biomarker concentrations (geometric mean (95% confidence interval)) for patients with simple infection or sepsis soon after Emergency Department admission (T0) and then 1 hour (T1), 3 hours (T3) and 12-24 hours later (T24).

<table>
<thead>
<tr>
<th>Control (n=29)</th>
<th>Simple Infection (n=23)</th>
<th>Sepsis (n=86)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Mean (95% CI)</td>
<td>Change from T0</td>
</tr>
<tr>
<td><strong>Syndecan-1 (pg/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>506 (423-605)</td>
<td>23 1052 (761-1455)</td>
</tr>
<tr>
<td>T1</td>
<td>- 1080 (758-1539)</td>
<td>0.99</td>
</tr>
<tr>
<td>T3</td>
<td>- 1153 (814-1635)</td>
<td>0.23</td>
</tr>
<tr>
<td>T24</td>
<td>- 1641 (1165-2312)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Syndecan-4 (pg/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>807 (704-926)</td>
<td>23 600 (458-785)</td>
</tr>
<tr>
<td>T1</td>
<td>- 581 (435-775)</td>
<td>0.82</td>
</tr>
<tr>
<td>T3</td>
<td>- 484 (282-829)</td>
<td>0.050</td>
</tr>
<tr>
<td>T24</td>
<td>- 634 (518-777)</td>
<td>0.62</td>
</tr>
<tr>
<td><strong>Hyaluronan (ng/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>39 (29-53)</td>
<td>23 36 (21-63)</td>
</tr>
<tr>
<td>T1</td>
<td>- 55 (33-92)</td>
<td>0.047</td>
</tr>
<tr>
<td>T3</td>
<td>- 54 (30-97)</td>
<td>0.001</td>
</tr>
<tr>
<td>T24</td>
<td>- 64 (34-123)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* P value given for the interaction term
3.4 References


CHAPTER 4 – Syndecan-1, hyaluronan and endocan in sepsis secondary to pneumonia

4.1 Gap in knowledge

In the previous chapter, syndecan-1 and hyaluronan were positively associated with Sequential Organ Failure Assessment (SOFA) score. Given that almost 40% of patients in that study had pneumonia as their source of sepsis, I was curious to know if these biomarkers measured on admission to the emergency department (ED) could predict respiratory failure or the need for mechanical ventilation. To further interrogate this possibility, endocan, a dermatan sulfate proteoglycan secreted from the endothelium and described as a biomarker of endothelial activation, was considered. The soluble form of endocan has been associated with respiratory failure in certain patient cohorts. Therefore, we compared the utility of three biomarkers, syndecan-1, hyaluronan and endocan, for predicting clinical outcomes in a subset of patients with pneumonia. During the peer review process, syndecan-4 data were removed from this study as it did not add sufficient value to the publication. The original data regarding syndecan-4 has been provided in Appendix A.

As previously described in Chapter 3, we observed differing patterns of endothelial glycocalyx shedding in sepsis. Syndecan-1 rose slowly over time whereas hyaluronan peaked much earlier during the observation period. Hyaluronan was associated with cumulative fluid volume whereas syndecan-1 was not. It is possible that these two biomarkers have different drivers for shedding, one of which is inflammatory cytokine release (see Section 2.3). Therefore, we also sought to explore relationships between all three endothelial biomarkers and biomarkers of acute inflammation.

The background information provided below reviews studies, including in vitro, animal model and clinical studies, on endocan that were published up to and including 2017, presented as a prologue to our observational study examining syndecan-1, hyaluronan and endocan over time in patients with pneumonia.
Results were published in *Journal of Critical Care* in 2018 and the manuscript is presented as published. At the time of publishing this paper, endocan was described as a cell surface proteoglycan i.e. part of the EG. However, upon reflection, it is likely more correct to describe this molecule as a secreted proteoglycan, as constitutive or stimulated endothelial surface expression has not been confirmed. A recent review article published after this paper, written by the group that originally characterised endocan, expressed this same sentiment.

### 4.2 Background Information

Endocan, originally named endothelial cell-specific molecule-1, was first identified from cultured endothelial cells in 1996 by Lassalle and others. It was found to be constitutively expressed in human lung and, weakly, kidney tissue but not in heart, brain, placenta, liver, muscle or pancreas. The Northern blot band for endocan identified from lung tissue was a similar band-width as that from human umbilical vein endothelial cells (HUVECs), indicating a comparable molecular weight, and therefore likely the same molecule. Furthermore, endocan mRNA was upregulated in HUVECs incubated with tumour necrosis factor-α (TNF-α), which peaked 18 hours after application. A similar pattern was shown with interleukin-1β, indicating that endothelial endocan expression can be stimulated by pro-inflammatory conditions.

Characterisation of endocan in the lung by immunohistochemistry showed constitutive expression in the cytoplasm of venular, arteriolar and capillary endothelial cells, and bronchial epithelium and submucosal glands. In the kidney, endocan was expressed in renal tubular epithelial cells and vascular endothelium. Endocan was also found in the capillaries and venules of the gut lamina propria. The endocan form expressed constitutively by HUVECS had a smaller MW (~20kDa) than the actively secreted endocan (~50kD), suggesting that endocan is further modified before it is secreted from the cell surface in its soluble form. In 2001, endocan was further characterised as a dermatan sulphate proteoglycan secreted by endothelial cells in a soluble form, which remains the current classification for this molecule.
Similar to endothelial glycocalyx biomarkers, endocan may be upregulated and released from the endothelium during systemic inflammation. Incubation of HUVECs with lipopolysaccharide (LPS) causes increased synthesis and release of endocan within 24 hours of stimulation, as does injection of LPS in mice. The authors replicated these results in a caecal ligation and puncture murine model of sepsis. Endocan was also shown in this study to increase vascular permeability in HUVEC cultures and vascular permeability in mice following injection with endocan. Endocan induced leuokocyte migration, both in vitro and in vivo, and increased the expression of interleukin(IL)-8, monocyte chemoattractant protein-1, TNF-α and mitogen-activated protein kinases in vitro. Another in vitro study showed sustained endocan release (96 hours), as well as increased endocan mRNA, from HUVECs after incubation with LPS and TNF-α. These studies highlight that endocan is not only upregulated and secreted in response to inflammatory cytokines but also that endocan itself acts as a proinflammatory molecule, drawing a parallel to the proinflammatory effects of soluble heparan sulfate and low molecular weight hyaluronan (see Section 1.4).

A clinical study published in 2006 followed on from this earlier work, showing that patients with sepsis had increased endocan concentrations (n=84) compared to controls, with higher concentrations seen in septic shock compared to sepsis, and in non-survivors compared to survivors. Weak correlations were found between endocan and IL-10 ($r^2=0.355$) and von Willebrand factor ($r^2=0.359$). The area under the receiver operating characteristic curve (ROC AUC) was 0.923 for endocan predicting sepsis versus systemic inflammatory response syndrome (n=7), which was higher than ROC AUCs for procalcitonin, C-reactive protein, intercellular adhesion molecule-1, IL -6, IL-10 and von Willebrand factor; however, AUCs were not significantly different between biomarkers. It was unclear in this study how patients with systemic inflammatory response syndrome were identified, beyond referring to the sepsis definitions available at the time, which is a limitation in addition to the small sample size.
Multiple studies since have demonstrated positive associations between endocan and scores of severity of illness, to varying degrees. One such study showed a trend of increasing endocan concentrations with severity of illness in intensive care unit (ICU) patients with sepsis (n=136) on day 1 and 3. In this study, endocan was also weakly correlated with Sequential Organ Failure Assessment (SOFA) score (\(\rho=0.3\)), Simplified Acute Physiology Score (SAPS) II score (\(\rho=0.25\)), Acute Physiology, Age, Chronic Health Evaluation (APACHE) II score (\(\rho=0.27\)) and white blood cell count (\(\rho=0.24\)). Endocan levels on day 1 were also associated with 30-day mortality, after adjustment for age, sex, creatinine, ICU days and APACHE II (hazards ratio 1.9, \(P=0.04\)). Endocan showed weak diagnostic discrimination between sepsis severity groups on day 1 of ICU hospitalisation (AUC 0.66) but performed better than C-reactive protein and white blood cell count. Conversely, procalcitonin and IL-6 showed better discrimination than endocan (AUC 0.83 and 0.85, respectively). Another study showed similar stratification of endocan according to sepsis severity (n=82), with increasing endocan concentrations across sepsis, severe sepsis and septic shock groups, respectively. However, in contrast to the previous study, endocan showed better discrimination in predicting sepsis severity than procalcitonin and IL-6.

Further studies have confirmed positive associations between endocan and illness severity. A study in adults with sepsis (n=60) found that endocan was significantly increased in patients that had organ failure on arrival to ICU, developed multiple organ failure within 48 hours or died, compared to patients that did not. Endocan was able to predict all three conditions with moderate discrimination (ROC AUCs 0.81, 0.67 and 0.71, respectively). A similarly-sized study in patients with sepsis (n=78) found endocan significantly decreased over time during hospitalisation. There was no correlation between endocan and either C-reactive protein or procalcitonin. Endocan was higher in patients with positive bacterial culture, with an ROC AUC of 0.662 for day 0-endocan predicting positive bacterial culture. High endocan concentrations were also identified post-mortem in patients that had died from sepsis. Furthermore, in a study of children with febrile neutropaenia, serum endocan concentration was
higher than in healthy controls and children with leukaemia, without fever or neutropaenia.  

In light of early work demonstrating lung-specific expression of endocan, several studies have measured circulating endocan concentration in patients specifically with lung disease. In hospitalised patients with pneumonia (n=82), plasma endocan concentration decreased after antibiotic treatment and was correlated with Pneumonia Severity Index (r=0.554), CURB-65 (r=0.51) and APACHE II (r=0.447) scores. In patients with pneumonia (n=42), those with acute respiratory distress syndrome (ARDS) had higher plasma endocan concentrations than those without ARDS, however, it appears that those without ARDS were not as sick, based on length of stay and mortality. In ICU patients with ARDS (n=96), 67% of which had sepsis, plasma endocan concentration was not different between survivors and non-survivors at ICU admission, but was significantly higher in non-survivors 24 hours later and in those mechanically ventilated for > 10 days. The ROC AUC was 0.662 (0.551-0.774) for predicting mechanical ventilation for >10 days. These studies focusing on patients with lung pathology provide evidence that endocan is positively associated with severity of illness and clinical outcomes in patients with infection or sepsis.

Counter-intuitively, two studies have identified a negative association between endocan concentration and respiratory failure. Although one such study published in 2016 found that endocan was higher in adults with septic shock (n=20), compared to healthy controls, endocan was negatively associated with respiratory failure (PaO₂/FiO₂ ratio less than 300). Endocan measured from blood samples drawn on day 1 predicted respiratory failure occurring by day 3 (ROC AUC 0.87). Further to this, a study in patients with major trauma (n=48) also found a negative association between endocan and respiratory failure. Endocan concentration measured in the ED was lower in patients that developed acute lung injury requiring mechanical ventilation within 5 days of admission, compared to those that did not. The association between endocan and acute lung injury was no longer significant after adjustment for units of packed red
blood cells transfused, therefore it is possible these lower concentrations were a result of haemodilution resulting from fluid resuscitation.

After finding that low concentrations of endocan in sepsis\textsuperscript{17} and trauma\textsuperscript{18} were associated with respiratory failure, the same authors explored whether endocan was being cleaved in circulation.\textsuperscript{19} They found that neutrophil-derived cathepsin G generated a 14kDa fragment and that concentrations of this fragment were increased in patients with sepsis, without having a correlation with endocan concentrations. It was suggested that the endocan fragment might be a useful biomarker in sepsis for exploring relationships with multiple organ failure, particularly lung failure.

Given that concentrations of other endothelial proteoglycans, syndecan-1 and syndecan-4, displayed different patterns of change over the time course of treatment of sepsis in our previous study (Chapter 3), I hypothesised that the same may be true for temporal patterns of endocan shedding. This may explain the inconsistencies between studies in regards to the type of association (positive versus negative) between endocan and respiratory failure.
4.3 Glycocalyx biomarker syndecan-1 is a stronger predictor of respiratory failure in patients with sepsis due to pneumonia, compared to endocan

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Conflicts of Interest and Source of Funding
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Abstract

\textit{Purpose}: Endocan, a component of the endothelial glycocalyx (EG), has been linked with respiratory failure in sepsis. This study explored the temporal patterns of three EG biomarkers, including endocan, and their relationships with inflammation and respiratory failure.
**Materials and Methods:** Plasma endocan, syndecan-1, and hyaluronan concentrations were measured in Emergency Department (ED) patients with sepsis due to pneumonia (n=44) on ED arrival (T0), 1 hour (T1), 3 hours (T3) and 12-24 hours (T24) later, with change over time tested using mixed regression models. Biomarker associations with inflammatory cytokine concentrations and with respiratory failure on days 1, 2 or 3, need for mechanical ventilation and 30-day mortality were also tested.

**Results:** Endocan concentration significantly decreased over time (T0-T24, \( P=0.003 \)) whereas both syndecan-1 (T0-T3, \( P=0.010 \); T0-T24, \( P<0.001 \)) and hyaluronan (T0-T1, \( P=0.010 \); T0-T3, \( P<0.001 \); T0-T24, \( P=0.003 \)) significantly increased over time. Increased syndecan-1 was significantly correlated with neutrophil activation biomarkers and significantly increased the odds of respiratory failure (OR 1.18, 95% CI 1.05-1.33, \( P=0.004 \)), need for mechanical ventilation (OR 1.24, 95% CI 1.04-1.48, \( P=0.014 \)) and 30-day mortality (OR 1.29, 95% CI 1.07-1.55, \( P=0.008 \)).

**Conclusion:** Syndecan-1, but not endocan, was associated with neutrophil activation and was the best EG biomarker predictor of adverse clinical outcomes.

**Introduction**

Pneumonia and sepsis are leading causes of respiratory failure in critically ill patients.\(^{20}\) Respiratory failure is characterized by pulmonary endothelial injury, including margination of leucocytes, hyperpermeability and protein-rich fluid leaking into the pulmonary interstitium.\(^{21}\) Pro-inflammatory conditions such as sepsis promote shedding of the endothelial glycocalyx (EG), which is a mesh-like complex lining the luminal surface of the endothelium.\(^{22-24}\) Shedding of the EG prompts endothelial activation and increases circulating concentrations of soluble EG components, such as glycosaminoglycans and proteoglycan fragments. Endothelial activation also upregulates synthesis and secretion of some of these EG components, therefore contributing further to circulating concentrations.\(^{4,6,7,25,26}\) Additionally, EG components such as endocan
and hyaluronan can have intrinsic pro-inflammatory properties and may contribute to organ injury when increased in circulation.6,27

Increased circulating EG biomarkers in patients with sepsis are associated with severity of illness,7,28-31 organ dysfunction28,29,31-34 and mortality.7,29,33,35 Endocan, in particular, appears heavily expressed in lung tissue.3,4 There is some evidence that plasma endocan concentration is positively associated with severity of pneumonia,14 as well as the need for mechanical ventilation for longer than 10 days in patients with Acute Respiratory Distress Syndrome (ARDS).16 However, other studies have reported negative associations between endocan concentration and clinical outcomes such as respiratory failure.17,18 It is difficult to know why these results are disparate, as timing of blood sampling in relation to time of illness onset and admission, and therapeutic intervention, is variable. The time course of endocan concentration in the early stages of sepsis treatment has not been described.

Studies conducted in the Intensive Care Unit (ICU) reporting the most frequently measured EG biomarkers, syndecan-1 and hyaluronan, typically describe decreasing concentrations over time in patients with sepsis.29,32,36 In contrast, previous work by our group found that patients with sepsis had increasing syndecan-1 and hyaluronan concentrations from Emergency Department (ED) admission through the first 24 hours.31 We also identified an association between increasing hyaluronan concentration and fluid volumes administered in the first 3 hours of treatment. However, increasing EG shedding early in the treatment of sepsis may also be related to the time course of systemic inflammation. Previous work by our group identified variation in the time course of inflammatory biomarker concentrations, where some biomarkers rapidly decreased while others increased during the first 24 hours of treatment.37 It is possible that the pattern of inflammatory biomarker release may explain some of the variations in EG biomarker shedding.

The aim of this study was to characterise the change over time in plasma endocan concentration in patients with sepsis due to pneumonia, and compare
this to patterns of shedding of two other EG biomarkers (syndecan-1 and hyaluronan). Given that we found different patterns of shedding over time between the EG biomarkers in this study, we explored associations with inflammatory and endothelial activation biomarker concentrations to further understand these differences. The secondary aim was to test associations between each EG biomarker and respiratory outcomes in patients with pneumonia, including respiratory failure, need for mechanical ventilation and mortality. Given that endocan is heavily expressed in the lung, we hypothesised that endocan concentration would be the best predictor of respiratory failure, compared to other EG biomarkers.

**Materials and Methods**

*Participant selection*

Patients meeting criteria for sepsis in the ED were identified from the Critical Illness and Shock Study (CISS) (HREC permit number 2009-080). Written informed consent was gained from the participant or next-of-kin for inclusion in the study. The CISS methodology has been described in detail elsewhere.\(^{38}\) In brief, CISS is an ED-based observational study of patients presenting to two urban EDs and meeting predefined physiologic criteria consistent with critical illness. Among the CISS cohort, sepsis was defined as having at least 2 of 4 Systemic Inflammatory Response Syndrome (SIRS) criteria;\(^{39}\) temperature >38°C or <36°C, heart rate >90 bpm, respiratory rate >20bpm or white cell count >12x10^9/L or <4x10^9/L, as well as clinical suspicion of infection as the primary admitting diagnosis and administration of intravenous antibiotics.

Participants enrolled in CISS from March 2011 to July 2013 with sepsis due to pneumonia were identified from a larger cohort in a previously reported study that measured EG biomarkers over time.\(^{31}\) Criteria for pneumonia included regional or lobar pulmonary infiltrates present on chest radiographs consistent with acute infection plus one or more of; cough, sputum production, chest pain or shortness of breath. Cases of suspected infective exacerbation of chronic airway disease without localising radiographic signs were excluded.
Healthy control samples were also utilised from the study previously described. As a part of the original study, these samples were selected based on age and sex-stratification to match the cohort with sepsis.

**Participant data collection**

The Sequential Organ Failure Assessment (SOFA) score and CURB-65 score were calculated from parameters collected on the first day of hospitalisation. Presence of respiratory failure, as defined by a PaO2/FiO2 (PF) ratio <300, an SpO2/FiO2 (SF) ratio <315 or SpO2 <90% on >6 L/min supplemental oxygen, was assessed on days 1, 2 and 3 of hospitalisation. Mechanical ventilation was defined as the use of invasive positive pressure ventilation at any point during the first 3 days of hospitalisation. Length of ICU stay did not include hospitalisation in high-dependency wards that provided non-invasive respiratory support. White cell count, C-reactive protein and lactate concentration were retrieved from medical records on the first day of hospitalisation. Charlson Comorbidity Score was retrieved from the CISS database.

**Biomarker analysis**

Samples collected at ED enrolment included serum and EDTA plasma, which were stored at -80°C until batched analysis. Plasma endocan was measured using a commercial ELISA kit (Lunginnov, Lille, France). Serum syndecan-1 and hyaluronan concentrations were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) as part of a previous study. Serum inflammatory and endothelial activation biomarker concentrations (interleukin-6, interleukin-10, neutrophil gelatinase-associated lipocalin (NGAL), intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1)) were measured for a previous biomarker study and were available from the CISS database. As an additional inflammatory biomarker, plasma neutrophil myeloperoxidase was measured using a commercial ELISA kit (R&D Systems, Minneapolis, MN). Healthy control samples (n=28) were available from the previous studies and were included...
to provide a frame of reference, with data either retrieved from the CISS database or biomarkers (endocan, myeloperoxidase) measured specifically for this study. Assays were performed according to manufacturer’s instructions and samples were only used if they had undergone no more than two freeze-thaw cycles.

**Statistical analysis**

Normality of data was assessed by visual inspection of histograms and Q-Q plots. Participant characteristics were summarised using number (percentage) for categorical variables, and median [Q1-Q3] or mean (95% confidence interval) for continuous variables depending on normality. Between-group differences for participant characteristics were tested using Student’s t-test for age, chi-square test for sex and negative binomial regression for Charlson Comorbidity Score.

Endothelial glycocalyx biomarker concentrations were log-transformed to generate normal or approximately normal distributions. For each EG biomarker, change in concentration over time was tested using linear mixed regression models incorporating maximum likelihood estimation. Raw data was summarised using predicted mean (95% confidence interval) generated by the regression model. A sensitivity analysis was performed for each model that included only participants with complete blood sampling to T24, to ensure that dropout of participants prior to the T24 did not significantly skew the pattern in change over time.

Associations between EG and inflammatory biomarker concentrations at T0 and T24 were tested using Spearman’s rank correlation, with only correlations at rho>0.5 being considered significant. Associations between EG biomarker concentrations at T0 and respiratory failure on days 1, 2 and 3 were tested using ordinal logistic mixed effects regression models. Where there was no significant interaction between respiratory failure and time (days 1, 2 and 3), a single odds ratio was reported for association across time. Associations between EG biomarker concentrations and mechanical ventilation, as well as 30-day mortality, were tested using logistic regression. Associations with the above
clinical outcomes were also tested for both CURB-65 score and lactate concentration as a frame of reference, as these clinical severity surrogates are predictive of mortality in ED patients with pneumonia.\textsuperscript{43-45}

All analyses were performed using Stata 14 (College Station, TX, USA), with significance set at $P<0.05$.

Results

Participant characteristics

This study included 44 patients with sepsis due to pneumonia and 28 healthy controls. Participant characteristics are provided in Table 1. There was no significant difference in age ($P=0.91$) or sex ($P=0.53$) between patients and controls. Patients had a significantly increased Charlson Comorbidity Score ($P<0.001$) compared to controls. Research blood samples were available at T0 and T1 for 44 participants, at T3 for 34 participants and at T24 for 24 participants.

Biomarker concentrations over time

Endocan concentration did not significantly change from T0 in the first 3 hours of the study (T0 to T1, $P=0.09$; T0-T3, $P=0.32$), however, endocan significantly decreased at T24 (T0-T24, $P=0.003$)(Figure 1). In contrast, both syndecan-1 and hyaluronan significantly increased over time (Figure 1). Specifically, syndecan-1 increased at T3 (T0-T3, $P=0.010$) and at T24 (T0-T24, $P<0.001$). Hyaluronan increased from T0 at all subsequent time points (T0-T1, $P=0.010$; T0-T3, $P<0.001$; T0-T24, $P=0.003$). Summarised healthy control biomarker concentrations are provided for reference in Supplementary data.

Participants that had respiratory failure on day 1 did not have any significant differences in the pattern of EG biomarker change over time, compared to participants that did not have respiratory failure on day 1 (data not shown).
Associations with inflammation and endothelial activation

Syndecan-1 concentration was the only EG biomarker to show moderate correlation with inflammatory biomarkers (Table 2). Syndecan-1 was moderately correlated with NGAL at both T0 (rho 0.52, \( P<0.001 \)) and T24 (rho 0.52, \( P=0.01 \)), with resistin at T0 (rho 0.54, \( P<0.001 \)) and T24 (0.53, \( P=0.007 \)), and with myeloperoxidase at T0 (rho 0.54, \( P<0.001 \)). Syndecan-1 also showed moderate correlation with endothelial activation biomarker VCAM-1 at T24 (rho=0.51, \( P=0.011 \)), as did endocan at T24 (rho=0.52, \( P=0.009 \)). Summarised inflammatory biomarker concentrations are provided in Supplementary data.

Associations with clinical outcomes

Associations between biomarker concentrations at T0 and respiratory failure on day 1 were not significantly different to days 2 and 3, therefore a single odds ratio over time is reported (Table 3). No significant associations were identified between endocan concentration and clinical outcomes. In contrast, increased syndecan-1 concentration was associated with higher odds of respiratory failure over the first 3 days (\( P=0.004 \)), mechanical ventilation (\( P=0.014 \)) and 30-day mortality (\( P=0.008 \)). Also, increased hyaluronan concentration was associated with higher odds of 30-day mortality (\( P=0.018 \)). Clinical severity surrogates, CURB-65 score and lactate concentration, were associated with 30-day mortality (\( P=0.004, P=0.035 \), respectively) but not respiratory failure over the first 3 days.

Discussion

This study found that patients with sepsis due to pneumonia admitted to the ED had decreasing endocan concentration over the first 24 hours of hospitalisation, whereas both syndecan-1 and hyaluronan concentration increased over time. Notably, syndecan-1 showed the most consistent association with inflammatory biomarkers, including neutrophil-derived cytokines NGAL, resistin and myeloperoxidase. Additionally, syndecan-1 concentration appeared superior to other EG biomarkers and clinical severity
surrogates for predicting three meaningful clinical outcomes: respiratory failure, mechanical ventilation and 30-day mortality.

It has been postulated that circulating endocan concentration is a useful indicator of severity of lung disease due to evidence of its strong expression in human lung tissue and upregulation in response to inflammation.\textsuperscript{3,6,46} In this study, endocan concentrations decreased over time, in contrast to other EG biomarkers, and the pattern in change over time did not differ between patients with respiratory failure, and those without. Additionally, endocan concentration was not a predictor of respiratory failure, in contrast to syndecan-1. It is possible that endocan secretion is not as 'lung-specific' as previously thought, and may simply represent systemic glycocalyx shedding or endothelial activation. Studies have identified endothelial endocan expression in tissues other than lung, such as kidney, intestine and fat.\textsuperscript{4,47} Positive associations reported by others between circulating endocan concentration and respiratory failure may simply reflect severity of systemic illness.\textsuperscript{6,7,9,10}

Circulating endocan may be present in multiple forms. It is likely that the type of endocan measured by ELISA in most previous studies,\textsuperscript{4,7,14,16,24} including this one, is that which is induced and actively secreted by endothelial cells. The molecular weight of endothelial cell surface endocan is in the range of 14-20kDa, whereas actively secreted endocan is \(~50\text{kDa}.\textsuperscript{4} The ELISA used in the present study quantifies the latter form and increased concentrations may more accurately reflect ongoing endothelial activation, rather than being a reliable indicator of real-time EG shedding. Measurement of smaller molecular weight endocan, such as 14kDa, would also not serve as an ideal EG biomarker, as it may simply have been cleaved in circulation.\textsuperscript{19} Regardless, given previous evidence that circulating endocan measured by assays currently available is associated with lung injury, our purpose was to describe the time course of this type of endocan, compared to other biomarkers associated with disruption of the endothelium.
Syndecan-1 concentration showed moderate correlation with markers of neutrophil activation; NGAL, resistin and myeloperoxidase. The increasing concentration of syndecan-1 concentration over time also parallels increasing concentrations of both NGAL and resistin (see Supplementary data, Table 1). It is well established that NGAL plays an important role in the neutrophil’s early response to bacterial infection\(^{46}\) and increased plasma NGAL concentration has been associated with severity of pneumonia.\(^{44}\) Neutrophils have also been recently identified as a major source of resistin in bacterial infection;\(^{49}\) a biomarker that has also been associated with severity of sepsis.\(^{37}\) We measured myeloperoxidase in this study as another marker of neutrophil activation, which is a neutrophil-derived cytotoxic enzyme that may contribute to lung injury in pneumonia.\(^{50}\) An association between these important neutrophil activation markers and syndecan-1 raises the possibility that activated neutrophils are a source of shed syndecan-1 ectodomains. Syndecan-1 belongs to a family of heparan sulfate proteoglycans, which have been identified as important binding sites on the surface of neutrophils for various inflammatory mediators.\(^{51,52}\) Neutrophil expression of syndecan-1 has also been shown to increase in patients with diabetes,\(^{53}\) and therefore may increase in other disease states as well. It is possible that syndecan-1 plays a dual role as an inflammatory biomarker and as an indicator of EG shedding. The relative contribution of neutrophil-derived syndecan-1 to total circulating concentrations in critically ill patients requires further exploration.

Further to the associations shown with neutrophil activation, syndecan-1 concentration in this study showed stronger associations with clinical outcomes compared to other EG biomarkers, CURB-65 score and lactate concentration. Other studies investigating sepsis have also shown strong positive associations between circulating syndecan-1 and organ failure.\(^{28,32}\) In another ED-based study, syndecan-1 concentration in patients with sepsis was also significantly associated with a higher odds of intubation.\(^{35}\) There is a paucity of studies directly comparing circulating EG biomarkers, but the results of this study suggest that syndecan-1 is superior for predicting clinical outcome in patients with sepsis secondary to pneumonia.
Examining relationships between EG biomarkers and inflammation is difficult in the clinical setting due to variability in the time of onset of disease. This study has identified some parallel patterns in biomarker change over time (e.g. syndecan-1 and NGAL) but, if a causal relationship exists, cannot differentiate between the instigator and the responder. For example, it has been demonstrated in vitro that shedding of the EG increases endothelial activation, leucocyte adhesion, upregulation of NFκb and inflammatory cytokine production.\textsuperscript{54-56} However, it is also well established that application of lipopolysaccharide (LPS) or inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), to endothelium can also cause shedding of the EG.\textsuperscript{3,4,6,7,24,25,57-60} It is likely that the process is dynamic and further work is needed to determine if therapy aimed at protecting the EG will reduce the pro-inflammatory response and, potentially, reduce the risk of multiple organ failure. Also, future studies should consider multiple modes of detecting glycocalyx shedding in clinical patients, beyond measurement of circulating biomarkers, as these soluble proteoglycan and glycosaminoglycans can be shed from various sources and may simply reflect cell activation.

Strengths of this study are the early enrolment in the ED, making it a point of difference to most other studies that have delayed enrolment in the ICU, as well as application of predefined inclusion criteria, and serial blood sampling. Limitations include convenience sampling, observational design and a relatively small sample size that may increase the risk of selection bias. Sensitivity analysis did not indicate a significant influence of participant drop-out before T24 blood sampling on the pattern of biomarker concentration change over time, however, we cannot excluded bias generated by participant drop-out. Although this study is exploratory in nature, multiple comparisons made between many biomarkers increases the risk of Type 1 error and results should be interpreted with caution. The findings of this study should be considered as hypothesis-generating only and prospective validation is required. Also, the respiratory failure criterion was expanded to include decreased Sp0₂, as arterial blood sampling was not always possible. Use of SF ratio has been validated in other studies\textsuperscript{61-64} but this
expansion of criteria may have led to some variability of classification of patients.

In summary, endocan and syndecan-1 showed different patterns of shedding in patients with sepsis due to pneumonia. Syndecan-1 showed the strongest associations with neutrophil activation biomarkers as well as relevant clinical outcomes. Further work is needed to better understand the various sources of EG biomarkers, as well as the mechanisms causing their rise during sepsis and acute lung injury.

Acknowledgements

The authors thank Drs Shelley Stone and Simon Brown for intellectual input in conception and design of the study. The authors also acknowledge the contributions of Ellen MacDonald, Sophie Damianopolous and the research nurses at the participating hospitals for patient recruitment and clinical data collection.
Figures and Tables

Figure 1. Endothelial glycocalyx biomarker concentrations (predicted mean, 95% confidence interval) in participants with sepsis due to pneumonia (n=44) at admission to an Emergency Department (0 hours, N=44), 1 hour (N=44), 3 hours (N=34) and 12-24 hours (N=24) later. Asterisks denote significant change ($P<0.05$) from 0 hours. Dashed lines represent median concentration of healthy controls.
### Table 1. Baseline characteristics of participants, either presenting to an Emergency Department with sepsis due to pneumonia or healthy control.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Pneumonia (N=44)</th>
<th>Control (N=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>66 (61-71)</td>
<td>65 (60-71)</td>
</tr>
<tr>
<td>Male</td>
<td>25 (57)</td>
<td>18 (64)</td>
</tr>
<tr>
<td>CCS</td>
<td>2 [1-4]</td>
<td>0 [0-1]</td>
</tr>
<tr>
<td>Infection severity*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simple infection</td>
<td>5 (11)</td>
<td>-</td>
</tr>
<tr>
<td>Sepsis</td>
<td>19 (43)</td>
<td>-</td>
</tr>
<tr>
<td>Septic shock</td>
<td>20 (46)</td>
<td>-</td>
</tr>
<tr>
<td>White cell count (10⁹/μL)</td>
<td>10.8 (8.7-13.3)</td>
<td>-</td>
</tr>
<tr>
<td>C-reactive protein (mg/mL)</td>
<td>200 [65-350]</td>
<td>-</td>
</tr>
<tr>
<td>SOFA Score</td>
<td>4 [2-8]</td>
<td>-</td>
</tr>
<tr>
<td>Respiratory failure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>30 (68)</td>
<td>-</td>
</tr>
<tr>
<td>Day 2</td>
<td>22 (55)</td>
<td>-</td>
</tr>
<tr>
<td>Day 3</td>
<td>12 (32)</td>
<td>-</td>
</tr>
<tr>
<td>CURB-65 score</td>
<td>3 [2-3]</td>
<td>-</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>2.1 [1.3-4.5]</td>
<td>-</td>
</tr>
<tr>
<td>Glasgow Coma Scale</td>
<td>15 [14-15]</td>
<td>-</td>
</tr>
<tr>
<td>Mechanical Ventilation</td>
<td>9 (21)</td>
<td>-</td>
</tr>
<tr>
<td>Admission to ICU</td>
<td>15 (34)</td>
<td>-</td>
</tr>
<tr>
<td>Length of ICU stay (days)</td>
<td>4.9 [0.5-11]</td>
<td>-</td>
</tr>
<tr>
<td>30-day mortality</td>
<td>11 (25)</td>
<td>-</td>
</tr>
<tr>
<td>1-year mortality</td>
<td>15 (34)</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are presented as either mean (95% confidence interval) or median [Q1-Q3] for continuous variables, or No. (%) for binary variables.
* Simple infection was defined as a SOFA score <2, sepsis as a SOFA score ≥2 and septic shock as systolic blood pressure <100mHg despite >20mL/kg of intravenous fluid.

Abbreviations: CCS, Charlson Comorbidity Score; SOFA, Sequential organ failure assessment; ICU, intensive care unit.
Table 2. Correlations between endothelial glycocalyx biomarkers (endocan, syndecan-1 and hyaluronan) and inflammatory biomarkers in patients with sepsis due to pneumonia, measured at admission to the Emergency Department (T0) and 12-24 hours later (T24).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Endocan</th>
<th></th>
<th></th>
<th></th>
<th>Syndecan-1</th>
<th></th>
<th></th>
<th></th>
<th>Hyaluronan</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0</td>
<td>T24</td>
<td>T0</td>
<td>T24</td>
<td>T0</td>
<td>T24</td>
<td>T0</td>
<td>T24</td>
<td>T0</td>
<td>T24</td>
<td>T0</td>
</tr>
<tr>
<td></td>
<td>rho</td>
<td>P</td>
<td>rho</td>
<td>P</td>
<td>rho</td>
<td>P</td>
<td>rho</td>
<td>P</td>
<td>rho</td>
<td>P</td>
<td>rho</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>0.26</td>
<td>0.09</td>
<td>0.36</td>
<td>0.09</td>
<td>0.25</td>
<td>0.11</td>
<td>0.41</td>
<td>0.048</td>
<td>0.35</td>
<td>0.019</td>
<td>0.21</td>
</tr>
<tr>
<td>Interleukin-10</td>
<td>0.24</td>
<td>0.11</td>
<td>0.29</td>
<td>0.17</td>
<td>0.10</td>
<td>0.54</td>
<td>0.40</td>
<td>0.052</td>
<td>0.15</td>
<td>0.34</td>
<td>0.25</td>
</tr>
<tr>
<td>NGAL</td>
<td>0.18</td>
<td>0.25</td>
<td>0.40</td>
<td>0.052</td>
<td>0.52</td>
<td>&lt;0.001</td>
<td>0.52</td>
<td>0.010</td>
<td>0.39</td>
<td>0.008</td>
<td>0.25</td>
</tr>
<tr>
<td>Resistin</td>
<td>0.19</td>
<td>0.21</td>
<td>0.37</td>
<td>0.079</td>
<td>0.54</td>
<td>&lt;0.001</td>
<td>0.53</td>
<td>0.007</td>
<td>0.45</td>
<td>0.002</td>
<td>0.32</td>
</tr>
<tr>
<td>MPO</td>
<td>0.07</td>
<td>0.64</td>
<td>0.14</td>
<td>0.50</td>
<td>0.54</td>
<td>&lt;0.001</td>
<td>0.38</td>
<td>0.067</td>
<td>0.42</td>
<td>0.005</td>
<td>0.48</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>0.18</td>
<td>0.24</td>
<td>0.20</td>
<td>0.35</td>
<td>0.21</td>
<td>0.17</td>
<td>0.32</td>
<td>0.13</td>
<td>0.27</td>
<td>0.079</td>
<td>0.09</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>0.34</td>
<td>0.025</td>
<td><strong>0.52</strong></td>
<td><strong>0.009</strong></td>
<td>0.24</td>
<td>0.13</td>
<td><strong>0.51</strong></td>
<td><strong>0.011</strong></td>
<td>0.32</td>
<td>0.034</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Values in bold indicate significant correlations at rho>0.5. Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; MPO, myeloperoxidase; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.
Table 3. Associations (odds ratio (95% confidence interval)) between variables at Emergency Department admission (glycocalyx biomarker, CURB-65 score or lactate) and clinical outcomes in patients with sepsis due to pneumonia.

<table>
<thead>
<tr>
<th></th>
<th>Respiratory failure</th>
<th>Mechanical ventilation</th>
<th>30-day mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P value</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Endocan (ng/mL)</td>
<td>1.04 (0.99-1.13)</td>
<td>0.053</td>
<td>1.04 (0.97-1.10)</td>
</tr>
<tr>
<td>Syndecan-1 (ng/mL)</td>
<td>1.18 (1.05-1.33)</td>
<td><strong>0.004</strong></td>
<td>1.24 (1.04-1.48)</td>
</tr>
<tr>
<td>Hyaluronan (μg/mL)</td>
<td>1.27 (0.96-1.69)</td>
<td>0.092</td>
<td>1.37 (0.91-2.07)</td>
</tr>
<tr>
<td>CURB-65 score</td>
<td>1.33 (0.97-1.83)</td>
<td>0.077</td>
<td>1.32 (0.72-2.43)</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>1.04 (0.92-1.16)</td>
<td>0.55</td>
<td>1.35 (1.07-1.70)</td>
</tr>
</tbody>
</table>

*Biomarker concentration log-transformed due to non-linearity

Odds ratios are for an increase of 1ng/mL for endocan and syndecan-1, 1μg/mL for hyaluronan and 1mmol/mL for lactate. Bold P values indicate a significant (P<0.05) association.

Respiratory failure was determined on days 1, 2 and 3 of hospitalization by a PaO₂/FiO₂ ratio<300, SpO₂/FiO₂<315 or SpO₂<90% on >6 L/min oxygen. As there was no
Supplementary Table 1. Inflammatory and endothelial activation biomarker concentrations (median, Q1-Q3) in patients with sepsis due to pneumonia, at enrolment in the Emergency Department (T0), then 1 hour (T1), 3 hours (T3) and 12-24 hours (T24) later.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Time point</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0</td>
<td>T1</td>
<td>T3</td>
<td>T24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin-6 (pg/mL)</td>
<td>1352 (230-7999)</td>
<td>680 (169-5833)</td>
<td>662 (151-3837)</td>
<td>126 (57-1522)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Interleukin-10 (pg/mL)</td>
<td>23 (13-106)</td>
<td>20 (13-100)</td>
<td>16 (13-67)</td>
<td>12 (9-17)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>NGAL (ng/mL)</td>
<td>289 (154-601)</td>
<td>264 (159-639)</td>
<td>285 (217-748)</td>
<td>303 (177-522)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Resistin (ng/mL)</td>
<td>65 (31-238)</td>
<td>64 (30-199)</td>
<td>71 (38-187)</td>
<td>94 (40-183)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>MPO (ng/mL)</td>
<td>128 (86-211)</td>
<td>125 (85-194)</td>
<td>152 (92-239)</td>
<td>125 (88-177)</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>ICAM-1 (ng/mL)</td>
<td>526 (384-722)</td>
<td>483 (352-661)</td>
<td>479 (318-721)</td>
<td>492 (297-814)</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>VCAM-1 (ng/mL)</td>
<td>1713 (1291-2272)</td>
<td>1607 (1213-2129)</td>
<td>1531 (1092-2146)</td>
<td>1407 (914-2168)</td>
<td>0.36</td>
<td></td>
</tr>
</tbody>
</table>

$P$ values represent change from T0 to T24, tested by linear mixed regression models on log-transformed data.

Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; MPO, neutrophil myeloperoxidase; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.
Supplementary Table 2. Serum or plasma biomarker concentrations (median, Q1-Q3) for healthy controls

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Healthy Control (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocan (ng/mL)</td>
<td>2.6 (2.3-3.6)</td>
</tr>
<tr>
<td>Syndecan-1 (ng/mL)</td>
<td>0.49 (0.37-0.74)</td>
</tr>
<tr>
<td>Hyaluronan (ng/mL)</td>
<td>42 (24-63)</td>
</tr>
<tr>
<td>Interleukin-6 (pg/mL)</td>
<td>13.8 (5.8-15.2)</td>
</tr>
<tr>
<td>Interleukin-10 (pg/mL)</td>
<td>6.4 (0.1-13.3)</td>
</tr>
<tr>
<td>NGAL (ng/mL)</td>
<td>62 (40-90)</td>
</tr>
<tr>
<td>Resistin (ng/mL)</td>
<td>11.1 (6.8-13.3)</td>
</tr>
<tr>
<td>MPO (ng/mL)</td>
<td>18.9 (14.2-23.1)</td>
</tr>
<tr>
<td>ICAM-1 (ng/mL)</td>
<td>449 (295-1034)</td>
</tr>
<tr>
<td>VCAM-1 (ng/mL)</td>
<td>1394 (937-2147)</td>
</tr>
</tbody>
</table>

Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; MPO, neutrophil myeloperoxidase; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.
4.4 References


5.1 Gap in knowledge

In Chapter 3, we found that rising serum hyaluronan concentration in patients with sepsis soon after admission to the emergency department (ED) was associated with the cumulative volume of intravenous fluid administered. We postulated that large volumes of crystalloid fluid resuscitation might exacerbate hyaluronan shedding, which may in turn promote further inflammation and endothelial dysfunction. We also found a positive association between serum syndecan-1 and biomarkers of neutrophil activation in patients with sepsis due to pneumonia (Chapter 4), suggesting that the degree of inflammatory response may drive syndecan-1 upregulation and shedding.

Similar to sepsis, patients with major trauma can experience an exaggerated systemic inflammatory response and often receive large volumes of crystalloid fluid in the ED soon after admission. Therefore, it was hypothesised that trauma patients may exhibit a similar pattern of endothelial glycocalyx (EG) biomarker shedding over time, to patients with sepsis. The temporal behaviour of EG biomarkers, syndecan-1 and hyaluronan, early in hospitalisation has not been well characterised in trauma. Furthermore, shedding of syndecan-4 has not been explored in trauma, to the author’s knowledge.

This chapter has been prepared in manuscript style and summarises studies on EG shedding in clinical trauma that were published up to, and including, 2018. Glycocalyx shedding in animal haemorrhagic shock models is covered in Chapter 6. The original work in this chapter describes a longitudinal observational study comparing EG biomarkers in patients with major trauma compared to the previously described cohort of patients with sepsis (Chapter 3). Patients from the previous study included those with sepsis and septic shock, grouped into a single group of 'sepsis', but did not include those with simple infection. Trauma patients were age and sex stratified to the sepsis cohort. Associations with cumulative fluid volumes and biomarkers of inflammation
were also explored. This paper has not been submitted for publication. Results have been presented in part in poster form at the International Shock Symposium, Tokyo in 2017.¹

**Background information**

The two earliest studies measuring EG biomarkers, published in 2011, reported increased circulating syndecan-1 concentration in trauma patients soon after ED admission. The first study combined an observational biomarker study of severely injured trauma patients (n=32) and an *in vitro* endothelial injury model.² In the clinical study, blood was sampled at ED admission and later at intensive care unit (ICU) admission (post-resuscitation). Plasma syndecan-1 concentration was increased on ED arrival, compared to healthy controls, and had declined by the time of admission to ICU. Syndecan-1 concentration was poorly positively correlated with interleukin(IL)-10 (goodness-of-fit $R^2=0.12$, $P=0.0002$), though all data were combined for the linear model (pre- and post-resuscitation, and healthy controls). The second study demonstrated increased serum syndecan-1 concentration in patients with trauma (n=75), with blood samples drawn at a median of 43 minutes after injury.³ The authors further dichotomised patients into low and high syndecan-1, according to the median concentration, and compared characteristics of the two groups. Although there was no significant difference in Injury Severity Score (ISS) between these two groups, patients with high syndecan-1 had significantly lower Sp₀₂ and higher lactate, glucose, inflammatory biomarkers (IL-6, IL-10), danger-associated molecular patterns, coagulation-related proteins (soluble thrombomodulin, D-dimers, tissue plasminogen activator, urokinase-type plasminogen activator), as well as higher mortality. Patients with high syndecan-1 also had significantly higher plasma adrenaline and noradrenaline concentrations, than patients with low syndecan-1. The authors hypothesised that the catecholamine surge observed with severe trauma may cause EG shedding.

The same group further explored the concept of sympathoadrenal activation and syndecan-1 shedding in a larger cohort of severely injured patients (n=424).⁴ Blood for biomarker analysis was collected on admission to
the ED. Syndecan-1 was weakly correlated with plasma adrenaline (r=0.38, P<0.001) and noradrenaline (r=0.23, P<0.001) concentrations. Higher syndecan-1 was significantly associated with higher ISS and lower Glasgow Coma Score, systolic blood pressure, base excess, platelet count and haemoglobin. Syndecan-1 was associated with 24-hour (hazards ratio 1.06 [95% CI 1.03-1.09], P<0.001), 7-day (hazards ratio 1.04 [95% CI 1.02-1.06], P<0.001), and 28-day mortality (hazards ratio 1.04 [95% CI 1.02-1.06], P<0.001), as were a range of other variables associated with injury severity. The association between sympathoadrenal activation and syndecan-1 was also explored by a second group, along with a host of other coagulation and inflammatory biomarkers, in 159 patients with moderate to severe TBI. At admission, syndecan-1 was associated with catecholamine concentrations and poor 6-month Glasgow Outcome Scale (GOSE). A range of biomarkers, including adrenaline, noradrenaline, thrombin-antithrombin complexes, tissue factor, tissue plasminogen activator, vascular adhesion protein-1, IL-6, -8 and -10, tumour necrosis factor-α and monocyte chemoattractant protein-1, measured at either admission or 24 hours later, were also positively associated with unfavourable 6-month GOSE.

Multiple studies have also demonstrated associations between syndecan-1 and endothelial activation, coagulation activation/impairment or inflammatory biomarkers in patients with trauma. This includes positive associations with soluble thrombomodulin, neutrophil gelatinase-associated lipocalin (NGAL), and adrenaline concentrations. Syndecan-1 has also been associated with lower colloid osmotic pressure, albumin concentration and hypocoagulability, increased lactate, need for transfusion, multiple organ dysfunction and mortality.

Although most studies have examined a single measurement of serum or plasma syndecan-1 soon after injury, some studies have assessed syndecan-1 levels over time. A recent study of 30 polytrauma patients, with and without haemorrhagic shock, were sampled daily, for the first 5 days from admission. Many biomarkers were assessed over time, including syndecan-1, heparan
sulfate, NGAL, IL-6, and C-reactive protein. Syndecan-1 showed a pattern of increasing concentration over time and was significantly higher in patients with haemorrhagic shock, compared to those without. In contrast, there was no apparent pattern over time in heparan sulfate concentration. In regards to inflammation, NGAL increased over time in patients with haemorrhagic shock, compared to those without shock, as did IL-6, which peaked on day 1. In a second study, syndecan-1 was measured pre-hospital and at some point within 16 hours of admission but did not show a clear pattern over time.\textsuperscript{13} The same group explored the timing of syndecan-1 shedding after trauma in a follow-up study, within which trauma patients (n=91) were sampled once in the pre-hospital period and a second time, 4-12 hours later.\textsuperscript{11} These samples were used from a previous study investigating early immune responses in trauma.\textsuperscript{12} Although there was no difference in syndecan-1 concentration between paired samples, a generalised additive model using all data predicted that increased syndecan-1 concentration, beyond healthy control concentrations, was likely to occur between 5 and 8 minutes of injury, peaking between 20 to 30 minutes after injury.

There is a paucity of clinical trauma studies examining relationships between EG shedding and fluid resuscitation strategies. However, a study in major burns patients (n=39) found that serum syndecan-1 concentration on admission was weakly correlated with fluid volume administered in the first 24 hours (\textit{rho}=0.38, \textit{P}=0.017).\textsuperscript{14} Patients received a median (Q1-Q3) of 167 (114-257) mL/kg of fluid during the first 24 hours. Using a multivariate analysis, syndecan-1 was independently associated with fluid volume (\textit{P}=0.04) and burn-induced compartment syndrome (\textit{P}=0.03) but not mortality, after adjusting for age, sex, percentage of total body surface area burned and inhalation injury.
5.3 Pattern of endothelial glycocalyx shedding biomarkers over time in emergency department patients with major trauma

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Abstract

**Objective:** Endothelial glycocalyx (EG) shedding occurs early during emergency department (ED) treatment of sepsis. It is unknown if the same pattern occurs in patients with major trauma. This study describes temporal changes in EG and inflammatory biomarkers in major trauma from ED arrival, and compares these patterns with sepsis.

**Materials and Methods:** This prospective observational study included 24 trauma patients and 86 patients with sepsis. Serum EG biomarkers measured were syndecan-1, syndecan-4 and hyaluronan. Inflammatory biomarkers were interleukin-6, interleukin-10, resistin and neutrophil gelatinase-associated lipocalin (NGAL). Samples were collected at enrolment in the ED (T0), 1 hour (T1), 3 hours (T3) and 12-24 hours (T24) later. Change in concentration over time within-group (trauma) and between-groups (sepsis vs trauma) was tested using linear mixed regression models. Correlation between EG biomarker and cumulative fluid volume in the trauma group at each time point was tested using Spearman’s correlation. Significance was set at P<0.05.
**Results:** Syndecan-1 concentration was significantly higher in the trauma group, compared to healthy controls, at all time points (all $P<0.001$), however it did not show a significant change over time. Syndecan-4 and hyaluronan concentrations in the trauma group were not significantly different to healthy controls at any time point.

Two biomarkers had significantly different temporal patterns in the trauma group compared to sepsis; syndecan-1 increased in sepsis from T0-T24 (difference to trauma, $P<0.001$) and hyaluronan increased in sepsis from T0-T3 (difference to trauma, $P=0.001$).

Interleukin-6, interleukin-10, NGAL and resistin were all significantly higher in the trauma group, compared to healthy controls, across multiple time points. Interleukin-6 did not significantly decrease over time in trauma, as it did in sepsis ($P<0.001$). Resistin increased from T0-T24 by a greater magnitude in trauma, compared to sepsis ($P=0.003$).

There was no association between EG biomarkers and cumulative fluid volume, except for a negative correlation with syndecan-4 at T0 ($\rho=-0.55$, $P=0.006$) and T1 ($\rho=-0.51$, $P=0.01$).

**Conclusions:** Patterns of EG shedding were distinctly different in trauma patients compared to sepsis patients during the first 24 hours of treatment.

**Introduction**

Major trauma, causing direct tissue injury and hypoperfusion, can be associated with an exaggerated systemic inflammatory response, leading to complications such as multiple organ failure. Damage to the endothelium is thought to be an early player in this clinical syndrome, and can lead to propagation of inflammatory mediators, increased vascular permeability, fluid extravasation and activation of coagulation. The endothelial glycocalyx (EG) is the most superficial layer of the endothelial cells, and provides a barrier between the circulation and the sub-endothelial matrix. Damage, or shedding, of the EG is one of the first responses to injury or inflammation and leads to leukocyte margination and increased vascular permeability.
Previous studies in major trauma have demonstrated increased serum or plasma concentrations of syndecan-1, a component of the EG, within an hour of injury.\textsuperscript{2,11} Increased syndecan-1 is positively associated with transfusion requirements, Extended Outcome Glasgow Scale, Sequential Organ Failure Assessment (SOFA) score, and mortality.\textsuperscript{5,6,8} Syndecan-1 is also positively associated with inflammatory biomarker concentrations in trauma patients,\textsuperscript{2,3,5} therefore early shedding of syndecan-1 may contribute to the inflammatory response in trauma. Other biomarkers of EG shedding, such as syndecan-4 and hyaluronan, have not been as well studied in trauma.

Early stabilisation of major trauma patients presenting in shock includes initial rapid administration of crystalloid fluid, with blood products and haemorrhage control emphasised in the setting of ongoing blood loss. Crystalloid fluid given during resuscitation for haemorrhagic shock is associated with increased biomarker concentrations of EG shedding and inflammation in rodent models, compared to administration of colloid fluids.\textsuperscript{19-22} Large volumes of crystalloid fluid may cause EG shedding by diluting plasma components or causing release of natriuretic peptides secondary to atrial stretch.\textsuperscript{22-26} An association between EG shedding and volumes of crystalloid fluid administered during the resuscitation phase has not been explored in trauma patients. Such an association has been demonstrated in people with sepsis. The syndromes of sepsis and trauma share some similarities in that fluid resuscitation within hours of hospital admission is a cornerstone of initial treatment, and that the first 24 hours of stabilisation often includes an ‘inflammatory storm’. Previous work by our group demonstrated that EG biomarkers syndecan-1 and hyaluronan increased over time during emergency department (ED) treatment of sepsis, and that hyaluronan was associated with the cumulative volume of fluid delivered during the same time period.\textsuperscript{27} It is unknown if the same patterns may be found in patients with major trauma.

This observational study aimed to describe the pattern of EG shedding and inflammatory biomarker concentrations over the first 24 hours of treatment.
in patients with trauma, and compared this to patients with sepsis from a previous study. The second objective was to determine if cumulative fluid volume or measures of inflammation were correlated with increased EG shedding in trauma patients, as is found in sepsis patients.

**Materials and Methods**

**Study design**

This observational study compared patients with trauma to a historical cohort of patients with sepsis from previously published studies. Selection criteria for patients with sepsis have been detailed elsewhere and are summarised in Figure 1. The trauma cohort included patients presenting to the ED of a Level 1 trauma centre participating in the Critical Illness and Shock Study (CISS)(HREC permit number 2009-080). As in patients with sepsis, trauma patients underwent real-time data collection and research blood sampling during the initial 24 hours from enrolment, and were then followed for clinical outcomes. Recruitment into CISS occurred during rostered research nurse hours 0700-2100, up to 7 days of the week. Thirty participants presenting after traumatic injury and meeting CISS criteria for critical illness were selected from the CISS database, using age and sex strata to match the sepsis cohort (Figure 1). Six were excluded due to insufficient blood samples available, leaving 24 participants for the study. Bio-banked healthy control samples were also selected based on age and sex-stratification to match the sepsis group. Biomarker data from the healthy control cohort have been previously published but are also provided in this study for comparison.

**Data collection**

Participant characteristics and outcome data were retrieved from medical records. Charlson Comorbidity Score (CCS) was calculated on the first day of hospitalisation and the Injury Severity Score was retrieved from the State Trauma Centre Database. Cumulative fluid volume at each blood sampling time point was calculated for each patient in the same manner as for the sepsis patients and included isotonic or hypertonic crystalloid and gelatine colloid
fluid. The calculated volume at each time point included all fluids given up until that time, including pre-hospital fluids.

**Biomarker analysis**

Both cohorts followed the same plan for blood sample timing, as per the CISS protocol. Blood samples were collected as soon as practicable after enrolment in the ED (T0) and then 1-2 hours (T1), 3-5 hours (T3) and 12-24 hours later (T24). Samples were collected into a serum clot tube then centrifuged at 3000rpm at 4°C for 10 minutes. Serum was then aliquoted into cryogenic tubes and stored at -80°C. Biomarkers syndecan-1, syndecan-4 and hyaluronan were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Inflammatory cytokines interleukin-6, interleukin-10, resistin and neutrophil gelatinase-associated lipocalin (NGAL) were also measured at each time point using either a commercial multiplex cytometric bead array kit (interleukin-6, interleukin-10) (Cytometric Bead Array Flex sets, Becton Dickinson, USA) or commercial ELISA kit (resistin, NGAL)(R&D Systems, Minneapolis, MN, USA). Biomarker data was retrieved from the CISS database for the sepsis cohort and the laboratory techniques were replicated for measurement of biomarker concentrations for the trauma cohort.

**Statistical analysis**

Normality of data was assessed by visual inspection of histograms and Q-Q plots. Trauma participant characteristics were summarized using proportions (percentage) for categorical variables, and median [Q1-Q3] or mean (95% confidence interval) for continuous variables depending on normality. Between-group differences (trauma versus sepsis) for participant characteristics were tested using Student’s t-test (normal continuous data), Kruskall Wallis test (non-normal continuous data) or chi-square test (categorical data).

Biomarker concentrations were log-transformed to generate normal or approximately normal distributions. Difference in biomarker concentration between control and trauma was tested at each time point using Student’s t test.
For each biomarker, change in concentration over time within-group (trauma) and between-groups (sepsis vs trauma) was tested using linear mixed regression models incorporating maximum likelihood estimation. This model compared the difference between trauma and sepsis in the slope of the change between T0 and later time points (T0-T1, T0-T3 and T0-T24). Change in biomarker concentration over time within the sepsis group has been previously described, therefore this study only compares the pattern in the change over time between the two groups. Raw data was summarised as geometric mean (95% confidence interval). A sensitivity analysis was performed for each model that included only participants with complete blood sampling to T24, to ensure that dropout of participants prior to T24 did not significantly skew the pattern in change over time. Correlations between EG and inflammatory biomarker concentrations, as well as cumulative fluid volume, in the trauma group were tested using Spearman’s rank correlation.

All analyses were performed using Stata 14 (College Station, TX, USA) with significance set at P<0.05.

Results

Participant characteristics

This study included 24 patients with major trauma (Figure 1). The median Injury Severity Score of the trauma group was 18 (Q1-Q3, 12-38) with most patients presenting with blunt (n=23) not penetrating (n=1) trauma. The median time from injury to ED arrival was 81 minutes (Q1-Q3, 55-233). The sepsis group had a significantly higher proportion of comorbidities than the trauma group (Table 1).

Glycocalyx biomarkers

Syndecan-1 concentration was significantly increased in the trauma group, compared to control, at all time points (all P<0.001)(Panel 1), however there was no significant change over time in the trauma group. This lack of change over time was significantly different to the change over time in the sepsis
group (P=0.004), where syndecan-1 increased from T0 to T24. Syndecan-4 concentration was not significantly different in the trauma group, compared to the control, at any time point, and did not significantly change over time, nor was this change over time different to the sepsis group (Panel 1). Hyaluronan concentration in the trauma group was also not significantly different to control at any time point, and did not significantly change over time. This lack of change over time was significantly different to the change over time in the sepsis group (P=0.001), where hyaluronan increased from T0 to T3.

Inflammatory biomarkers

Interleukin-6 concentration was significantly higher in the trauma group, compared to control, at all time points (all P<0.001)(Panel 2), however there was no significant change over time. This lack of change was significantly different to the change over time in the sepsis group (P<0.001), whereby interleukin-6 decreased from T0 to T24. Interleukin-10 concentration was significantly higher in trauma patients, compared to control, at T0 (P<0.001), T1 (P<0.001) and T3 (P=0.012), and significantly decreased over time from T0 to T24 (P=0.033)(Panel 2). There was no significant difference in the change over time between sepsis and trauma. Neutrophil gelatinase-associated lipocalin concentration was significantly higher in trauma patients, compared to control, at all time points (T0, P=0.002; T1, P=0.001; T3, P<0.001; T24, P<0.001), but there was no significant change over time, nor was there any difference in the change over time between sepsis and trauma (Panel 2). Resistin concentration was also in increased in trauma patients, compared to control, at all time points (all P<0.001), and significantly increased from T0 to T24 (P=0.018)(Panel 2). This increase from T0 to T24 was of significantly greater magnitude than the increase observed in the sepsis group (P=0.003).

Only two significant correlations were identified between EG and inflammatory biomarkers at either T0 or T24; both were weak associations (Table 2).

Cumulative fluid volume
Median cumulative fluid volume in the trauma group at T0 was 1000 mL (Q1-Q3, 750-2875), at T1 was 1500 mL (1000-3125), at T3 was 2750 mL (1500-3625) and at T24 was 3500 mL (2250-5500). At both T0 and T1, syndecan-4 was significantly, but weakly, negatively correlated with cumulative fluid volume (Table 3). There were no other significant correlations between EG biomarkers and cumulative fluid volume.

Blood products were administered to a small proportion of trauma patients over the time period of research blood sampling, including packed red cells (n=6), fresh frozen plasma (n=6), albumin (n=1), cryoprecipitate (n=5), and platelet concentrate (n=6).

**Discussion**

This study found that the EG biomarkers, syndecan-1 and hyaluronan, showed a different pattern of expression over time in trauma patients, compared to patients treated for sepsis. This difference was most apparent in hyaluronan concentration. While there was no significant difference to healthy control concentrations in trauma, this biomarker showed a rapid rise during early resuscitation in patients with sepsis. The trauma and sepsis groups showed similar patterns in inflammatory biomarker concentrations over time, however the sepsis group showed much higher concentrations during this early phase of treatment.

The different patterns of EG biomarker shedding between the trauma and sepsis patients in this study may be explained by differences in severity of the inflammatory response, timing of insult, timing of fluid administration or the underlying pathophysiology. Trauma studies have shown that syndecan-1 is increased at admission, with one study predicting that the peak of syndecan-1 shedding to be within 20 to 30 minutes of injury, using mathematical modelling. The results of our study suggest that this increase in trauma patients changes little over the first 24 hours of hospitalisation. However, in patients with sepsis, syndecan-1 increased over time. Sepsis patients also
demonstrated marked elevations in all four inflammatory biomarkers measured in this study (Panel 2). Inflammation is known to not only stimulate EG shedding but also upregulate endothelial expression and increase cell turnover of syndecan-1.\textsuperscript{33-35} Lipopolysaccharide, a component of gram-negative bacteria, has also been shown to upregulate and shed syndecan-1 from murine endothelium.\textsuperscript{36,37} Therefore, it is possible that increasing concentrations of syndecan-1 not only represent increased EG shedding in sepsis but also increased expression related to the pathology of sepsis. A contribution of shed syndecan-1 from high numbers of circulating neutrophils or tissue macrophages in sepsis should also be considered.\textsuperscript{38,39} In contrast, major trauma patients at risk of multiple organ dysfunction may take longer to manifest these changes in syndecan-1 concentration.

The trauma patients in this study did not show increasing hyaluronan concentrations during ED treatment. This was in contrast to sepsis, whereby hyaluronan sharply increased in the first three hours. Previously, we had found that this increase, in the same cohort of patients, was associated with cumulative volume of fluids administered.\textsuperscript{27} Therefore, we hypothesised that we would see a similar pattern of hyaluronan shedding in patients with trauma, as these patients also receive fluid loading during this time period. In our previous paper, the median cumulative volume given at T0 in patients with sepsis was 250 mL (Q1-Q3, 0-1000).\textsuperscript{27} However, in the trauma group, median cumulative volume at T0 was 1000 mL (Q1-Q3, 750-2875). This means that a large bolus of fluid had been delivered to most trauma patients in the pre-hospital setting. There was also variability in time from injury to hospital admission (median 81 minutes [55-233]). Therefore, it is possible that the peak of hyaluronan shedding in relation to rapid fluid administration was missed in this study. An immediate rise in hyaluronan concentration after fluids is supported by a study performed in healthy men, where a peak in hyaluronan concentration was observed 15 minutes after the end of a 30-minute infusion of crystalloid.\textsuperscript{40} The delay in blood sampling after some degree of fluid loading in our study may also explain the lack of peak in syndecan-1 at admission in the trauma patients.
Another possibility for trauma patients failing to show a rise in hyaluronan and syndecan-1 during resuscitation in the ED may have been the provision of plasma during this treatment phase. Bolus therapy with blood products containing plasma is associated with less EG shedding, compared to administration of crystalloid fluids. Provision of proteins and avoiding haemodilution appears to be protective of the EG. Although only 6 out of 24 patients received plasma during the time period when research blood sampling was performed, it is possible that provision of plasma proteins in these patients may have skewed results. Unfortunately, the small sample size did not allow for further sub-group analysis.

One unique aspect of this study is the inclusion of resistin as a measure of inflammation in trauma. Although resistin is described as an adipokine, it is also secreted by monocytes and is an important pro-inflammatory mediator. This biomarker is associated with multiple organ failure in sepsis, and given data on resistin concentration was already available for the sepsis cohort, we thought it relevant to compare to patients with major trauma. Plasma resistin concentration has been found to increase over the first 24 hours of hospitalisation for traumatic brain injury, which is consistent with the increase observed in our study. Increased resistin concentration was also associated with poorer outcomes in the aforementioned study. Otherwise, most studies demonstrating an association between increased resistin and organ failure are in patients with sepsis, though two studies have also shown an association with illness severity in pancreatitis. Further work needs to be done to elucidate the relationship between activated endothelial cells, secretion of resistin and organ dysfunction in critical illness, including sepsis and trauma.

A major strength of this study is the comparison of patient cohorts that were age stratified. The syndrome of sepsis is more frequent in patients of advanced age with chronic comorbidities. Age is well known to induce some form of chronic endothelial dysfunction, including altering the structure and thickness of the EG. Comorbidities more common in older age are also associated with altered EG thickness and increased blood concentrations of
EG biomarkers.\textsuperscript{50-52} Given that major trauma is more common in young adults with a low rate of comorbidities, it was important to ensure that we selected a cohort that was at least close to the sepsis cohort in age. Unfortunately, this also created a significant limitation of small sample size, as samples available in the biobank for older participants were not plentiful. There was also a limitation of lack of repeat blood sampling in the trauma patients due to logistical reasons, for example, the patient being in theatre. Also, although the mean age of the trauma patients was no different to the sepsis patients, there were significantly fewer comorbidities in the trauma patients, which may have altered EG biomarker concentrations or the time course of EG shedding, compared to the sepsis patients with a higher frequency of comorbidities. The small sample size also precluded utilising mixed effects linear models to explore the associations between EG biomarkers and cumulative fluid over time, as was done for the previous paper demonstrating this association.\textsuperscript{27}

**Conclusion**

There are differing patterns of EG shedding in trauma compared with sepsis during the first 24 hours of hospitalisation. Differences in the degree of inflammation or timing of crystalloid fluid therapy may contribute to this observation.

**Footnotes**

\textsuperscript{a} Department of Health Western Australia, Royal Perth Hospital Trauma Registry Report 2014, Perth, Western Australia
Figures and Tables

Figure 1. Study enrolment flow chart

Enrolment into the Critical Illness and Shock Study at Emergency Department admission
- Shock (hypotension OR shock index>1 OR lactate>4mmol/l),
- OR Hypoxemia
- OR Sepsis
  - Likely infection,
  - AND at least two out of four SIRS criteria,
  - AND shock OR hypoxemia OR organ dysfunction

Participants with trauma meeting CISS criteria (shock, hypoxemia) between March 2010 and July 2013 (n=30)

Excluded
- Only T0 samples collected (n=6)

- T0 (n=24)
- T1 (n=24, except n=23 for IL-6 and IL-10)
- T3 (n=20)
- T24 (n=14, except n=15 for NGAL)

Sepsis Cohort (n=86)
- T0 (n=86, except n=85 for Syn-4)
- T1 (n=86)
- T3 (n=81)
- T24 (n=70)

Consecutive participants with sepsis between January 2012 and July 2013* (n=109)*

Excluded
- Simple infection (n=23)

Age/sex stratified

Trauma Cohort (n=24)

Table 1. Baseline characteristics of emergency department patients with sepsis or trauma. Healthy control group provided for reference.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control (n=29)</th>
<th>Sepsis (n=86)</th>
<th>Trauma (n=24)</th>
<th>P value*</th>
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<tbody>
<tr>
<td>Age (years)</td>
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<td>Male, n (%)</td>
<td>18 (62)</td>
<td>51 (59)</td>
<td>15 (63)</td>
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<td>CCS</td>
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<td>2 [1-4]</td>
<td>1 [0-1]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Comorbidities, n (%)</td>
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<tr>
<td>Pulmonary disease</td>
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<td>Renal disease</td>
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</tr>
<tr>
<td>Prior MI</td>
<td>2 (7)</td>
<td>13 (15)</td>
<td>2 (8)</td>
<td>0.37</td>
</tr>
<tr>
<td>CHF</td>
<td>2 (7)</td>
<td>10 (12)</td>
<td>2 (8)</td>
<td>0.64</td>
</tr>
<tr>
<td>Admission to ICU n (%)</td>
<td>-</td>
<td>38 (44)</td>
<td>13 (57)*</td>
<td>0.29</td>
</tr>
<tr>
<td>Length of stay (days)</td>
<td>-</td>
<td>7 [4-13]</td>
<td>16 [5-30]</td>
<td>0.057</td>
</tr>
<tr>
<td>30-day mortality n (%)</td>
<td>-</td>
<td>16 (19)</td>
<td>5 (21)</td>
<td>0.81</td>
</tr>
<tr>
<td>Time-to-first-research sample (mins)</td>
<td>-</td>
<td>47 [30-108]</td>
<td>30 [16-67]</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Data is presented as either median [Q1-Q3], mean (95% confidence interval) or number (percentage). *P values for difference between sepsis and trauma. # One patient in the Trauma group died in the ED before admission to ICU. Time-to-first-research sample was calculated from time of ED admission to the time the first blood sample was taken for the study.

Abbreviations: CCS, Charlson Comorbidity Score; MI, myocardial infarction; CHF, congestive heart failure; ICU, intensive care unit; ED, emergency department.
Table 2. Spearman correlation (P value) between glycocalyx biomarkers (syndecan-1, syndecan-4, hyaluronan) and inflammatory biomarker (interleukin-6, interleukin-10, resistin and neutrophil gelatinase-associated lipocalin (NGAL). concentrations in patients with trauma, measured at emergency department admission (T0)(n=24) and 12 to 24 hours later (T24)(n=14).

| Biomarker | Syndecan-1 | | | | Syndecan-4 | | | | | Hyaluronan | | |
|-----------|------------|--------|----------------|------------|--------|----------------|------------|--------|----------------|------------|
|           | T0         | T24    | rho            | P           | T0         | T24    | rho            | P           | T0         | T24    | rho            | P           |
| Interleukin-6 | 0.25       | 0.24   | 0.51           | 0.078       | -0.17      | 0.44   | -0.18           | 0.55         | 0.0       | - | 0.35           | 0.25         |
| Interleukin-10 | 0.13       | 0.54   | -0.01          | 0.97        | -0.29      | 0.17   | -0.29           | 0.33         | 0.24      | 0.26           | 0.06         | 0.85       |
| NGAL       | -0.15      | 0.48   | -0.28          | 0.33        | 0.18       | 0.40   | 0.56           | 0.039        | -0.40      | 0.085          | -0.54         | 0.047      |
| Resistin   | -0.32      | 0.12   | 0.06           | 0.85        | 0.11       | 0.61   | 0.11           | 0.71         | -0.49      | 0.014          | -0.27         | 0.36       |
Table 3. Spearman’s correlation ($P$ value) between cumulative fluid volume and glycocalyx biomarker concentrations in patients with trauma, measured at emergency department admission (T0)(n=24), then 1 hour (T1)(n=24), 3 hours (T3)(n=20) and 12 to 24 hours later (T24)(n=14).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cumulative fluid volume</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T0</td>
<td>T1</td>
<td>T3</td>
<td>T24</td>
</tr>
<tr>
<td></td>
<td>rho</td>
<td>P</td>
<td>rho</td>
<td>P</td>
<td>rho</td>
</tr>
<tr>
<td>Syndecan-1</td>
<td>-0.15</td>
<td>0.47</td>
<td>0.11</td>
<td>0.61</td>
<td>0.20</td>
</tr>
<tr>
<td>Syndecan-4</td>
<td>-0.55</td>
<td>0.006</td>
<td>-0.51</td>
<td>0.011</td>
<td>-0.33</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>0.02</td>
<td>0.93</td>
<td>0.19</td>
<td>0.37</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Panel 1. Geometric mean (95% confidence interval) of endothelial glycocalyx biomarker concentrations (syndecan-1, syndecan-4 and hyaluronan) in patients with either sepsis or trauma, measured at Emergency Department admission (T0), then 1 hour (T1), 3 hours (T3) and 12 to 24 hours later (T24). Shaded area represents the 95% confidence interval of the healthy control group.
Panel 2. Geometric mean (95% confidence interval) of inflammatory biomarker concentrations in patients with either sepsis or trauma, measured at Emergency Department admission (T0), then 1 hour (T1), 3 hours (T3) and 12 to 24 hours later (T24). Shaded area represents the 95% confidence interval of the healthy control group.
5.4 References


49. Gorshkov AY, Klimushina MV, Boytsov SA, Kots AY, Gumanova NG. Increase in perfused boundary region of endothelial glycocalyx is associated with higher prevalence of ischemic heart disease and lesions of microcirculation and vascular wall. *Microcirculation* 2018;25:e12454.


CHAPTER 6 – Effect of fluid resuscitation on endothelial glycocalyx shedding and inflammation

6.1 Gap in Knowledge
In Chapter 3, we found that an increase in serum hyaluronan concentration over time in patients with sepsis soon after admission to the emergency department (ED) was positively associated with the cumulative volume of intravenous fluid administered. In Chapter 5, we found that serum hyaluronan concentration did not increase over time during fluid resuscitation of major trauma patients in the ED. Further, although serum syndecan-1 concentration increased over 24 hours in patients with sepsis, we did not observe a change over time in patients with trauma. We proposed that the peak in endothelial glycocalyx (EG) shedding might have been missed in trauma patients, given that the trauma cohort received a median of 1000mL of fluid in the pre-hospital setting before research blood sampling.

We sought to explore the relationship further between large-volume crystalloid fluid administration and EG shedding in a canine haemorrhagic shock model, with controlled timing of blood sampling. Both major trauma and sepsis can cause EG shedding and alter normal kinetics of fluid redistribution,1 therefore we chose to use a simple atraumatic haemorrhagic model to explore EG shedding, without these confounding variables. We opportunistically employed a model already in use to assess adverse effects of synthetic colloid fluids, compared to fresh whole blood and crystalloid fluid. The benefit of using this model was that the study design ensured similarities in effective circulating blood volume after fluid redistribution and restoration of tissue perfusion.

The background information below summarises studies on EG shedding in haemorrhagic shock animal models that were published up to, and including, 2018. This background information is followed by an original study of a canine haemorrhagic shock study comparing the effect of four different fluid strategies on concentrations of hyaluronan, atrial natriuretic peptide and inflammatory
cytokines. The results of this study were, in part, published in abstract form (Critical Care 2018, 22(Suppl 1):P293) as a part of the International Symposium of Intensive Care and Emergency Medicine, Brussels, Belgium (March, 2018) and have subsequently been published in full in Inflammation.

6.2 Background

Administration of intravenous isotonic crystalloid fluid improves effective circulating blood volume; however, a proportion of the fluid infused redistributes to the interstitial space within approximately 30 minutes. Therefore, in order to achieve normalisation of circulating blood volume after haemorrhage, a volume larger than the existing blood volume deficit is usually required. This creates a period of time whereby blood volume is expanding quickly before dissipation and redistribution. During this same time, blood constituents are rapidly diluted and the cardiac chambers are potentially stretched beyond homeostatic wall limits, which may cause release of natriuretic peptides. These two consequences of rapid, large-volume crystalloid fluid administration; haemodilution and release of natriuretic peptides, are proposed as harmful to the EG (see Section 1.3.2 and 1.3.3). Other strategies of blood volume expansion include use of products with large molecules that are designed to have less extravascular redistribution, such as natural protein solutions (albumin or plasma) or synthetic colloid solutions (hydroxyethyl starch (HES) or gelatine). Because these fluids tend to stay in the intravascular space, a smaller volume is used to expand blood volume, compared with crystalloid fluids. Studies assessing harmful effects of crystalloid fluid administration on the EG include comparison to one or more of these types of colloid products. Comparison of natural plasma-derived colloid solutions with synthetic colloid solutions has the added benefit of keeping the volume of infused fluid the same, avoiding variations in cardiac chamber stretch, while adding the variable of haemodilution that is created by synthetic colloids. This review will include weight-average molecular weight and degree of substitution, as denoted by \( x/y \), for HES products mentioned, as these properties affect the pharmacokinetics and pharmacodynamics of individual products.
Most experimental studies assessing EG shedding in haemorrhagic shock have used rodent models. A series of rodent studies published by the Damage Control Resuscitation group at the U.S Army Institute of Surgical Research compared the effects of different resuscitation fluids on EG biomarkers, EG thickness and leucocyte activation.\textsuperscript{8-11} Their first study showed that rats that received 75 mL/kg of lactated Ringer’s solution (LRS) or 15 mL/kg of HES 670/0.75 for haemorrhagic shock had decreased EG thickness, two hours after the start of the fluid bolus, compared to those given 15 mL/kg of fresh frozen plasma (FFP).\textsuperscript{8} Endothelial glycocalyx thickness was estimated by a dye exclusion technique. Plasma syndecan-1 concentration was also significantly higher in the rats receiving LRS and HES, compared to FFP. Although they reported no differences in endothelial wall shear rate between treatment groups, it was only measured once, two hours after the start of the bolus. A follow-up study with a similar design compared multiple parameters after 15 mL/kg of fresh whole blood (FWB), 15 mL/kg of packed red blood cells (PRBC)/LRS (1:1), 15 mL/kg of washed PRBC/LRS (1:1) or 75 mL/kg of LRS.\textsuperscript{9} Blood samples were taken 30 minutes after fluid resuscitation. The LRS group showed significantly increased plasma heparan sulfate concentration and decreased glycocalyx thickness, compared to baseline. Interestingly, the PRBC/LRS product that underwent a preparatory washing procedure also showed these differences, whereas the PRBC/LRS preparation that wasn’t washed did not show these changes from baseline. The unwashed PRBC/LRS group also did not have a decrease in total plasma protein, indicating that this group received more proteins than the washed group. Therefore, the results of this study support the proposal that provision of proteins protects the EG. A third study of similar design conducted by the same group randomised rats to a range of different treatments, including 15 mL/kg of FWB, PRBC, FFP or 5% albumin, 8 mL/kg of 3% saline, 45 mL/kg of 0.9% saline or 75 mL/kg of LRS.\textsuperscript{10} Measurements were made 30 minutes after the end of the fluid bolus. Rats that received either 5% albumin, LRS, 3% saline or 0.9% saline had a significantly increased shock index after the fluid bolus, whereas rats that received FWB, PRBC and FFP did not have a shock index different from baseline. Shock index was not defined but was presumably calculated as heart rate divided by either mean or systolic arterial
blood pressure, as per standard definitions. The greatest increases in syndecan-1 and heparan sulfate were observed in the LRS and 0.9% saline group, with the lowest concentrations observed in the FWB and FFP groups. Both isotonic crystalloid groups also showed higher vascular permeability and lower EG thickness, based on the dye exclusion method. Given that the LRS, 0.9% saline and 3% saline groups all had an increased shock index at the time of biomarker measurement, it is hard to separate the effects of fluid versus persistent shock. However, rats that received 5% albumin also had an increase in shock index but less EG shedding than the crystalloid groups, again supporting the proposal that protein-poor crystalloid fluids wash-out the EG. Finally, these investigators published a later study using the same design that randomised rats to receive either 45 mL/kg of LRS or 0.9% saline, or 15 mL/kg of 5% albumin or FFP. Data was collected two hours after fluid resuscitation. Both crystalloid groups had reduced EG thickness and increased syndecan-1 concentration, compared to the albumin and FFP groups. The 0.9% saline group also showed significantly increased microvascular permeability, and leukocyte rolling and adhesion, compared to the albumin and FFP groups, whereas the LRS group was not significantly different. Cardiovascular monitoring did not extend beyond basic parameters; however, microvascular haemodynamics such as blood flow and endothelial wall stress appeared worse in the 0.9% saline group, based on a significant difference compared to baseline, indicating poorer peripheral perfusion at two hours.

Another series of rodent studies with a military focus were published from the University of Texas. Mice had pressure-targeted atraumatic haemorrhage and then pressure-targeted fluid resuscitation with either fresh plasma or LRS. Volumes administered were a mean of ~30 mL/kg or ~150 mL/kg, respectively, over two hours. Mice that either experienced shock alone or received LRS showed higher histologic lung injury scores, compared to those allocated to anaesthesia-only or fresh plasma. Also, the shock and LRS groups both had thinner EG measurements on electron microscopy, and decreased syndecan-1 mRNA expression and surface syndecan-1 expression. This study demonstrated that shock alone caused shedding of the EG, which was
ameliorated by plasma infusion but not by LRS. In the authors’ second study, mice were given a laparotomy incision to induce surgical trauma before being bled to a pressure target of a mean arterial blood pressure (MAP) of 35mmHg for 90 minutes. Mice were then given either three times the shed volume in LRS or the shed volume in FFP, or no fluids (shock alone). Mean arterial pressure was only measured over the 15 minutes that the fluid bolus was being delivered, and the LRS group was trending down before the end of this time period, therefore it is possible that the LRS group suffered poorer perfusion in the hours following fluid resuscitation. Mice that had either shock alone or received LRS had significantly increased plasma syndecan-1 concentration, increased leucocyte binding to endothelial cells, increased lung vascular permeability and lung myeloperoxidase expression, at three hours after the start of the experiment. This study also had an in vitro component, where application of LRS to pulmonary endothelial cell culture produced greater endothelial permeability and leucocyte adhesion, compared to cells incubated with FFP.

Most studies have included 0.9% saline or LRS as the isotonic crystalloid fluid of choice for studies assessing effects on the EG. However, one recent study focused on Ringer’s acetate, an alternative balanced isotonic crystalloid comparable to LRS. This study in mice showed that plasma concentration of heparan sulfate, but not syndecan-1, was significantly increased in mice that received 135 mL/kg of Ringer’s acetate, compared to mice that received 25 mL/kg of 5% albumin or 31mL/kg of FFP, measured two hours after the end of the fluid bolus. There were no differences in haematocrit at the end of the experiment; however, the Ringer’s acetate group did have lower MAP and higher lactate concentration, suggesting that volume loading was not as effective in this group. Blood volume measured by an indicator dilution technique was also lower in the Ringer’s acetate group. When EG biomarker concentrations of heparan sulfate and syndecan-1 were calculated per mL of plasma volume, there were no significant differences between treatment groups. This study was limited since the delayed measurement of EG biomarkers two hours after the end of fluid resuscitation would have missed any peak that occurred immediately after the fluid bolus.
The challenge with many of the rodent models is that they are unable to easily measure the degree of shock, such as assessment of oxygen extraction ratio. Most rodent studies rely on crude parameters such as MAP or heart rate, and often at quite infrequent time points. Crystalloid fluid may not maintain effective circulating blood volume adequately, compared to colloid fluids, therefore it is hard to separate effects of a persisting perfusion deficit on the EG, compared to effects solely related to crystalloid infusion. Shock alone, without fluid resuscitation, has been shown to shed the EG or increase EG biomarkers, in haemorrhagic shock animal models.\textsuperscript{8,13,16-18} Therefore, it is important that differential effects of fluid resuscitation on cardiovascular parameters and oxygen utilisation are monitored closely in studies assessing EG shedding. Large animal models, such as pigs or dogs, afford the ability of advanced invasive monitoring and are best directed at differentiating the effects of fluid resuscitation.

There are few large animal model studies assessing EG shedding after crystalloid infusion. One swine study mentioned above examining traumatic brain injury and controlled haemorrhage showed increased syndecan-1 concentration during shock; however, a fluid intervention was not delivered.\textsuperscript{17} Another swine study demonstrated increased heparan sulfate concentration after ischaemic-reperfusion via aorta occlusion, and also showed that sevoflurane prevented further increases, compared to propofol infusion.\textsuperscript{18} Interestingly, in sham pigs, heparan sulfate concentration also increased over time in the propofol group, compared to sevoflurane, though to a lesser degree than the pigs experiencing ischaemic-reperfusion. The authors speculated that this shedding of heparan sulfate might have been due to surgical stimulation or a direct effect of propofol.

Ensuring equitable blood volume expansion when comparing fluid resuscitation strategies is challenging. The correct colloid to crystalloid ratio may vary according to the products compared, as well as patient factors associated with pattern of redistribution, such as species, initial blood volume...
(euvolaemia versus hypovolaemia)\(^1\) and vascular permeability.\(^1\) Controlled animal models of shock use a ratio of approximately 1:3.\(^1,13,20,21\) That is, 1 mL of synthetic colloid fluid is considered equivalent to administering 3 mL of isotonic crystalloid based on measures of cardiovascular response. We found in our previous work that this ratio may be closer to 1:4 in greyhounds to produce similar haemodilution.\(^22\) Of course, the debate of the correct colloid to crystalloid ratio is fuelled further by blinded clinical trials in people showing that the ratio is closer to 1:1.5;\(^23\) however, measuring effectiveness of blood volume expansion in the clinical setting is challenging. Thus, volumes of fluid administered tend to gravitate towards accepted clinical practice of what should be ‘adequate’. To add further complexity, there is no precise method for measuring efficacy of the product (i.e. degree of blood volume expansion) or effectiveness (i.e. perfusion). Most techniques used to measure degree of blood volume expansion use tracer dilution, either of haemoglobin, albumin or injected molecules, each with varying accuracy.\(^24\) Some authors have generated equations using change in haemoglobin to measure blood volume expansion,\(^4,25,26\) however, this method presumes a standard blood volume before haemorrhage, which can vary between individuals.\(^27\) Using albumin as a measure of haemodilution can be inaccurate because of extravasation of albumin, especially under conditions of increased vascular permeability.\(^24,28\) Given the lack of data on what is truly equitable volume when for colloids versus crystalloids in any single animal model, it is important to ensure that at least haemoglobin concentration and invasive measures of cardiovascular performance are not different between fluid groups.

A further consideration for assessing effects of different fluid resuscitation strategies on EG shedding is the interaction of the endothelium with inflammation. It is likely EG shedding precedes inflammation in states of circulatory shock, based on early increases in syndecan-1 concentration in people with major trauma.\(^29-33\) The exact time course of EG shedding in relation to inflammatory cytokine release and fluid resuscitation has not been examined in detail in shock models. However, measures of inflammation have been compared between different fluid strategies in studies using animal models. In a
porcine study, 28 mL/kg of blood was withdrawn over 15 minutes via the carotid artery and shock was maintained for 60 minutes. The pigs then received either 84 mL/kg of LRS, 10.9 mL/kg of 7.5% saline or the volume of withdrawn blood returned. A comparison group did not undergo haemorrhage but received 84 mL/kg of LRS. Both groups that received LRS, bled and not bled, showed increased neutrophil activation at the end of resuscitation, compared to baseline, and this was maintained at 180 minutes beyond resuscitation in the bled group. In a similar follow-up porcine study by the same group, pigs underwent haemorrhage followed by administration of either LRS (84 mL/kg over 60 minutes, 84 mL/kg over 180 minutes, or 28 mL/kg over 60 minutes), 28 mL/kg of colloid (dextran, 6% HES 600/0.75, 5% human albumin), 5.6 mL/kg of 25% human albumin, 9.3 mL/kg 7.5% saline or 28 mL/kg of autologous FWB. There was increased neutrophil activation over baseline after 60 minutes of resuscitation in the LRS and synthetic colloid groups but no significant increase in the FWB, albumin or hypertonic saline groups. In mice, blood was withdrawn to maintain a MAP of 50mmHg for 45 minutes and then 7.14 mL/kg HES 260/0.45 + shed blood or two times shed volume of LRS + shed blood was administered. The LRS group showed increased neutrophil rolling and adherence, as well as increased permeability index, compared to the HES group. Another study in rats reported an association between crystalloid fluid and increased concentrations of inflammatory biomarkers, compared to colloids. These studies provide evidence that large-volume isotonic crystalloid fluid resuscitation amplifies inflammation, but it is unknown if EG shedding precedes this effect.

The proinflammatory effect of crystalloid fluid may also be related to the speed at which it is delivered. In one study, rats had 40% of their blood volume removed and then were given three times the shed volume of LRS, administered over either 30 minutes or 12 hours. The rapid bolus resulted in increased tumour necrosis factor-α and interleukin-10 concentrations at one and three hours after the bolus, compared to rats that had the slow bolus. At 24 hours, the rats that received a rapid bolus had lower MAP and higher lactate concentration, compared to both the slow bolus group and rats that received no fluid at all. The
rapid bolus was associated with leukocyte infiltration, haemorrhage and interstitial oedema of the lung. Despite rapid bolus fluid therapy normalising cardiovascular parameters promptly in states of shock, it is possible that these adverse effects of rapid fluid administration are due to EG shedding, exacerbating inflammation, and might contribute to the post-resuscitation morbidity of shock.

Synthetic colloid fluids, such as HES and gelatine products, can confound any inflammatory effect of infusion. Hydroxyethyl starch preparations reduce chemotaxis of polymorphonuclear leukocytes in vitro. In an isovolemic haemodilution study in people undergoing cardiopulmonary bypass, dilution with HES 130/0.4 was associated with lower interleukin-8 and interleukin-10 concentrations, compared to gelatine. Conversely, HES also increased the activity of circulating neutrophils at 12 hours, compared to gelatine and a balanced crystalloid containing acetate/gluconate. One study in mice showed that return of shed blood after haemorrhage was associated with increased interleukin-6 concentrations and oxidative stress, whereas restoration of blood volume with HES 130/0.4 mitigated this proinflammatory response. Given that synthetic colloid products may have differential effects on inflammation, it is prudent to include both types when assessing the effects of fluid resuscitation on the EG and inflammation.

Finally, the constituents of isotonic crystalloid preparations may alter the release of inflammatory mediators. Balanced isotonic crystalloids contain anions such as lactate, acetate and gluconate, which are converted to bicarbonate in vivo. These fluids can avoid the dilutional hyperchloraemic acidosis associated with high-chloride solutions, such as 0.9% saline. Acetate is present in balanced crystalloids, such as Plasmalyte 148®, and some synthetic colloids, such as Volulyte®, however it has potential as a proinflammatory mediator. In an endotoxaemia model, rats were administered either lipopolysaccharide or phosphate buffered solution, and then one of five fluids; 75 mL/kg of either LRS or Ringer's acetate, or 25 mL/kg of either HES 130/0.42 in 0.9% saline solution, HES 130/0.42 in acetate solution or 4% gelatine in 0.9% saline solution.
Inclusion of an acetate buffer significantly increased the fold-change in inflammatory cytokine mRNA expression in the kidney and liver, compared to LRS. Gelatine also increased the fold-change of some inflammatory mediators’ mRNA expression. In a caecal ligation-and-puncture sepsis model, rats were administered one of the following; 75 mL/kg of LRS or 25 mL/kg of either HES 130/0.42 in balanced solution or HES 130/0.42 in 0.9% saline. The HES/balanced group had increased mRNA expression of inflammatory cytokines in the kidney as well as the liver. The authors also found that application of HES/balanced or acetate solution increased cytokine secretion from endothelial cells in vitro, compared to HES/saline or LRS. Conversely, a rodent haemorrhagic shock model did not show any difference in inflammatory or oxidative stress biomarkers in rats administered either 0.9% saline versus Ringer’s acetate in a pressure-targeted approach. Interestingly, both fluid groups showed an increase in plasma hyaluronan concentration. Finally, another murine haemorrhagic shock model showed that Ringer’s acetate administration was associated with a higher blood pH and base excess, and conferred a survival benefit, compared to LRS and 0.9% saline. Therefore, although there is some evidence that acetate may be proinflammatory in some rodent models, it is unclear if this might significantly affects morbidity or mortality.
6.3 Large-volume crystalloid fluid is associated with increased hyaluronan shedding and inflammation: a prospective pilot canine trial

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Abstract

Shedding of the endothelial glycocalyx precedes leukocyte activation and adherence in acute inflammation. Rapid administration of crystalloid or colloid fluids for treating hemorrhagic shock may cause endothelial glycocalyx shedding, thereby increasing inflammation. This study aimed to compare the effect of different fluid treatments in a canine shock model on glycocalyx biomarker, hyaluronan, and inflammatory biomarkers. Greyhound dogs under general anesthesia subject to hemorrhage for 60 minutes were given 20 mL kg⁻¹ of either fresh whole blood (FWB), hydroxyethyl starch (HES) 130/0.4, 4% succinylated gelatin (GELO), or 80 mL kg⁻¹ of isotonic crystalloid (CRYST) over 20 minutes (n=6 per group). Plasma biomarkers hyaluronan, interleukin (IL) 6, 8, 10, tumor necrosis factor-α, monocyte chemoattractant protein-1, keratinocyte chemokine-like and atrial natriuretic peptide were measured at baseline, end of hemorrhage (Shock), end of fluid administration (T20), and then 40 (T60), 100 (T120) and 160 (T180) minutes later. Biomarker concentrations were compared between groups using the Kruskal-Wallis test or Fisher’s exact test (measurable versus unmeasurable) (significance set at P<0.05). Hyaluronan concentration peaked early in the CRYST group at T20, compared to HES (P=0.005) and GELO (P=0.018), and later in the GELO group at T60, compared to FWB (P<0.001). The CRYST group had significantly more samples with measurable IL6 at T180 (P=0.015), compared to GELO, and IL10 at T60, T120 and T180 (all P=0.015), compared to FWB. There were no significant differences in other biomarker concentrations. In conclusion, rapid large-volume crystalloid administered for hemorrhagic shock was associated with increased hyaluronan and a greater inflammatory response.
Introduction

Hemorrhagic shock induces systemic inflammation and increases risk for later organ dysfunction. Organ dysfunction may be, in part, due to widespread shedding of the endothelial glycocalyx, which is the surface layer of the endothelium rich in proteins, proteoglycans and glycosaminoglycans. Shedding of this surface layer may promote systemic inflammation in two ways: endothelial activation and circulation of glycocalyx components. One component in particular, hyaluronan, can independently stimulate chemokine and cytokine production. Therefore, conditions that increase circulation of hyaluronan may contribute to risk of complications in the post-shock phase of treatment.

Rapid fluid administration, central to the treatment of hemorrhagic shock, can promote glycocalyx shedding and release of hyaluronan. Proposed mechanisms include hemodilution of plasma components, essentially ‘washing out’ the glycocalyx, and release of atrial natriuretic peptide (ANP). Natriuretic peptides are released during atrial stretch and ex vivo studies have shown that direct application of natriuretic peptides to the endothelium causes glycocalyx shedding. Fluid bolus-associated glycocalyx shedding may then stimulate inflammation. There is some evidence that administration of large-volume crystalloid and of synthetic colloid fluids increases neutrophil activation compared to fresh whole blood (FWB). However, studies directly comparing crystalloid with colloid fluids in inducing inflammation show inconsistent results. This disparity may be explained by varying degrees of glycocalyx shedding, and therefore variable increases in circulating hyaluronan concentration, associated with these fluids.

This study compared the effect of different resuscitation fluids on glycocalyx shedding, release of ANP, and inflammation. To achieve this, we designed a canine hemorrhagic shock model in which the degree of shock during the experiment and the cardiovascular response to fluid intervention were not different between treatment groups. We compared a balanced crystalloid fluid
and two synthetic colloid fluids, at doses known to cause similar hemodilution after redistribution, as well as autologous FWB as a non-hemodilution control. We hypothesized that crystalloid fluid would induce the most hyaluronan shedding, resulting in the highest concentration of ANP and inflammatory biomarkers.

**Methods**

**Animals**

This study was approved by the Institutional Animal Ethics Committee (R2666/14) and was conducted by specialist anesthesia and critical care veterinarians in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes. This work represents part of a larger study that also included assessment of coagulation and acute kidney injury after fluid resuscitation. Twenty-seven ex-racing greyhounds (23 male, 4 female) that were unable to be rehomed were used. Three dogs (2 male, 1 female) were excluded due to equipment malfunction at the start of, or during, the experiment. Dogs were deemed healthy based on veterinary assessment of physical examination, basic clinicopathologic parameters (packed cell volume, total plasma protein, platelet count, urinalysis), and ultrasound of the kidneys and urinary bladder. In accordance with animal ethics requirements, blood removed during the experiment that was not re-infused was made available to a veterinary hospital blood bank, therefore blood bank stock levels influenced study fluid allocation. Dogs were housed overnight in a kennel environment with free access to water before the day of the experiment.

**Anesthesia and instrumentation**

Dogs were premedicated with 0.3 mg kg\(^{-1}\) methadone IM. Thirty minutes later, anesthesia was induced with alfaxalone, titrated up to 3 mg kg\(^{-1}\) via a cephalic venous cannula, to allow sufficient anesthetic depth for orotracheal intubation. Anesthesia was maintained with isoflurane delivered by a rebreathing system, titrated to achieve an end-tidal concentration of 1.4%. Dogs were mechanically ventilated with a 0.3 fraction of inspired oxygen.
concentration, with ventilation rate adjusted before baseline measurements to achieve an end-tidal carbon dioxide partial pressure of 35-40 mmHg. Body temperature was monitored via an esophageal thermistor probe and was maintained between 36-38°C by a heating mat and warmed air delivery system. Infusions of compound sodium lactate (CSL) at 10 mL kg⁻¹ hr⁻¹ and fentanyl at 2 μg kg⁻¹ hr⁻¹ were administered IV throughout the experiment, with the exception of suspending CSL during study fluid infusion. Heart rate, arterial blood pressure, pulse oximetry, end-tidal carbon dioxide and isoflurane, body temperature, and anesthetic depth (eye position, facial reflexes and jaw tone) were monitored continuously and recorded at 5-minute intervals throughout anesthesia.

Percutaneous 0.5% bupivacaine was instilled adjacent to the femoral nerve via ultrasound guidance and a 14G 88 mm cannula was inserted into the femoral artery after surgical exposure. This cannula was used for continuous arterial pressure monitoring, arterial blood sampling and blood removal during the shock phase. A 14G 133 mm cannula was inserted percutaneously into the distal right jugular vein and advanced so that the tip was approximately located between the thoracic inlet and right atrium. This cannula was used for continuous central venous pressure monitoring, venous blood sampling, and study fluid administration. A urethral Foley catheter was placed for urine output monitoring and to collect samples for the acute kidney injury arm of the project. At the end of instrumentation, dogs were moved from left lateral to dorsal recumbency. Blood pressure transducers were adjusted to the approximate level of the heart base (scapulohumeral joint) and calibrated to atmospheric pressure. After repositioning and prior to baseline data collection, if mean arterial blood pressure (MAP) decreased to <60 mmHg for longer than 10 minutes, up to 10 mL kg⁻¹ of CSL was infused until MAP increased to >60mmHg. A minimum of 10 minutes elapsed after fluid infusion before baseline data and blood samples were collected.

Experimental procedure

Baseline data and blood samples were collected immediately before hemorrhage. Hemorrhage was achieved initially by passive flow of blood from
the femoral arterial catheter into blood collection bags, containing citrate-phosphate-dextrose-adenine anticoagulant. Once a MAP of 50 mmHg was reached, blood flow into collection bags was stopped. Blood was then intermittently removed via a 60 mL syringe to maintain MAP less than 60 mmHg for a 60-minute time period (hypotensive shock). The total amount of blood removed was calculated from the change in weight of collection bags and volume removed by syringe. Blood collection bags were stored at room temperature until they were either re-infused into the subject (FWB group) or transferred to the blood bank.

At the end of the 60-minute hypotension phase, dogs were administered one of four study fluids (n=6 per group) IV over 20 minutes including; 20 mL kg⁻¹ of autologous FWB, balanced hydroxyethyl starch (HES) 130/0.4 (Volulyte 6%, Fresenius Kabi) or 4% succinylated gelatin (GELO)(Gelofusine, B. Braun Medical), or 80 mL kg⁻¹ of a balanced isotonic crystalloid (CRYST)(Plasmalyte-148, Baxter Healthcare). Dogs were then monitored for a further 3 hours before euthanasia with intravenous pentobarbitone to effect. Cadavers were retained for other teaching and research purposes within the university.

**Cardiovascular measurements**

Cardiovascular variables (heart rate, arterial and central venous pressure, pulse pressure variation and cardiac output) were recorded at baseline, after 60 minutes of hypotension (Shock), at the end of study fluid administration (T20), and then 40 (T60), 100 (T120) and 160 (T180) minutes later. Cardiac output was measured by the lithium dilution technique, as previously described. Arterial and venous blood samples were drawn for biomarker measurement and blood gas, electrolyte and lactate measurement at the same time points. Calculated variables included body surface area, oxygen content, stroke volume index, systemic vascular resistance index, oxygen delivery index, oxygen consumption index, and oxygen extraction ratio, using equations previously described.

**Biomarker measurements**
Collected blood samples were immediately placed into lithium heparin and serum tubes, and then placed on ice. Within 60 minutes of collection, samples were processed by centrifugation at 1350g, with resulting serum and plasma separated into aliquots and stored at -80°C for later batched analysis. All biomarker assays were validated for use in dogs and performed according to manufacturer’s instructions. Plasma hyaluronan (R&D Systems, Minneapolis, MN, USA) and ANP (US Biologicals, Salem, MA, USA) were measured using commercial ELISA kits. Plasma interleukin-6 (IL6), interleukin-8 (IL8), interleukin-10 (IL10), monocyte chemoattractant protein-1 (MCP) and tumor necrosis factor-α were measured using a commercial magnetic bead multiplexed assay kit (Millipore, Billerica, MA, USA). Plasma keratinocyte chemokine-like (KC) was also measured by magnetic bead assay but as a single analyte, due to requirement for a higher dilution. Assays were repeated with pooled greyhound plasma diluted in vitro with saline, HES and GELO to ensure assays were not influenced by the presence of colloid molecules.

Statistical methods

Normal distribution of baseline characteristics, cardiovascular parameters and biomarker concentrations were assessed by visualisation of histograms and Q-Q plots. Log-transformation of data was performed, if required, to achieve normality. Data are described as either mean (95% confidence interval (CI)) or geometric mean (95% CI) when log-transformed. Differences between group baseline characteristics were tested using either analysis of variance (age, body surface area, blood volume removed) or Fisher’s exact test (sex). Differences in cardiovascular data between groups were tested using mixed effects models with post-hoc pair-wise comparisons performed if there was a significant time by treatment interaction. To minimise Type I error, only select cardiovascular variables were chosen a priori for comparison between groups that best reflected differences in cardiovascular performance (oxygen extraction ratio, pulse pressure variation, venous-arterial carbon dioxide difference).
Given most data distributions for biomarker concentrations were non-normal, differences in biomarker concentrations between groups at each time point were tested using the Kruskal Wallis test, with post-hoc pairwise comparisons (Mann-Whitney) performed if indicated. If more than 50% of data were below the limit of detection, then differences between groups were explored using a Fisher’s exact test (4x2 table) at each time point on categorized data (measurable versus unmeasurable). When significant, post-hoc pairwise comparisons were performed using Fishers exact test (2x2).

All data was analyzed using SAS (SAS Institute, Cary, NC). All tests were considered significant at P<0.05. The significance level for post-hoc pairwise comparisons were not adjusted due to the exploratory nature of the study.

**Results**

**Baseline and cardiovascular data**

There were no significant differences in age, sex, body weight or body surface area between groups. Two dogs received additional CSL for hypotension prior to baseline: 6.8 mL kg⁻¹, GELO group and 2.2 mL kg⁻¹, CRYST group. Mean blood volume removed during the hypotension phase was 47 mL kg⁻¹ (95% CI 37-57) for the FWB group, 55 mL kg⁻¹ (95% CI 44-66) for the CRYST group, 48 mL kg⁻¹ (95% CI 39-58) for the HES group and 44 mL kg⁻¹ (95% CI 35-54) for the GELO group, and there were no significant differences between groups. There were some differences in venous-arterial partial pressure of CO₂ between groups directly after fluid administration but these differences resolved by T60 (Table 1). The CRYST group showed greater hemodilution, based on hemoglobin concentration, at T20 compared to FWB, HES and GELO (P<0.001, P<0.001, P=0.001, respectively)(Table 1). The only other significant differences in hemodilution were between FWB and all other groups after T20. All other measured cardiovascular, acid-base and electrolyte variables are available in Table 1, Supplementary data.

**Hyaluronan and ANP concentrations**
Two distinct patterns of increased hyaluronan concentration, indicative of glycocalyx shedding, were observed across the four groups. The highest peaks in hyaluronan were observed early in the CRYST group, and later in the GELO group (Figure 1). To a lesser extent, the FWB group peaked at T20 whereas the HES group followed a similar pattern to GELO, peaking at T60. Specifically, the CRYST group had significantly higher hyaluronan concentration at T20, compared to HES (P=0.005) and GELO (P=0.018). Both the FWB and GELO group had significantly higher hyaluronan concentration at T20, compared to HES (both P=0.008). The GELO group had significantly higher hyaluronan concentration at T60 and T120, compared to FWB (P<0.001, P=0.034, respectively). The HES group also had significantly higher hyaluronan concentration at T120, compared to CRYST (P=0.026) and FWB (P=0.006).

As natriuretic peptides can directly shed the glycocalyx, we also measured plasma ANP concentration, however, there were no significant differences between groups at any time point (Figure 1).

**Inflammatory cytokine and chemokine concentrations**

Some increases in inflammatory cytokine concentrations were observed in the CRYST group, specifically IL6 and IL10, which occurred later than the peak of hyaluronan concentration (Figure 2). More than 50% of samples for both IL6 and IL10 measurement were below the limit of detection for the assay, therefore differences were tested between numbers of measurable versus unmeasurable samples. The CRYST group had a trend of increasing IL6 concentration over time and, at T180, there were significantly more samples with measurable IL6 concentration in the CRYST group, compared to the GELO group (Fisher’s exact test, P=0.015). The CRYST group also had significantly more samples with measurable IL10 concentration, compared to the FWB group, at T60, T120 and T180 (Fisher’s exact test, all P=0.015)(Figure 2). There was no significant difference in IL8 between any of the treatment groups (Figure 2). Tumor necrosis factor-α concentration was below the limit of detection in 92% of samples (data not reported).
A trend of increasing concentrations of chemokine, KC, was observed in all groups after fluid administration (Figure 3). However, observed differences between treatment groups were not statistically significant. There were also no significant differences between groups in MCP concentrations.

See Table 2 in Supplementary data for summarized biomarker concentrations and number of measurable samples per time point.

Discussion

This pilot experimental canine study showed that large-volume crystalloid fluid given for hemorrhagic shock was associated with higher concentrations in hyaluronan immediately after the fluid bolus, compared to other fluid types, suggesting greater systemic glycocalyx shedding. This change was observed despite greater dilution of blood with crystalloid fluid at this time point. Dogs treated with CRYST also had evidence of an enhanced inflammatory response, as indicated by significantly higher plasma concentrations of IL6 and IL10, compared to other fluid types.

This hemorrhagic shock model was designed to minimize variability in degree of shock response to fluid intervention. Previous work with the greyhound hemorrhagic shock model had demonstrated variability in degree of shock amongst individuals using a volume-guided approach. Therefore, we paired pressure-targeted hemorrhagic shock over 60 minutes, followed by a fixed-volume fluid intervention. This approach resulted in uniform cardiovascular responses to both shock and fluid intervention across the groups. The doses of fluid chosen for this study were based on previous work by our group, which showed that oxygen extraction ratio and hemoglobin concentration were no different with 20 mL kg\(^{-1}\) of HES 130/0.4 compared to 80 mL kg\(^{-1}\) of 0.9% sodium chloride at 1 hour after the fluid bolus i.e. after fluid redistribution. Severity and length of time of shock would likely alter the subsequent inflammatory response, and the achievement of uniformity from T60 in this hemorrhagic shock study is a particular strength.
There is growing evidence that rapid infusion of intravenous fluids causes shedding of the glycocalyx, both in rodent models\textsuperscript{8-10,13,14} and people.\textsuperscript{25,51,55} Laboratory studies have shown that both dilution of plasma components\textsuperscript{49} and infusion of natriuretic peptides\textsuperscript{50,51} can cause glycocalyx shedding. It is possible that rapid infusions of large-volume crystalloid may cause substantial atrial stretch and release of ANP, which may directly cause glycocalyx shedding from the endothelial surface. In this study, it was expected that dogs in the CRYST group would have a greater increase in plasma ANP concentration, compared to other fluid types, given the rapid blood volume expansion achieved by large-volume crystalloid and the potential for atrial stretch. However, our pilot data does not support that large-volume crystalloid fluid causes a significant increase in ANP, therefore this mechanism may not be as important for glycocalyx shedding secondary to fluid administration.

The dogs that received a rapid infusion of autologous fresh whole blood also demonstrated a rise in hyaluronan at T20. This may be due to glycocalyx shedding from sheer stress created by the rapid infusion or, more likely, redistribution of hyaluronan that was shed from the vascular beds during the shock stage. There is little information on glycocalyx shedding during shock, as opposed to after fluid resuscitation; however, pulmonary endothelial glycocalyx shedding has been demonstrated in mice subjected to hemorrhagic shock alone.\textsuperscript{13,14} Our study also found a large increase in plasma hyaluronan concentration in the GELO group, 40 minutes after the peak observed for the CRYST and FWB group. The HES group showed a similar pattern, though somewhat blunted. This may reflect the differences in pharmacokinetics of synthetic colloids, and speed at which maximum blood volume expansion is obtained, compared to infusion of crystalloid.

The CRYST group showed the largest changes in inflammation-related biomarkers compared to other fluid types, especially IL10, which has mostly anti-inflammatory properties. Other animal studies have also observed an association between rapid high-volume crystalloids and enhanced inflammation,
compared to autologous blood, albumin, or slow crystalloid administration.21,34,35,37 This exaggerated inflammatory response after crystalloid administration may be due to preceding glycocalyx shedding. Both shedding of the glycocalyx and exposure of cells to hyaluronan in vitro have been linked with increased production of IL10 and pro-inflammatory cytokines in laboratory studies.48,56,57 Rapid IL10 production, in particular, forms an important part of the immune response to hemorrhagic shock and trauma, and has been associated with increased infection susceptibility in trauma patients.58 Variations in glycocalyx shedding or increases in circulating glycocalyx components may, in part, explain some of the patient morbidity related to post-resuscitation immunomodulation. This study was limited to a 2-hour post-hemorrhage sampling period, therefore we have no information regarding changes in inflammatory cytokines or chemokines beyond this period. However, these results suggest important differences in immune responses in dogs receiving large-volume crystalloid in the early treatment phase.

The clinical relevance of glycocalyx shedding in critically ill patients, and its association with enhanced inflammation, is currently an avid area of research. Increased biomarkers of glycocalyx shedding, including hyaluronan, have been associated with worse clinical outcomes, both in trauma30,59 and sepsis patients.60-64 Previous work in patients with sepsis showed that increasing hyaluronan concentration during initial treatment was associated with fluid volumes administered and organ failure scores.65 Further studies are required to determine if strategies aimed at mitigating glycocalyx shedding, such as reducing the use of crystalloid fluid boluses, provide clinically relevant benefits to critically ill patients.

A limitation of this study was the inter-dog variation in biomarker concentrations and the restricted sensitivity of the assays. As there is limited information on many of these biomarker assays for dogs, it was difficult to anticipate the performance of these assays or sample size needed, especially in the setting of shock. The data produced in this study for a range of inflammatory biomarkers in the setting of acute canine inflammation is important for
informing future work in this area, whether that includes canine experimental models or canine clinical studies that are relevant to human disease. A second limitation is that two of our fluid choices included acetate, which has been shown to increase inflammatory cytokines in mice,\textsuperscript{42,43} and therefore may have influenced the results. We chose these fluids in preference to 0.9% saline in order to avoid administration of high-chloride fluid, both in the CRYST and HES group. Although we did not observe an increase in inflammatory biomarkers in the HES group, compared to other fluid types, we cannot rule out the influence of acetate on the results of the CRYST group.

A unique aspect of this study is the inclusion of multiple sampling time points soon after fluid intervention, demonstrating the time course of hyaluronan shedding and subsequent cytokine release. Our study would have been strengthened with inclusion of other biomarkers of glycocalyx shedding, such as syndecan-1. There is limited availability of this assay homologous to dog and those we tested in pilot studies were unacceptable. Also, hyaluronan may not solely represent endothelial glycocalyx shedding as it is a ubiquitous molecule present on many cell types.\textsuperscript{66} This is true of proteoglycans as well, such as syndecan-1, which are also expressed on leukocytes. However, hyaluronan itself has pro-inflammatory properties\textsuperscript{46-48} and an increase in circulating concentration, regardless of the contributing sources, is considered significant. Further studies focusing on imaging the endothelial glycocalyx, as well as biomarker measurement, are needed to increase understanding in this area.

In conclusion, this pilot study found higher concentrations of hyaluronan and biomarkers of inflammation in dogs with hemorrhagic shock given a large volume of balanced crystalloid fluid, compared to dogs given synthetic colloid fluids and autologous FWB. Further work is needed to characterize the clinical relevance of glycocalyx shedding secondary to rapid infusion of large doses of crystalloid fluid.
Figures and Tables

Figure 1. Plasma hyaluronan and atrial natriuretic peptide concentration (median, Q1-Q3) in dogs (n=6 per group) with hemorrhagic shock given 20 mL kg⁻¹ of either fresh whole blood (FWB), hydroxyethyl starch 130/0.4 (HES), 4% succinylated gelatin (GELO) or 80 mL kg⁻¹ of balanced isotonic crystalloid (CRYST).

+, significantly different to HES; & , significantly different to FWB; #, significantly different to GELO
Significance was set at P <0.05.
Figure 2. Plasma interleukin-6, -10 and -8 concentrations (median, Q1-Q3) in dogs (n=6 per group) with hemorrhagic shock given 20 mL kg\(^{-1}\) of either fresh whole blood (FWB), hydroxyethyl starch 130/0.4 (HES), 4% succinylated gelatin (GELO) or 80 mL kg\(^{-1}\) of balanced isotonic crystalloid (CRYST).
Figure 3. Plasma keratinocyte-like chemokine and monocyte chemoattractant protein-1 (median, Q1-Q3) in dogs (n=6 per group) with hemorrhagic shock given 20 mL kg\(^{-1}\) of either fresh whole blood (FWB), hydroxyethyl starch 130/0.4 (HES), 4% succinylated gelatin (GELO) or 80 mL kg\(^{-1}\) of balanced isotonic crystalloid (CRYST).
Table 1. Mean (95% confidence intervals) of cardiovascular shock parameters and hemoglobin concentration in dogs (n=6 per group) given 20 mL kg⁻¹ of either fresh whole blood (FWB), hydroxyethyl starch 130/0.4 (HES), 4% succinylated gelatin (GELO) or 80 mL kg⁻¹ of balanced isotonic crystalloid (CRYST). Fluid was delivered directly after the Shock time point.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Shock (0 mins)</th>
<th>20 mins</th>
<th>60 mins</th>
<th>120 mins</th>
<th>180 mins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen extraction ratio (%)</td>
<td>FWB</td>
<td>14 (11-16)</td>
<td>46 (38-55)</td>
<td>21 (13-29)</td>
<td>18 (12-23)</td>
<td>17 (12-21)</td>
</tr>
<tr>
<td></td>
<td>CRYST</td>
<td>10 (5-14)</td>
<td>49 (38-60)</td>
<td>9 (4-14)</td>
<td>13 (10-17)</td>
<td>16 (12-21)</td>
</tr>
<tr>
<td></td>
<td>HES</td>
<td>11 (8-14)</td>
<td>51 (39-62)</td>
<td>13 (9-17)</td>
<td>8 (6-11)</td>
<td>15 (12-19)</td>
</tr>
<tr>
<td></td>
<td>GELO</td>
<td>11 (7-14)</td>
<td>39 (30-47)</td>
<td>12 (6-17)</td>
<td>13 (6-21)</td>
<td>17 (12-23)</td>
</tr>
<tr>
<td>Pulse pressure variation (%)</td>
<td>FWB</td>
<td>23 (14-32)</td>
<td>44 (31-56)</td>
<td>33 (21-46)</td>
<td>35 (22-49)</td>
<td>31 (21-42)</td>
</tr>
<tr>
<td></td>
<td>CRYST</td>
<td>26 (16-35)</td>
<td>38 (35-41)</td>
<td>19 (11-26)</td>
<td>35 (24-46)</td>
<td>40 (32-48)</td>
</tr>
<tr>
<td></td>
<td>HES</td>
<td>30 (19-42)</td>
<td>43 (34-52)</td>
<td>26 (19-33)</td>
<td>30 (18-42)</td>
<td>39 (22-56)</td>
</tr>
<tr>
<td></td>
<td>GELO</td>
<td>24 (14-35)</td>
<td>44 (36-52)</td>
<td>26 (22-30)</td>
<td>31 (23-40)</td>
<td>33 (27-39)</td>
</tr>
<tr>
<td>Venous - arterial CO₂ (mmHg)</td>
<td>FWB</td>
<td>4.0 (2.7-5.3)</td>
<td>8.0 (4.9-11.0)</td>
<td>7.3 (5.2-9.4)</td>
<td>5.4 (4.1-6.6)</td>
<td>4.0 (3.0-4.9)</td>
</tr>
<tr>
<td></td>
<td>CRYST</td>
<td>3.8 (2.8-4.9)</td>
<td>11.9 (6.4-17.4)</td>
<td>3.8 (2.6-5.1)</td>
<td>3.9 (2.6-5.1)</td>
<td>4.6 (3.1-6.2)</td>
</tr>
<tr>
<td></td>
<td>HES</td>
<td>3.3 (2.3-4.4)</td>
<td>8.5 (5.7-11.3)</td>
<td>4.2 (2.0-6.4)</td>
<td>4.1 (3.3-5.0)</td>
<td>4.5 (3.4-5.7)</td>
</tr>
<tr>
<td></td>
<td>GELO</td>
<td>3.1 (2.2-3.9)</td>
<td>6.7 (5.3-8.1)</td>
<td>3.7 (2.7-4.8)</td>
<td>4.5 (3.7-5.3)</td>
<td>4.5 (3.7-5.3)</td>
</tr>
<tr>
<td>Hemoglobin concentration (g L⁻¹)</td>
<td>FWB</td>
<td>147 (128-167)</td>
<td>155 (140-172)</td>
<td>142 (126-159)</td>
<td>134 (119-152)</td>
<td>126 (111-142)</td>
</tr>
<tr>
<td>Group</td>
<td>Value (Range)</td>
<td>Value (Range)</td>
<td>Value (Range)</td>
<td>Value (Range)</td>
<td>Value (Range)</td>
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</tr>
<tr>
<td>CRYST</td>
<td>157 (148-166)</td>
<td>168 (157-179)</td>
<td>80 (74-87) &amp;+,#</td>
<td>96 (88-104) &amp;</td>
<td>99 (92-108) &amp;</td>
<td>106 (98-115)</td>
</tr>
<tr>
<td>HES</td>
<td>160 (150-169)</td>
<td>166 (153-179)</td>
<td>106 (99-113) &amp;</td>
<td>91 (80-104) &amp;</td>
<td>92 (81-105) &amp;</td>
<td>102 (91-113)</td>
</tr>
<tr>
<td>GELO</td>
<td>153 (144-163)</td>
<td>153 (143-164)</td>
<td>96 (89-104) &amp;</td>
<td>89 (79-98) &amp;</td>
<td>87 (78-97) &amp;</td>
<td>89 (80-99) &amp;</td>
</tr>
</tbody>
</table>

Symbols indicate significant difference (P<0.05) between groups according to the following symbols: +, significantly different to HES; &, significantly different to FWB; #, significantly different to GELO.
Supplementary File 1

Table. Cardiovascular, blood gas and electrolyte parameters (median, Q1-Q3) in dogs (n=6 per group) with hemorrhagic shock given 20 mL kg\(^{-1}\) of either fresh whole blood (FWB), hydroxyethyl starch 130/0.4 (HES), 4% succinylated gelatin (GELO) or 80 mL kg\(^{-1}\) of balanced isotonic crystalloid (CRYST). Fluid was delivered directly after Shock time point.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Shock (0 minutes)</th>
<th>20 minutes</th>
<th>60 minutes</th>
<th>120 minutes</th>
<th>180 minutes</th>
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<tr>
<td><strong>Cardiac index</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(mL min(^{-1}) m(^{-2}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FWB</td>
<td>2.9 (2.5-3.2)</td>
<td>1.5 (1.4-1.5)</td>
<td>3.3 (3.0-3.7)</td>
<td>4.1 (3.3-4.4)</td>
<td>4.0 (3.1-4.5)</td>
<td>4.1 (3.7-4.1)</td>
</tr>
<tr>
<td>CRYST</td>
<td>3.6 (3.3-4.0)</td>
<td>1.5 (1.2-1.9)</td>
<td>4.6 (4.2-5.7)</td>
<td>3.8 (3.6-4.2)</td>
<td>3.5 (3.4-3.8)</td>
<td>3.6 (3.5-3.8)</td>
</tr>
<tr>
<td>HES</td>
<td>3.5 (3.4-3.7)</td>
<td>1.8 (1.5-2.1)</td>
<td>4.9 (4.3-5.1)</td>
<td>4.2 (3.8-4.6)</td>
<td>4.3 (3.9-4.6)</td>
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<tr>
<td>GELO</td>
<td>3.2 (2.2-3.4)</td>
<td>1.5 (1.1-2.1)</td>
<td>3.9 (3.6-4.1)</td>
<td>3.5 (3.4-4.0)</td>
<td>3.7 (3.4-4.3)</td>
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<td><strong>Stroke volume index</strong></td>
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<tr>
<td>(mL m(^{-2}))</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>FWB</td>
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<td>9 (8-10)</td>
<td>23 (22-27)</td>
<td>28 (24-30)</td>
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<td>34 (28-35)</td>
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<tr>
<td>CRYST</td>
<td>43 (34-52)</td>
<td>8 (7-13)</td>
<td>45 (32-48)</td>
<td>27 (26-38)</td>
<td>24 (22-30)</td>
<td>24 (21-29)</td>
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<tr>
<td>HES</td>
<td>37 (33-38)</td>
<td>11 (8-13)</td>
<td>35 (32-37)</td>
<td>30 (26-33)</td>
<td>29 (28-30)</td>
<td>28 (26-33)</td>
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<tr>
<td>GELO</td>
<td>35 (26-48)</td>
<td>9 (7-11)</td>
<td>27 (26-33)</td>
<td>26 (25-27)</td>
<td>25 (24-26)</td>
<td>32 (28-36)</td>
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<tr>
<td><strong>Heart rate (bpm)</strong></td>
<td></td>
<td></td>
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<tr>
<td>FWB</td>
<td>82 (54-104)</td>
<td>164 (162-180)</td>
<td>137 (134-142)</td>
<td>142 (136-150)</td>
<td>144 (123-146)</td>
<td>127 (116-147)</td>
</tr>
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<td>CRYST</td>
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<tr>
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<tr>
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<td>Systemic vascular resistance index</td>
<td>Central venous pressure</td>
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## Oxygen consumption (mL min\(^{-1}\))

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## Blood pH

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## Partial pressure CO\(_2\) (mmHg)

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## Base excess (mmol L\(^{-1}\))

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<td>-2.9 (-5.5 - -0.1)</td>
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## Lactate (mmol L\(^{-1}\))

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<th>Potassium (mmol L(^{-1}))</th>
<th>Chloride (mmol L(^{-1}))</th>
<th>Ionised calcium (mmol L(^{-1}))</th>
<th>Glucose (mmol L(^{-1}))</th>
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<td>HES</td>
<td>GELO</td>
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<td>144 (143-146)</td>
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<td>146 (146-148)</td>
<td>145 (144-147)</td>
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<th>Chloride (mmol L(^{-1}))</th>
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All blood parameters were measured on arterial heparinized blood.
Supplementary File 2

Table. Biomarker concentrations (median (range)) in dogs (n=6 per group) with hemorrhagic shock given 20 mL.kg\(^{-1}\) of either fresh whole blood (FWB), hydroxyethyl starch 130/0.4 (HES), 4% succinylated gelatin (GELO) or 80 mL.kg\(^{-1}\) of balanced isotonic crystalloid (CRYST). Table includes number of samples with concentration above the lower limit of detection (LLOD) of the assay (n).

<table>
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<tr>
<th>Biomarker</th>
<th>Baseline</th>
<th>n</th>
<th>Shock (0 mins)</th>
<th>n</th>
<th>20 mins*</th>
<th>n</th>
<th>60 mins*</th>
<th>n</th>
<th>120 mins*</th>
<th>n</th>
<th>180 mins*</th>
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<tr>
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MCP (pg mL⁻¹)
**Table:** Biomarker concentrations (pg/mL) for different fluid administrations

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<th>Fluid</th>
<th>LLOD (pg/mL)</th>
<th>Median</th>
<th>1st Quartile</th>
<th>3rd Quartile</th>
<th>1st Quartile</th>
<th>3rd Quartile</th>
<th>1st Quartile</th>
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<td>169 (21-295)</td>
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</tbody>
</table>

* Fluid was delivered after the Shock time point. Biomarker concentrations were corrected for change in hemoglobin concentration in order to account for effects of hemodilution.

**Abbreviations:** ANP, atrial natriuretic peptide; KC, keratinocyte chemokine-like; MCP, Monocyte chemoattractant protein-1
6.4 References


22. McBride D, Raisis AL, Hosgood G, Smart L. Hydroxyethyl starch 130/0.4 compared with 0.9% NaCl administered to greyhounds with haemorrhagic shock. *Vet Anaesth Analg* 2017;44:444-51.


53. McBride D, Raisis AL, Hosgood G, Smart L. Hydroxyethyl starch 130/0.4 compared with 0.9% NaCl administered to greyhounds with haemorrhagic shock. *Vet Anaesth Analg* 2017;44(3):444-451.


CHAPTER 7 – The effect of hypertonic saline on endothelial glycocalyx shedding and inflammation in sepsis

7.1 Gap in knowledge

It was reported in previous chapters that a) crystalloid fluid volume was associated with increasing endothelial glycocalyx (EG) biomarker concentrations in emergency department (ED) patients with sepsis (Chapter 3), b) syndecan-1 was positively associated with biomarker concentrations of neutrophil activation in patients with pneumonia (Chapter 4), and c) large-volume crystalloid fluid resuscitation increased hyaluronan and inflammatory biomarker concentrations in a canine haemorrhagic shock model (Chapter 6). The effect of alternative fluid strategies to large-volume crystalloids for fluid resuscitation in sepsis on EG shedding and inflammation is unknown.

Hypertonic saline is a low-volume hyperosmolar fluid resuscitation strategy that may have beneficial immunomodulatory effects in patients with trauma. One study in mice with haemorrhagic shock, reviewed in Chapter 6, provided some evidence that 3% saline was associated with less EG shedding, compared to a larger volume of 0.9% saline.1 The effect of administration of hypertonic saline on biomarker concentrations of EG shedding and inflammation in patients with sepsis is unknown. The background information to follow summarises studies on the use of hypertonic saline in sepsis published up to, and including, 2018. This literature review focuses on studies that deliver only hypertonic saline, and will not address its use in combination with synthetic colloids. The background section provides an introduction to the final manuscript presented in this thesis. This paper describes a pilot randomised controlled trial comparing hypertonic saline to isotonic saline for fluid resuscitation in ED patients with suspected sepsis. The results of this study were, in part, published in abstract form (Critical Care 2018, 22(Suppl 1):P303)2 as a part of the International Symposium on Intensive Care and Emergency Medicine, Brussels, Belgium (March, 2018) and have subsequently been published in full in Journal of Critical Care.3 The manuscript is presented as published.
7.2 Background

In vitro studies

Early in vitro studies show that hypertonic solutions have immunosuppressive effects on polymorphonuclear (PMN) leukocytes, characterised by blunted or reduced responses to pro-inflammatory stimuli. This includes decreased intracellular signalling, expression of surface receptors and cytokine production. Some of these findings are particularly relevant to sepsis, such as the reduced response to lipopolysaccharide (LPS) described in several of these studies. Hypertonic solution at a concentration of 180 mmol/L (equivalent osmolarity of 360 mmol/L) inhibited cytoskeletal reorganisation and cytotoxic signalling of human neutrophils, as well as causing a reduction in the oxidative respiratory burst. Another study showed that hypertonic solutions decreased the release of inflammatory mediators and oxidants from neutrophils. Maximal suppression was observed at 400 mmol/kg. When observing duration of suppression, an increase of 40 mmol/kg from baseline for 10 minutes provided 40-50% suppression of superoxide formation, but for only one hour. Conversely, human PMNs incubated with Escherichia coli showed increased antimicrobial activity, rather than suppression of function, at an osmolarity of 360 mmol/L and 400 mmol/L, but not at 280 mmol/L or 320 mmol/L.

Some responses to hypertonicity may be particular to sodium rather than other concentrated solutions. At an osmolarity of 350 and 410 mmol/L, saline solution inhibited the N-formyl-methionyl-leucylphenylalanine (fMLP)-stimulated expression of adhesion receptors on the surface of human PMN cells, but inhibition was not achieved by hypertonic choline, mannitol or sucrose solutions. The response may also be time-dependent; PMNs showed decreased L-selectin expression when incubated in hypertonic saline at 350 mmol/L for

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1 Osmolarity is the number of particles per litre of solvent whereas osmolality is the number of particles per kilogram of solvent. Studies will differ in the type of measurement, though numerical difference is very small. The type of measurement is reflected in the units reported (mmol/L versus mmol/kg). The reference range of human blood osmolarity is generally 280 to 300 mmol/L.
four hours, but not when incubated for only two hours. Clinically, it may be difficult to maintain this level of hyperosmolarity safely for such extended periods.

In addition to leukocyte suppression, endothelial cells can also be affected by hyperosmolar conditions. In a study using cultured human pulmonary microvascular endothelial cells (HMVECS), tumour necrosis factor-α (TNF-α) was added to cultures to stimulate intercellular adhesion molecule-1 (ICAM-1) expression. At osmolarities greater than 340 mmol/L, a concentration-dependent reduction in ICAM-1 expression was observed. There was no effect on constitutive HMVEC ICAM-1 expression. These effects were observed for sodium and sorbitol solutions. At an osmolarity greater than 360 mmol/L, TNF-α-stimulated interleukin-8 production was reduced and there was less PMN adhesion to TNF-α-exposed HMVECs. Neutrophils also show reduced adhesion to TNF-α-treated endothelial cells when pre-incubated at an osmolality of 350 mmol/L. Furthermore, when a neutrophil and endothelial cell co-culture was incubated with plasma from people with sepsis, there was reduced neutrophil activation, adhesion and leukoaggregation after the addition of a combined solution of dextran 70 and 7.5% hypertonic saline.

In addition to the immunomodulatory effects on endothelial cells, hypertonic solutions also affect epithelial cells. Similar to endothelial cells, pulmonary epithelial cells show attenuated TNF-α-stimulated ICAM-1 expression when incubated with hypertonic saline, as well as altered pro-inflammatory intracellular signalling. In a second study, hypertonic solution (400 mmol/kg) interfered with TNF-α-mediated activation of the nuclear factor-κB pathway in pulmonary epithelial cells and inhibited the release of a range of inflammatory cytokines. Interestingly, a third study of epithelial cell responses showed that extreme hyperosmolality of 700 mmol/L, which would be lethal in vivo, caused shedding of both syndecan-1 and syndecan-4 ectodomains, as measured by dot immunoassay of cell-culture medium. Although hypertonic solutions may be beneficial for suppressing inflammation at the tissue level, extreme hyperosmolarities would not be tolerated systemically. Therefore, these
studies may only be relevant for local application of hypertonic solutions, such as those in wound dressings.

The studies mentioned above provide evidence of the immunomodulatory effects of hypertonic solutions above an osmolarity or osmolality of approximately 340 mmol/L. In order to improve the translation of these in vitro studies to in vivo models, a separate study aimed to determine the sodium concentration achieved in vivo after hypertonic saline administration, before proceeding with their in vitro study. Anaesthetised pigs were administered 4 mL/kg of 7.5% saline over 4 minutes, with serial measurements of sodium concentration. During the infusion, sodium concentration increased to a mean of 184 mmol/L, decreasing to 157 mmol/L by the end of the 4-minute infusion. Given this peak effect equated to a serum osmolarity of approximately 370 mmol/L, the authors then investigated the effects of incubating human PMNs in hypertonic saline at an osmolality of 360 mmol/kg for 5 minutes, to emulate the in vivo condition. They used a two-hit model of neutrophil pro-inflammatory priming then further neutrophil activation. This was to simulate conditions in trauma whereby injury primes neutrophils (first hit) and then a second insult, such as infection (second hit), activates neutrophils further to create an inflammatory storm. They found that if exposure to hypertonic saline occurred before or after immune priming of the cells, then PMN activation was reduced, compared to no hypertonic saline. However, if incubation with hypertonic saline occurred after both priming and activation of the cells, as a two-hit model approach, then elastase release was actually enhanced. An increase in elastase release from neutrophils likely propagates local tissue injury. The authors proposed that hypertonic saline may only be beneficial if given early in resuscitation in trauma, but may augment pro-inflammatory responses if given later in hospitalisation when a patient is already in a pro-inflammatory state. The limitation of this study is that these levels of hyperosmolarity rapidly disappear in vivo, as demonstrated in their porcine model, therefore conclusions should be delayed until work in in vivo models provides evidence.

Laboratory animal models
Haemorrhagic shock models have provided much of the evidence to explain the immunomodulatory effects of hypertonic saline. Several rodent models, where rats or mice were administered 4 mL/kg of 7.5% saline after haemorrhage, showed decreased neutrophil activation and adhesion, and decreased vascular permeability compared to a crystalloid fluid or shed blood control. A haemorrhagic shock model in mice compared 10 mL/kg of 3% saline over 10 minutes with 4 mL/kg of 7.5% saline over 5 minutes, as well as including a group that received 33 mL/kg of lactated Ringer’s solution (LRS) over 15 minutes. Two hypertonic saline groups (7.5% and 3%) were included as only 3% saline had approval for human use in the authors’ country. All mice simultaneously received half the shed volume. Thirty minutes after the start of the fluid bolus, the 7.5% saline group reached an osmolality of 302 mmol/kg, an increase of ~8 mmol/kg from shock, and the 3% saline group reached an osmolality of 297 mmol/kg, an increase of ~7 mmol/kg from shock. Although this increase in osmolality appears modest, mice in the hypertonic groups had significantly increased mean arterial blood pressure (MAP) at one hour and lower plasma TNF-α concentration at three hours after infusion, compared to the LRS group. The hypertonic saline groups also had significantly lower lung and intestinal injury scores.

Several large animal haemorrhagic shock models have also investigated the immunomodulatory effects of hypertonic saline. In one study, conscious pigs were bled, then randomised to receive either shed blood, 84 mL/kg of LRS, 10.9 mL/kg of 7.5% saline or sham haemorrhage plus the same volume of LRS. There were no significant differences in cardiovascular parameters between groups over time. Serum osmolality went from ~300 mmol/kg to ~320 mmol/kg in the hypertonic saline group, which was maintained for the following three hours. The LRS group showed an increase in neutrophil fluorescence after fluid administration, indicating oxidative burst activity, whereas this returned to baseline in the hypertonic saline and shed blood group. The sham LRS group also had an increase in neutrophil fluorescence at this time, similar to the haemorrhaged LRS group; however, this decreased over subsequent time points. Finally, in a canine haemorrhagic shock model, dogs were given 6 mL/kg of
either hypertonic saline at 2400 mmol/L or isotonic saline at 300 mmol/L over 3-5 minutes. The isotonic group had little response to the fluid and all died by 3 hours whereas the hypertonic group showed some cardiovascular improvement and survived to be euthanased at 6 hours. It is unclear if the improved survival after hypertonic saline was related to better tissue perfusion or immunomodulatory benefits, however the former is suspected.

Given the immunomodulatory potential of hypertonic saline shown in some models of haemorrhagic shock, more recent studies have investigated the potential benefits of hypertonic saline during sepsis. Most of these studies have used rodent models. In studies using an LPS challenge, 4 mL/kg of 7% or 7.5% saline was associated with decreased inflammatory cytokine concentrations, reduced oxidative stress, and improved survival. In one LPS model, mice that received 4 mL/kg of 7.5% saline showed better cardiovascular performance and lower indicators of organ injury, compared to mice that received the same volume of 0.9% saline; however these results may have been obtained by better blood volume expansion created by hypertonic saline. Better cardiovascular performance after hypertonic saline, compared with the same volume of 0.9% saline, was also demonstrated in a rat model with a similar design. Hypertonic saline has also been beneficial in other rodent models of sepsis, including a dengue fever model, which showed reduced vascular permeability, lower inflammatory cytokine concentrations and improved survival, compared to 0.9% saline. In an Escherichia coli injection model, lower levels of bacterial colonisation of organs was observed in mice that received hypertonic saline, compared to those that received a larger volume of 0.9% saline.

Two large animal sepsis models have also demonstrated beneficial effects following treatment with hypertonic saline, although effects on the immune system were not evaluated. Dogs injected with Escherichia coli intravenously that then received 4 mL/kg of 7.5% hypertonic saline over 5 minutes, showed lower oxygen extraction ratio and improved arterial oxygen saturation, compared to dogs that received 32 mL/kg of LRS over 30 minutes. The increase in serum sodium concentration was only an average of 10 mmol/L to achieve this benefit,
and there was no significant difference in haemoglobin concentration over time, indicating similar haemodilution. In an LPS model, macaques administered either 4 mL/kg of 0.9% saline or 4 mL/kg of 3% saline did not show a significant difference in most cardiovascular variables. However, the serum sodium concentration increased similarly in both groups of macaques, with a peak osmolarity of 336 mmol/L in the isotonic saline group, much higher than the osmolarity of 0.9% saline itself. Therefore it appears that a free water deficit was created in both fluid groups.

Similar to the proposal of the benefits of hypertonic saline for trauma being time-sensitive, hypertonic saline may be ineffective if administration is given after a delay from the start of infection. Mice administered hypertonic saline 15 minutes after LPS injection showed improved pulmonary mechanics and lower mortality, compared to those receiving isotonic crystalloid; however the benefit of hypertonic saline was not conferred if it was administered 90 minutes after LPS injection. The translation of the experimental immunomodulatory benefits of hypertonic saline to naturally occurring sepsis may be impeded by delayed diagnosis and administration in a clinical setting.

Hypertonic saline has also been tested in models of pro-inflammatory states other than sepsis. In a model of acute pancreatitis, mice that received 2 mL/kg of 7.5% saline, 24 and 48 hours after induction of pancreatitis, showed lower histologic scores of pancreatitis and lung injury, and lower lung myeloperoxidase. In a murine model of induced burn injury, hypertonic saline combined with LRS given as 24-hour fluid replacement, was compared to LRS alone. Mice that received the hypertonic combination fluid achieved mild hypernatraemia and had significantly less intestine and lung myeloperoxidase activity with less tissue oxidative stress, compared to mice that received LRS alone. However there was no significant difference in serum cytokine concentrations.

*Clinical trials*
Several pilot randomised controlled trials in people with traumatic injury have shown that hypertonic saline can reduce markers of inflammation, compared to isotonic crystalloid therapy. In a pre-hospital setting, a single 250 mL bolus of either 7.5% saline (n=9), 7.5% saline combined with dextrans (n=8) or 0.9% saline (n=17) was delivered to people with major trauma. Patients that received 7.5% saline had significantly lower markers of neutrophil and endothelial activation over the first 24 hours of hospitalisation, compared to the other fluid groups. These patients also had a significantly higher systolic blood pressure on arrival, compared to those that received 0.9% saline. A second pilot study, again in a pre-hospital setting, randomised patients with traumatic brain injury to receive 250 mL either 7.5% saline in dextrans (n=30) or 0.9% saline (n=35), as a single bolus. Patients that received the combination of 7.5% saline with dextrans showed lower activation of PMN cells and monocytes, lower TNF-α and interleukin-10 concentration, as well as a decrease in some markers of endothelial activation. A third pilot study in patients undergoing femoral fracture repair found that those that received 4 mL/kg of 7.5% saline (n=10) immediately before induction of general anaesthesia showed less PMN cell activation, compared to those randomised to 4 mL/kg of 0.9% saline (n=10). These three studies support that hypertonic saline may attenuate the pro-inflammatory response that occurs after traumatic injury.

There are few studies assessing the immunomodulatory effects of hypertonic saline in patients with sepsis. One pilot randomised controlled blinded trial in intensive care unit (ICU) patients with septic shock compared two fluid strategies; hydroxyethyl starch (HES) suspended in 7.2% saline (n=12) or HES suspended in 0.9% saline (n=12). The hypertonic group showed improved MAP, relative to norepinephrine dose, and stroke volume index post-bolus, while the isotonic group did not. A follow-up study examined inflammation in the same patients and found no difference in inflammatory cytokine concentrations. However, the inclusion of HES in both fluid groups may have attenuated inflammatory cytokine release in this study. Although this study was able to demonstrate some improvement in cardiovascular parameters with hypertonic fluid administration in these septic patients, another study in
patients with septic shock did not show a significant difference in cardiovascular parameters in patients that received 5 mL/kg of 0.9% saline (n=32) versus those that received 5 mL/kg of 3.5% saline (n=30). Another study also failed to demonstrate immunomodulatory effects of hypertonic saline, albeit not in sepsis but in women undergoing hysterectomy. Patients were randomised to 4 mL/kg of 7.5% saline (n=19), 4 mL/kg of 0.9% saline (n=20) or 32 mL/kg of 0.9% saline (n=19) as a single bolus immediately before surgery. There was no difference between groups in multiple measures of leucocyte activation; however, given the planned surgical procedure likely incited less insult than sepsis or trauma, these results are less relevant.

Two studies have compared clinical outcomes after administration of hypertonic versus isotonic saline; one for major trauma and the other for major surgical blood loss. A single 250 mL bolus of 7.5% saline in the pre-hospital setting of major trauma did not reduce mortality compared to the same volume of either 7.5% saline in dextrans or 0.9% saline. This trial was stopped early after 853 patients were enrolled due to safety concerns, as a sub-group of patients receiving blood transfusion in the 0.9% saline group had lower 28-day mortality, compared to other groups. An observational case-control study on patients with major surgical blood loss (n=114) found that 3% saline administered perioperatively resulted in fewer post-operative infections, compared to administration of isotonic crystalloid. There was no difference in mortality. The relatively small sample size in both of these studies may preclude identification of any beneficial treatment effect on mortality, given the scale of sample size usually required to impact this outcome.

Given the concern for the adverse effects of fluid volume overload and interstitial oedema related to administration of large volumes of isotonic crystalloid fluid, several studies have assessed the effects of adding hypertonic saline as a fluid resuscitation strategy to decrease the subsequent volume of fluid administration. In the sepsis study previously mentioned, patients with septic shock that received HES suspended in 7.2% saline received less fluid over the subsequent 24 hours, compared to patients that received HES suspended in 0.9%
saline (mean 2.8L versus 4.1L, respectively). A second study found that patients with septic shock randomised to receive 4% saline had significantly less total volume of fluids administered, compared to groups that received either 0.9% saline or hydroxyethyl starch. However, results could not be further evaluated as the article is written in Chinese. In a paediatric study, children with sepsis were randomised to receive either 20 mL/kg of 0.9% saline or 15 mL/kg of 3% saline, followed by 0.9% saline titrated to effect for both groups. Children that received a single bolus of 3% saline had a significantly lower total volume of fluids administered (mean 28 mL/kg) compared to those that received 0.9% saline only (mean 69 mL/kg). A similar result was demonstrated in a second pilot trial in paediatric sepsis, with a significantly higher PaO2/FiO2 ratio also observed in children that received 3% saline, compared to 0.9% saline. Finally, an observational study in trauma patients that underwent temporary abdominal closure compared a constant rate infusion of 3.5% saline, as an adjunct to fluid therapy, to isotonic crystalloid alone. Patients in the hypertonic saline group had a significantly lower total volume of fluid administered in the first 48 hours (8.5 L versus 11.8 L). These studies support the use of hypertonic saline to reduce crystalloid fluid volume in the fluid loading phase of sepsis and trauma. However, this effect has failed to be verified. There was no difference in subsequent fluid volume administered in mechanically ventilated patients in septic shock that were randomised (blinded) to bolus therapy of either 3% saline (n=218) or 0.9% saline (n=224). If hypernatraemia developed, the blinded fluid was switched to open-label 0.9% saline, which occurred in 39% of patients in the hypertonic saline group. The pre-planned sample size was not reached as the study was stopped early due to a concurrent interventional arm, hyperoxia, showing signs of harm.

Finally, there is little information on the level of blood osmolality that can be achieved clinically with a single bolus of hypertonic saline. Given that laboratory studies have demonstrated immunomodulatory effects at supraphysiologic concentrations, for example, above 340 mmol/L (see Section 6.2.1), whether or not this can be achieved in the clinical setting, or for how long is uncertain. Some of the aforementioned studies also measured serum
osmolality or sodium concentration as a secondary outcome. Studies using 4 mL/kg, or 250 mL total, of 7.5% saline showed an increase in osmolality of ~10-20 mmol/kg or a transient increase in sodium concentration of ~8-10 mmol/L. There is little published data on osmolality after 3% saline; however, patients with septic shock receiving 7.2 mL/kg of 5% saline over 2 hours had a mean serum osmolality of 310 mmol/kg at the end of the infusion. It is unclear if these increases in osmolality are sufficient to cause measurable immunomodulatory effects, especially in the setting of sepsis. Although some beneficial effects on the endothelium have been shown in laboratory studies, the effects of hypertonic saline on the endothelial glycocalyx has not been explored.
7.3 Bolus therapy with 3% hypertonic saline or 0.9% saline in emergency
department patients with suspected sepsis: a pilot randomised controlled
trial

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Clinical Research in Emergency Medicine.
Abstract

**Objective and design:** Hypertonic saline administered during fluid resuscitation may mitigate endothelial glycocalyx (EG) shedding and inflammation. The objective of this pilot randomised controlled trial was to measure the effect of hypertonic saline, compared to isotonic saline on biomarkers of EG shedding and inflammation in emergency department patients with suspected sepsis.

**Methods:** Patients received either 5 mL/kg of 3% saline (hypertonic group, n=34) or 10 mL/kg of 0.9% saline (isotonic group, n=31). Serum biomarker concentrations of syndecan-1, hyaluronan, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, interleukin-6, -8, -10, interferon-γ, neutrophil gelatinase-associated lipocalin and resistin were compared at baseline (T0), after fluid (T1), 3 hours (T3) and 12-24 hours (T24) later, as was serum osmolality, using linear mixed effects models.

**Results:** The hypertonic group had significantly increased mean serum osmolality compared to the isotonic group at T1 (P<0.001) and T3 (P=0.004). Minor differences were found in some biomarker outcomes, including a decreased fold-change in syndecan-1 at T1 (P=0.012) and in interleukin-10 at T24 (P=0.006) in the isotonic group, compared to the hypertonic group.

**Conclusions:** Although a single bolus of hypertonic saline increased serum osmolality, it did not reduce biomarkers of EG shedding or inflammation, compared to patients that received isotonic saline.

**Keywords:** Crystalloid fluids, sepsis, endothelium, inflammation

**Abbreviations:**
ED Emergency department
EG Endothelial glycocalyx
ICU Intensive care unit
IL Interleukin
SIRS Systemic Inflammatory Response Syndrome
SOFA Sequential Organ Failure Assessment
Introduction

Sepsis is characterised by a dysregulated host response to an infection. This can lead to microcirculatory alteration, reduced tissue perfusion and organ dysfunction. Current recommendations for improving perfusion in the early stages of treatment include bolus fluid administration, up to 30 mL/kg for patients with hypoperfusion. However, patient fluid requirements and fluid responsiveness can be difficult to assess in regards to volume titration, and positive fluid balance has been associated with increased mortality in intensive care unit (ICU) patients with sepsis.

An association between large volumes of crystalloid and poorer outcomes in septic patients is currently an active area of investigation. One proposed mechanism for this association is shedding of the endothelial glycocalyx (EG), causing fluid extravasation and interstitial oedema. Shedding of the EG precipitates endothelial and leucocyte activation, and increased vascular permeability. Our recent work in patients with pneumonia has shown positive associations between circulating EG and neutrophil activation biomarker concentrations. Increased EG shedding in patients with sepsis has also been associated with cumulative fluid volumes, degree of organ failure and mortality. Therefore, strategies aimed at mitigating shedding of the EG, such as modification of current resuscitation practices, may improve outcomes for patients with sepsis.

Hypertonic saline increases serum osmolality and draws water from the interstitial, therefore it may re-establish blood flow while avoiding administration of large volumes of fluid. In turn, this may reduce EG shedding and endothelial activation. Hypertonic saline has been shown in in vitro and rodent models to reduce inflammatory cytokine release, cell activation and adhesion. Studies in trauma patients have shown evidence that hypertonic saline reduced endothelial activation and inflammation, compared to isotonic crystalloid. However, there is currently little information on the
endothelial and immunomodulatory effects of hypertonic saline, compared to isotonic crystalloid, when used in adult patients with sepsis.

In this randomised controlled pilot trial we sought to compare the effect of two initial fluid bolus strategies, 5 mL/kg of hypertonic saline or 10 mL/kg of isotonic saline, in adult patients with suspected infection meeting Systemic Inflammatory Response Syndrome (SIRS) criteria. We hypothesised that patients who received hypertonic saline would have an increased serum osmolality and reductions in biomarkers of endothelial activation and inflammation over time, compared to patients who received isotonic saline. We also hypothesised that hypertonic saline would reduce the volume of fluids subsequently given over the following 24 hours, compared to isotonic saline.

Methods

Study Design

This study was approved by the Royal Perth Hospital Human Research Ethics Committee (HREC 2011-091) and was registered with the Australian and New Zealand Clinical Trials Registry (ACTRN12611001021965) before commencement. The Sepsis-Saline Trial (SST) was a pragmatic, investigator-initiated, single-center, randomised controlled open-label pilot trial of adult patients presenting to the emergency department (ED) with suspected infection, who met SIRS criteria and required a fluid bolus as judged by the treating clinician. The HREC restricted enrolment to patients that could provide written informed consent. Patients were randomised via password-protected web-based interface to receive either intravenous 3% saline (hypertonic group) or 0.9% saline (isotonic group). Patients were recruited between November, 2011 and April, 2015. The primary outcome of this study was fold-change in endothelial activation and inflammatory biomarker concentrations. Secondary outcomes included serum osmolality, volume of fluids administered and development of organ failure.

Patients
Patients admitted to the ED were included if they met 2 of 4 SIRS criteria (temperature >38 °C or <36 °C, heart rate >90 bpm, respiratory rate >20 bpm or white cell count >12 x10⁹/L or <4 x10⁹/L) as well as clinical suspicion of infection and a clinical requirement for fluid administration. Patients were excluded if there was a contraindication to fluid volume loading, a plasma sodium concentration <135 or >145 mmol/L, acidosis (venous pH<7.25), established renal failure (eGFR <45 mL/min/1.73m²), pregnancy, age <18 years, patient deemed to receive palliative care only or they had received >500 mL total of pre-hospital crystalloid fluid. Patients that required immediate resuscitation were also excluded, as research processes including the ethics committee requirement for informed patient consent may have delayed treatment.

**Intervention**

Patients were randomised to receive either 5 mL/kg of 3% saline or 10 mL/kg of 0.9% saline, to be given over less than 1 hour intravenously. Blinding of the study intervention was not possible due to the different volumes required. The dose of 0.9% saline was chosen to be consistent with the usual practice in the authors’ ED, with a maximum dose of 1000 mL total allowed. The dose of 3% saline was equated to deliver twice the sodium load equivalent of one litre of 0.9% saline at a maximum dose of 600 mL. Any treatment given after the study fluid, including additional crystalloid fluid, was open-label and not restricted. All fluids given after enrolment were recorded, including volume and type of fluid, up until the last blood sampling time point.

**Data collection**

Heart rate, mean arterial blood pressure and haemoglobin concentration were collected from the medical records, aligned with the research blood sampling time points below. The Sequential Organ Failure Assessment (SOFA) score⁶⁵ was slightly modified for the ED setting (Table 1) and calculated from data collected during three time periods; during ED stay, on day 1 (first 24 hours in wards or ICU) and day 2 (second 24 hours in wards or ICU). A single clinician blinded to treatment allocation reviewed medical records and assigned a discharge diagnosis category of either ‘not infection’, ‘suspected infection’ or
confirmed infection’, informed by review of diagnostic test results and subsequent clinical course. Charlson Comorbidity Score\textsuperscript{66} was calculated from data at time of admission.

\textit{Laboratory parameters}

Blood samples were taken prior to administration of the study fluid (T0), and 1 hour (T1), 3 hours (T3) and 12-14 hours after fluid administration (T24), and were chosen to be consistent with the sampling protocol of a parallel study.\textsuperscript{67} Samples were collected into a serum clot tube then centrifuged at 3000 rpm at 4°C for 10 minutes. Serum was then aliquoted and stored at -80°C. Inflammatory biomarkers, neutrophil gelatinase-associated lipocalin and resistin, as well as glycocalyx biomarkers, syndecan-1 and hyaluronan, were measured using commercial enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA). Inflammatory cytokines interleukin (IL)-6, IL-8, IL-10, interferon-\textgamma and endothelial biomarkers, soluble intercellular adhesion molecule-1 and soluble vascular cell adhesion molecule-1, were measured using a commercial multiplex cytometric bead array kit (BD Biosciences, San Jose, CA, USA). Serum osmolality was measured by freezing point depression (6M Osmometer, Löser Messetechnik, Berlin, Germany).

\textit{Statistical methods}

Due to the exploratory nature of this study and lack of previous data to inform potential differences, a power calculation to estimate sample size was not performed. Normality of data was assessed by visual inspection of histograms and Q-Q plots. Patient characteristics were summarised using number (percentage) for categorical variables, and median [Q1-Q3] or mean (95% confidence interval) for continuous variables depending on normality. Between-group differences for baseline characteristics were tested using linear regression for normally distributed continuous variables, Fisher’s exact test for dichotomous variables, negative binomial regression for count variables and Wilcoxon rank sum test for continuous variables not normally distributed.
Changes in clinical variables from baseline were compared using linear random effects regression models. Volumes of fluid delivered between each time point were compared using Wilcoxon rank sum test. Biomarker concentrations were log-transformed to produce normal or approximately normal distributions and summarised using geometric mean (95% confidence interval). Comparisons of change in biomarker concentration over time were made using either linear random effects regression or Tobit random effects regression models. For each biomarker, change from T0 was expressed as fold-change (95% confidence interval). Patients that had completed research blood sampling at T24 were further followed for clinical progression by comparing components of their SOFA score. Proportion of patients with organ failure (SOFA score >0) (Table 1) was compared between groups using Fisher’s exact test for each time period. Hospital length of stay was compared between groups using Wilcoxon rank sum test.

All analyses were performed using Stata 14 (College Station, TX, USA) with significance set at two-sided $P<0.05$.

**Results**

**Baseline characteristics**

This study included 31 patients randomised to the isotonic group and 34 patients randomised to the hypertonic group (Figure 1). There were no significant differences between baseline characteristics (Table 2). Of patients with confirmed infection, 21 had positive bacterial culture (13 in the isotonic group, 8 in the hypertonic group), 9 had infection confirmed on imaging results (3 in the isotonic group, 6 in the hypertonic group), and 4 patients had positive viral serology results (2 in each group). The most frequent source of infection was lung (n=15), followed by urinary tract (n=15) and soft tissue (n=9).

**Change in biomarker concentrations over time**

Summarised biomarker concentrations and fold-change from T0 for each subsequent time point are presented in Table 4. There was a significantly
decreased fold-change in syndecan-1 at T1 in the isotonic group, compared to the hypertonic group ($P=0.012$). There were no significant differences between the two groups in fold-change for hyaluronan, soluble intercellular adhesion molecule-1 or soluble vascular cell adhesion molecule-1 at any time point.

There was a significantly decreased fold-change in IL-10 at T24 in the isotonic group, compared to the hypertonic group ($P=0.006$). Otherwise, there were no significant differences between the two groups in fold-change for inflammatory cytokines IL-6, IL-8, neutrophil gelatinase-associated lipocalin or resistin. Interferon-$\gamma$ was below the detectable limit for 78% of samples. There was no significant difference in number of samples with measurable interferon-$\gamma$, versus unmeasurable, at any time point between the two groups (data not shown).

Clinical variables

Serum osmolality in participants in the hypertonic group was significantly increased from baseline at T1 ($P<0.001$) and T3 ($P=0.004$), compared to the isotonic group, but there was no significant difference between groups by T24 ($P=0.59$) (Figure 2). There were no significant changes from baseline between the two groups in heart rate, mean arterial blood pressure or haemoglobin concentration over time (Table 3).

Fluids administered

Only patients in the hypertonic group received 3% saline during the study period (Figure 3). The isotonic group received significantly more 0.9% saline and total volume of fluids between T0 and T1 (both $P<0.001$), compared to the hypertonic group, but there were no significant differences between groups in volume of 0.9% saline or total fluid volume administered between T1 to T3 ($P=0.37$, $P=0.68$, respectively) or T3 to T24 ($P=0.94$, $P=0.14$, respectively) (Figure 3). One patient in the hypertonic group reported pain above the cannula insertion site therefore the study fluid rate was halved.
The most frequent fluid type administered after the study fluid was 0.9% saline. Eight patients in the isotonic group received an additional fluid type, compared to 3 patients in the hypertonic group \((P=0.068)\). Fluid types other than 0.9% saline included compound sodium lactate, 4% succinylated gelatine and albumin solution.

**Clinical outcomes**

There were significantly more patients with cardiovascular failure on day 1 in the isotonic group, compared to the hypertonic group \((P=0.014)\) (Table 5). On day 1, 4 patients in the isotonic group required at least 20 mL/kg of intravenous fluids to maintain blood pressure \((n=1)\) and/or required vasopressor support \((n=3)\) whereas none of the patients in the hypertonic group required either types of blood pressure support. Also, there were significantly more patients with haematologic failure in the isotonic group on day 1, compared to the hypertonic group \((P=0.026)\), confluent with decreases in platelet count (Table 5). Event rate was too low for central nervous system and renal failure to be compared (data not shown).

There was no significant difference in length of hospitalisation between the isotonic group (median 2.4 days, Q1-Q3 1.2-4.2) and hypertonic group (3.1 days, Q1-Q3 1.9-5.2) \((P=0.26)\). Only one patient was admitted to the ICU (isotonic group) and only one patient died in each of the groups.

**Discussion**

This pilot study demonstrated that a single bolus of hypertonic saline given to ED patients with suspected sepsis resulted in a significant increase in serum osmolality, compared to a single bolus of isotonic saline. However, administration of hypertonic saline did not reduce biomarker concentrations of EG shedding, endothelial activation or inflammatory biomarker concentrations, though small differences were found in fold-change of syndecan-1 and IL-10 at isolated time points. These minor differences were considered of low relevance given the distribution of the raw biomarker data at each time point. Additionally,
hypertonic saline did not significantly reduce the volume of subsequent crystalloid administered.

Studies in trauma patients that have received hypertonic saline have found less leucocyte and endothelial activation, lower inflammatory cytokine concentrations and fewer post-operative infections, compared to isotonic crystalloid.31-33,38 This effect has not been replicated thus far in patients with sepsis. Although one pilot study found that hypertonic saline reduced the volume of subsequent fluid administration,34 there were no significant differences in gene expression for inflammatory cytokines compared to an isotonic fluid.68 These results are difficult to interpret as the fluids used in this study were combined with hydroxyethyl starch, known to suppress cytokine production.69-71

Lack of treatment effect observed in this study may have been due to only achieving a modest increase of ~10 mmol/kg in serum osmolality (Figure 3). A greater change in osmolality may have been required in order to mitigate inflammation. For the purpose of simplicity, studies that have measured osmolarity are described here as osmolality, with recognition that these measurements have slight differences. Previous studies demonstrating immunomodulatory effects of hypertonic saline in vitro have used concentrations of saline at an osmolality of ~340 mmol/kg or higher.4,6,8,10,12,13,16 In animal models demonstrating anti-inflammatory effects of hypertonic saline, studies have achieved a minimum of ~325 to 350 mmol/kg within 1 hour of infusion.8,19 Lack of treatment effect in this study may also be due to the timing of hypertonic saline delivery in relation to injury. Two different studies support that hypertonic saline has little effect if given after leucocyte activation9 or is delayed beyond the onset of inflammation.24 This may negate the immunomodulatory effects of hypertonic saline in the setting of sepsis, whereby there is little opportunity to deliver a fluid intervention close to the time of illness onset.

In this study, there was no difference in volumes of subsequent crystalloid administered, due to additional open-label administration of 0.9%
saline to patients in the hypertonic group. We were unable to determine from clinical data in medical records what prompted further fluid administration but this may have been driven by lack of blinding and clinician bias against withholding 0.9% saline. This additional fluid administration may have blunted any treatment effect on biomarker outcomes. The other reason for lack of treatment effect may have been due to the relatively mild severity of illness of this cohort; a consequence of the HREC requirement to restrict the trial only to those who could provide written consent. It is possible that inclusion of sicker patients or stricter protocolisation of subsequent fluid administration may have yielded different results.

Strengths of this study include delivery of the intervention fluid early in the treatment of suspected sepsis, as well as frequent blood sampling during initial patient stabilisation. This study demonstrates the feasibility of achieving hyperosmolality with a single dose of 3% saline, which has not been previously reported in this type of patient cohort. Patients in the hypertonic group also achieved similar blood volume expansion, based on no difference in haemoglobin concentration at T1, supporting the effect of hypertonic saline drawing fluid from the extravascular space. Limitations include the lack of blinding and mild severity of illness in this patient cohort. Although the finding of more patients in the isotonic group having cardiovascular failure on day 1 of hospitalisation is provocative, the result may be coincidental in such a small sample size. This study would have also been strengthened by assessment of serum sodium, chloride and base excess concentrations, however lack of these measurements at T24 for most patients precluded this analysis. The biomarkers used in this study were chosen on the basis of previous work demonstrating their association with severity of sepsis, however, broadening the spectrum of biomarker assessment may have been more informative. Given the small sample size, variations in baseline biomarker concentrations may have influenced the results, despite only statistically comparing fold-change in biomarker concentrations. Recommendations for future research in this area include selection of patients with a higher severity of illness, blinding of the intervention fluid and
protocolisation of subsequent fluid administration aimed at achieving clinical endpoints.

In conclusion, delivery of a single bolus of 3% hypertonic saline in ED patients with suspected sepsis increased serum osmolality but did not reduce biomarkers of endothelial glycocalyx shedding, endothelial activation or inflammation, compared to patients that received 0.9% saline.
Figures and Tables

Figure 1. Study flow chart
Figure 2. Serum osmolality (mean, 95% confidence interval) in Emergency Department patients with suspected sepsis that received either 10 mL/kg of 0.9% NaCl (Isotonic) or 5 mL/kg of 3% NaCl (Hypertonic) measured at baseline (T0), 1 hour after start of fluid intervention (T1), and then 3 hours (T3) and 12-24 hours (T24) later. Asterisks denote significant ($P<0.05$) differences between groups in change from T0.
Figure 3. Fluid volumes (median, interquartile range) of 3% NaCl, 0.9% NaCl and all crystalloid fluid delivered to Emergency Department patients with suspected sepsis that were randomised to receive either 10 mL/kg of 0.9% NaCl (Isotonic) or 5 mL/kg of 3% NaCl (Hypertonic) between 0 and 1 hour.
Table 1. Modified Sequential Organ Failure Assessment score scheme for Emergency Department patients with suspected sepsis.

<table>
<thead>
<tr>
<th>Organ system</th>
<th>Criteria</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Respiratory</strong></td>
<td>PaO₂/FiO₂</td>
<td>&gt;400</td>
<td>&lt;400</td>
<td>&lt;300</td>
<td>&lt;200</td>
<td>&lt;100</td>
</tr>
<tr>
<td></td>
<td>SpO₂/FiO₂</td>
<td>&gt;400</td>
<td>&lt;400</td>
<td>&lt;315</td>
<td>&lt;235</td>
<td>&lt;150</td>
</tr>
<tr>
<td></td>
<td>SpO₂</td>
<td>&gt;94% on 0.21</td>
<td>&gt;94% on 6 LPM</td>
<td>&gt;90% on &gt;6 LPM</td>
<td>+ resp support</td>
<td>+ resp support</td>
</tr>
<tr>
<td><strong>Cardiovascular</strong></td>
<td>Systolic blood pressure (mmHg)</td>
<td>SBP&gt;90</td>
<td>SBP&gt;90 after fluid bolus &gt;20mL/kg</td>
<td>Dopamine ≤5</td>
<td>Dopamine &gt;5</td>
<td>Dopamine &gt;15</td>
</tr>
<tr>
<td></td>
<td>Vasopressor</td>
<td></td>
<td></td>
<td>Noradrenaline ≤0.1</td>
<td>Noradrenaline &gt;0.1</td>
<td></td>
</tr>
<tr>
<td><strong>Haematologic</strong></td>
<td>Platelet count (x10⁹/L)</td>
<td>&gt;150</td>
<td>100-150</td>
<td>50-99</td>
<td>20-49</td>
<td>&lt;20</td>
</tr>
<tr>
<td><strong>GIT</strong></td>
<td>Bilirubin (µmol/L)</td>
<td>&lt;20</td>
<td>20-32</td>
<td>33-101</td>
<td>102-204</td>
<td>&gt;204</td>
</tr>
<tr>
<td><strong>CNS</strong></td>
<td>GCS score</td>
<td>15</td>
<td>13-14</td>
<td>10-12</td>
<td>6-9</td>
<td>3-5</td>
</tr>
<tr>
<td><strong>Renal</strong></td>
<td>Creatinine (µmol/L)</td>
<td>&lt;110</td>
<td>110-170</td>
<td>171-300</td>
<td>301-440</td>
<td>&gt;440</td>
</tr>
<tr>
<td></td>
<td>Urine output (mL/kg/hr)</td>
<td></td>
<td>&lt;0.5 for 2 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Abbreviations: CNS, central nervous system; GCS, Glasgow Coma Scale; GIT, gastrointestinal; Fio₂, inspired oxygen concentration; LPM, litres per minute of oxygen; PaO₂, arterial partial pressure oxygen concentration; SBP, systolic blood pressure; SpO₂, pulsatile blood oxygen saturation.
Table 2. Characteristics of emergency department (ED) patients with suspected sepsis that received either 10mL/kg of 0.9% NaCl (Isotonic) or 5mL/kg of 3% NaCl (Hypertonic).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Isotonic group (n=31)</th>
<th>Hypertonic group (n=34)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45 [39-52]</td>
<td>41 [35-47]</td>
<td>0.83</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>18 (58)</td>
<td>21 (62)</td>
<td>0.76</td>
</tr>
<tr>
<td>Body weight (kg)*</td>
<td>89 [75-95]</td>
<td>85 [70-95]</td>
<td>0.81</td>
</tr>
<tr>
<td>CCS</td>
<td>0 [0-1]</td>
<td>0 [0-1]</td>
<td>0.45</td>
</tr>
<tr>
<td>Discharge diagnosis, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not infection</td>
<td>3 (10)</td>
<td>3 (9)</td>
<td>-</td>
</tr>
<tr>
<td>Suspected infection</td>
<td>9 (29)</td>
<td>11 (32)</td>
<td>-</td>
</tr>
<tr>
<td>Confirmed infection</td>
<td>19 (61)</td>
<td>20 (59)</td>
<td>0.96*</td>
</tr>
<tr>
<td>Admission parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>104 [97-111]</td>
<td>102 [97-108]</td>
<td>0.58</td>
</tr>
<tr>
<td>Respiratory rate (bpm)</td>
<td>21 [19-23]</td>
<td>22 [20-23]</td>
<td>0.50</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>38.3 [37.9-38.7]</td>
<td>38.2 [37.8-38.6]</td>
<td>0.65</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>92 [86-98]</td>
<td>90 [86-95]</td>
<td>0.67</td>
</tr>
<tr>
<td>WBCC (x10⁹/L)</td>
<td>10.6 [8.9-14.0]</td>
<td>12.7 [10-17]</td>
<td>0.16</td>
</tr>
<tr>
<td>SOFA score in ED</td>
<td>0 [0-1]</td>
<td>0 [0-1]</td>
<td>0.33</td>
</tr>
<tr>
<td>C-reactive protein (mg/mL)</td>
<td>97 [13-180]</td>
<td>99 [38-150]</td>
<td>0.91</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>1.5 [1.0-2.1]</td>
<td>1.4 [0.9-2.2]</td>
<td>0.96</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>75 [69-81]</td>
<td>77 [70-84]</td>
<td>0.66</td>
</tr>
<tr>
<td>Time admit-to-fluid (mins)*</td>
<td>116 [90-133]</td>
<td>102 [88-117]</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Data is presented as either median [Q1-Q3], mean (95% confidence interval) or number (percentage). * Body weight was available for 26 in the isotonic group and 33 in the hypertonic group. # Fisher’s exact test across groups. ^ Time from ED admission to delivery of intervention fluid.
Abbreviations: CCS, Charlson Comorbidity Score; GCS, Glasgow Coma Score; MAP, mean arterial blood pressure; SOFA, sequential organ failure assessment; WBCC, white blood cell count.
Table 3. Clinical variables (mean (95% confidence interval)) of emergency department patients with suspected sepsis that received either 10mL/kg of 0.9% NaCl (Isotonic) or 5mL/kg of 3% NaCl (Hypertonic), measured at baseline (T0), 1 hour after start of fluid intervention (T1), and then 3 hours (T3) and 12-24 hours (T24) later.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>T0</th>
<th>n</th>
<th>T1</th>
<th>n</th>
<th>T3</th>
<th>n</th>
<th>T24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isotonic</td>
<td>30</td>
<td>104 (97-111)</td>
<td>30</td>
<td>98 (92-104)</td>
<td>24</td>
<td>93 (87-100)</td>
<td>24</td>
<td>85 (79-91)</td>
</tr>
<tr>
<td>Hypertonic</td>
<td>34</td>
<td>102 (96-108)</td>
<td>31</td>
<td>95 (89-102)</td>
<td>27</td>
<td>87 (87-99)</td>
<td>26</td>
<td>82 (76-88)</td>
</tr>
<tr>
<td><em>P</em> value</td>
<td></td>
<td>0.80</td>
<td></td>
<td>0.85</td>
<td></td>
<td>0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isotonic</td>
<td>30</td>
<td>92 (86-98)</td>
<td>30</td>
<td>87 (82-93)</td>
<td>24</td>
<td>86 (80-92)</td>
<td>24</td>
<td>85 (80-90)</td>
</tr>
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*P* values are for differences between groups in change from baseline.
Table 4. Concentrations (geometric mean, 95% CI) of endothelial activation, glycocalyx shedding and inflammation biomarkers in Emergency Department patients with suspected sepsis that received either 10mL/kg of 0.9% NaCl (Isotonic) or 5mL/kg of 3% NaCl (Hypertonic) measured at baseline (T0), 1 hour after start of fluid intervention (T1), and then 3 hours (T3) and 12-24 hours (T24) later. Comparison between groups in change from T0 (fold change (95% CI) is also provided.

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Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular adhesion molecule-1
Table 5. Number of participants that received either 10mL/kg of 0.9% NaCl (Isotonic) or 5mL/kg of 3% NaCl (Hypertonic) with organ failure detected in the emergency department (ED) or on day 1 or 2 of hospitalization. Organ failure for each category was defined as a Sequential Organ Failure Assessment (SOFA) score >0.

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7.4 References


activation marker profiles in severe traumatic brain injured patients. *J Neuroinflammation* 2010;7:5.


CHAPTER 8 – Discussion

The key focus of this thesis is characterising endothelial glycocalyx shedding in the clinical setting of early treatment of sepsis and trauma. The results of these studies have revealed variable temporal patterns between different types of glycocalyx biomarkers during stabilisation of patients in the emergency department (ED). Each biomarker itself, however, has shown consistency in its temporal pattern of shedding between studies involving patients with sepsis. These observations will be discussed as per the study objectives of this thesis, focusing on each biomarker where comparisons can be made across studies.

The second focus of this thesis was assessing the effect of fluid bolus therapy on glycocalyx biomarker shedding and inflammation, including an animal model study and a pilot randomised controlled trial. A brief discussion will be provided on the limitations of these studies and proposed future directions for research.

8.1 Temporal patterns in glycocalyx shedding

Thesis objectives:

• To describe the temporal pattern of circulating concentrations of three glycocalyx biomarkers during ED treatment of sepsis, and assess associations between biomarker concentrations and:
  o cumulative fluid volumes and organ failure scores (Chapter 3)
  o inflammatory biomarker concentrations, respiratory failure, need for mechanical ventilation and mortality (Chapter 4)

• To describe the temporal pattern of circulating concentrations of three glycocalyx biomarkers and inflammatory biomarkers during ED treatment of major trauma (Chapter 5), and:
  o compare these changes over time with that of patients with sepsis
The studies included in this thesis showed that the glycocalyx biomarkers; syndecan-1, syndecan-4 and hyaluronan, displayed different temporal patterns during ED treatment. Each of these biomarkers is discussed separately, with comparison made across the studies in this thesis and reflections made on how these differences in temporal patterns might be explained. The figures presented in this chapter are provided to add clarity to these comparative observations and have been created exclusively using data presented in previous chapters.

**Syndecan-1**

Both sepsis cohorts included in this thesis; the observational cohort (chapters 3 and 4) and the hypertonic saline trial cohort (chapter 7), demonstrated a similar pattern of increasing syndecan-1 concentration over time (Figure 1). This gradual increase over time in syndecan-1 concentration appeared different to the patterns of shedding of both hyaluronan and syndecan-4. It was hypothesised that syndecan-1 shedding was being amplified by inflammation, beyond promotion of endothelial glycocalyx shedding itself, especially given the associations found between this biomarker and those of neutrophil activation (Chapter 4). Another study has also found a weak association between syndecan-1 and neutrophil activation, specifically, myeloperoxidase concentration (Murphy et al). This is supported by the lack of increase in syndecan-1 concentration in the trauma cohort (Chapter 5), whereby an inflammatory response may take longer to develop. One study assessed syndecan-1 concentration over time in patients with polytrauma, and although it increased, this occurred over days rather than hours.
Figure 1. Temporal pattern of syndecan-1 shedding (geometric mean, 95% confidence interval) in an observational cohort of patients with sepsis or simple infection (A) and in an RCT cohort of patients with suspected infection (B). The grey area in graph A represents the confidence interval for an age-matched healthy control population. The asterisk indicates a significant (P<0.05) increase from baseline.

Inflammation may increase syndecan-1 shedding by promoting the upregulation of syndecan-1 on the endothelial surface or by causing shedding from the surface of leucocytes, specifically neutrophils. Endothelial surface expression of syndecan-1 can be upregulated by factors pertinent to critical illness, such as shear stress, tissue injury, inflammation or mediators that can cause endothelial activation.3-8 This upregulation of syndecan-1 may simply increase endothelial cell turnover of syndecan-1; increasing shedding from the endothelial surface without necessarily decreasing overall syndecan-1 expression on the surface or loss of glycocalyx structure. Secondly, shedding of syndecan-1 from activated leucocytes may contribute to circulating concentrations of syndecan-1 ectodomains.9-11 The upregulation of syndecan-1 on the surface of activated neutrophils is poorly documented, however one study in patients with diabetes showed increased cell surface expression relative to healthy controls.9

Syndecan-1 is the most prevalent endothelial glycocalyx biomarker utilised in clinical studies for assessment of glycocalyx shedding. The results
from this thesis highlight the need for future studies to enhance our understanding of the relevance of increases in syndecan-1 concentration, including contributions from cell surface upregulation. Further *in vivo* work is needed to explore relationships between cell surface expression of syndecan-1, shedding, blood concentrations of syndecan-1 and reduction in glycocalyx thickness, using technologies such as sidestream darkfield microscopy.

It would have been ideal to measure syndecan-1 in the canine haemorrhagic shock model in order to better examine immediate effects of fluid therapy on syndecan-1 shedding, however, the availability of commercial canine-specific syndecan-1 enzyme-linked immunosorbent assays is limited. One such kit was tested during pilot work with samples from the canine shock study, however, the standard curve for the assay was inconsistent and very little analyte was detected. Therefore, this work was not progressed.

**Hyaluronan**

Hyaluronan concentrations rapidly increased during initial treatment of sepsis, when compared to syndecan-1. In both sepsis cohorts (Chapter 3, 4 and 7), there was a sharp increase in hyaluronan from baseline during the fluid resuscitation phase in the ED (Figure 3). Given the association with cumulative fluid volumes in the first sepsis cohort, it was hypothesised that trauma patients would also show this pattern in hyaluronan concentration during ED treatment (Chapter 5). However this was not evident (Figure 4). The lack of change over time may have been due to hyaluronan shedding occurring during fluid loading in the pre-hospital setting, before study enrolment. When hyaluronan shedding was explored in a canine haemorrhagic shock model (Chapter 6), a sharp increase in plasma hyaluronan concentration was observed immediately after fluid administration, most noticeably in the large-volume crystalloid group (Figure 4). Results of the dogs that received hydroxyethyl starch, a fluid with little extravascular redistribution, have been included in Figure 4 for contrast.
Figure 3. Temporal pattern of hyaluronan shedding (geometric mean, 95% confidence interval) in an observational cohort of patients with sepsis or simple infection (A) and in an RCT cohort of patients with suspected infection (B). The asterisk denotes a significant increase from baseline. The grey area in graph A represents the confidence interval for an age-matched healthy control population. The asterisk indicates a significant (P<0.05) increase from baseline.

Figure 4. Temporal pattern of hyaluronan shedding (geometric mean, 95% confidence interval) in an observational cohort of major trauma patients compared with an observational cohort of sepsis patients (A) and in a shock model where dogs received crystalloid (CRYST) or hydroxyethyl starch (HES)(B). The grey area in graph A represents the confidence interval for an age-matched healthy control population.

These patterns of rapid increase in circulating hyaluronan concentration support the hypothesis that bolus administration of intravenous fluids
contributes to endothelial glycocalyx shedding. Given there was disparity in the timing of increases in hyaluronan (rapid) and syndecan-1 (more slowly), there may be alternative sources to hyaluronan shedding that are directly related to rapid fluid administration.

One possibility is that increased fluid load to the interstitium may be flushing hyaluronan into the lymphatic system and back into circulation, as shown in several animal model studies. Increased tissue efflux of hyaluronan was one of the first proposed mechanisms for increased circulating hyaluronan concentration in sepsis, before hyaluronan was recognised as an endothelial glycocalyx biomarker. Therefore, it is possible that the peak in hyaluronan concentration observed after crystalloid fluid loading may not be due to endothelial glycocalyx shedding in isolation but also due to increased lymphatic flow during crystalloid redistribution. Sustained increases in hyaluronan shedding, such as those observed in sepsis at 24 hours (Figure 3) may be due to ongoing fluid therapy or effects of inflammation.

Although these mechanisms of increasing hyaluronan shedding may seem benign, low molecular weight hyaluronan has pro-inflammatory properties, which may amplify the systemic inflammatory response in critical illness. Future studies are needed to further characterise the type of hyaluronan that is shed during critical illness, using techniques such as size-selective filtration and size exclusion chromatography. Similar to syndecan-1, it is important that temporal changes in circulating concentrations of hyaluronan are also compared with real-time visualisation of endothelial glycocalyx shedding.

**Syndecan-4**

In contrast to syndecan-1 and hyaluronan, syndecan-4 was not increased in sepsis or trauma patients (Chapters 3 and 5)(Figure 5), and was lower than healthy control concentrations at multiple time points. There were no associations found between syndecan-4 and severity of illness in patients with sepsis (Chapter 3) or clinical outcomes in patients with pneumonia (Chapter 4 – Appendix). There were also no associations found between syndecan-4 and
inflammatory or endothelial activation biomarkers in patients with pneumonia (Chapter 4 – Appendix A) or in trauma patients (Chapter 5).

Given these results across two patient cohorts, sepsis and trauma, it is possible that syndecan-4 is negatively associated with critical illness, in that its concentration is decreased in these circumstances. This is in agreement with two studies in patients with pneumonia, whereby syndecan-4 concentration was negatively correlated with pneumonia severity score\textsuperscript{27} and was lower in acute exacerbation of idiopathic interstitial pneumonia compared to stable disease\textsuperscript{28}. Although these authors suggested that syndecan-4 may have a protective effect in lung inflammation, ‘the source and kinetics of serum syndecan-4 in the human body have not yet been clarified.’\textsuperscript{28} It is unclear if syndecan-4 has utility as a marker of severity of illness in sepsis or trauma. The results of this thesis suggest marked discordance between patterns of syndecan-4 shedding and those of other endothelial glycocalyx biomarkers, syndecan-1 and hyaluronan. At this stage, furthering our understanding of syndecan-1 and hyaluronan shedding in critical illness is likely to be of more value than further pursuing syndecan-4. However, a role for this biomarker may be elucidated in the future given its well-documented constitutive and induced endothelial glycocalyx distribution\textsuperscript{5-8,27,29-36}

Figure 5. Temporal pattern of syndecan-4 shedding (geometric mean, 95% confidence interval) in an observational cohort of patients with sepsis or severe infection (A) and an observational cohort of major trauma patients compared
with sepsis patients (B). The grey area in graph A represents the confidence interval for an age-matched healthy control population.

8.2 The effects of bolus fluid therapy on endothelial glycocalyx shedding

Objectives:

- To measure the effect of four different fluid strategies on glycocalyx and inflammatory biomarker concentrations in a canine haemorrhagic shock model (Chapter 6).

- To compare the effect of hypertonic saline with isotonic saline on glycocalyx and inflammatory biomarker concentrations when used for fluid resuscitation in ED patients with suspected infection (Chapter 7).

Both fluid intervention studies showed increased endothelial glycocalyx biomarker shedding, as measured by hyaluronan concentration, immediately following fluid bolus therapy (Figure 3 and 4). Other contributors to increased circulating concentrations of hyaluronan have been proposed above. In the canine haemorrhagic shock study (Chapter 6), dogs that were administered a large volume of crystalloid fluid also showed higher concentrations of inflammatory biomarkers after fluid administration. Although this result is provocative and supports the hypothesis that large-volume crystalloid causes endothelial glycocalyx shedding and enhanced inflammation, the study was hampered by the relative insensitivity of available canine-specific ELISA products and inter-individual variability. These limitations restricted the statistical analysis of some biomarkers to fairly crude methods, such as categorical analysis (measurable versus unmeasurable biomarker concentration). Although the canine model presents many comparative benefits to human haemorrhagic shock, limited availability of commercial assays for biomarker analysis is a significant impediment. Future animal models exploring endothelial glycocalyx shedding after bolus fluid administration should include multiple biomarkers for comparison, as well as real-time visualisation of shedding.
Although a treatment difference was not observed between patients that received hypertonic saline and those that received isotonic saline (Chapter 7), some interesting trends were observed. The patients in the isotonic group showed a trend towards higher volume of crystalloid administered over 24 hours and more patients receiving fluid types other than 0.9% saline, such as balanced crystalloid, 4% succinylated gelatine and albumin solution. In the isotonic group, there was also a trend towards lower mean arterial pressure at 24 hours and significantly more patients that developed cardiovascular failure. Unfortunately, this study had significant limitations such as small sample size and patients with mild illness severity. There is a growing body of evidence that bolus crystalloid administration for treatment of sepsis, as well as a positive fluid balance, may be harmful\textsuperscript{37-40} and prospective randomised clinical trials comparing restrictive versus liberal fluid strategies are ongoing.\textsuperscript{41-43}

This thesis has not fully addressed the area of ‘glycoprotective’ therapies: drugs or fluids used to either prevent glycocalyx shedding or assist with rebuilding the glycocalyx. Such postulated therapies include provision of plasma proteins, such as fresh frozen plasma, albumin solutions, antithrombin and activated protein C, as well as antioxidants and hydrocortisone.\textsuperscript{44-50} The evidence for efficacy of these therapies for repairing the endothelial surface is preliminary. Further detail on these therapies was considered beyond the scope of this thesis but this is likely an expanding area of future research.

8.3 Final thoughts

The work contained in this thesis demonstrates the complexity of endothelial glycocalyx shedding and suggests that multiple factors associated with critical illness, such as illness severity, key inflammatory biomarkers and fluid interventions, may alter the temporal pattern of shedding. One of the key messages gained from this data examining temporal patterns early in the treatment of sepsis and trauma is that isolated measurements of single glycocalyx biomarkers do not tell the whole story. Many questions remain as to
the optimal method for measurement of this effect in the clinical setting. Undoubtedly, endothelial glycocalyx shedding plays a role in microcirculatory dysfunction and propagation of inflammation, and likely contributes to multiple organ failure. However, it is unclear if endothelial glycocalyx shedding is simply a bystander of the inflammatory storm or a key player in propagating organ dysfunction and clinical deterioration. Further, it is yet to be elucidated if 'glycoprotective' therapy, or therapy aimed at rebuilding the glycocalyx, will improve clinical outcomes. This is an exciting area of research with much scope for future pathways of investigation.

8.4 References


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APPENDIX

Appendix A

Syndecan-1, hyaluronan and endocan in sepsis secondary to pneumonia: Additional results for biomarker, syndecan-4 (omitted from publication).

Please refer to 4.1 Gap in Knowledge in regards to omission of this biomarker from the final publication. Also refer to Chapter 4 for Materials and Methods.

Figure 1. Endocan, syndecan-1, syndecan-4 and hyaluronan concentration (predicted mean, 95% confidence interval) in participants with sepsis due to pneumonia (n=44) at admission to an Emergency Department (0 hours, N=44), 1 hour (N=44), 3 hours (N=34) and 12-24 hours (N=24) later. Asterisks denote significant change ($P<0.05$) from 0 hours. Dashed lines represent median concentration of healthy controls.
Table 1. Correlations between endocan, syndecan-1, syndecan-4 and hyaluronan, and inflammatory biomarkers in patients with sepsis due to pneumonia, measured at admission to the Emergency Department (T0) and 12-24 hours later (T24).

| Biomarker | Endocan | | | | Syndecan-1 | | | | | Syndecan-4 | | | | Hyaluronan | | |
|-----------|---------|-------|-------|-------|---------|-------|-------|-------|-------|---------|-------|-------|-------|---------|-------|
|           | T0      | T24   | T0    | T24   | T0      | T24   | T0    | T24   | T0    | T24    | T0    | T24   | T0    | T24    | T0    | T24   |
|           | rho     | P     | rho   | P     | rho     | P     | rho    | P     | rho    | P     | rho    | P     | rho    | P     | rho    | P     |
| Interleukin-6 | 0.26 | 0.09 | 0.36 | 0.09 | 0.25 | 0.11 | 0.41 | 0.048 | -0.13 | 0.41 | -0.14 | 0.51 | 0.35 | 0.019 | 0.21 | 0.33 |
| Interleukin-10 | 0.24 | 0.11 | 0.29 | 0.17 | 0.10 | 0.54 | 0.40 | 0.052 | -0.07 | 0.63 | 0.40 | 0.052 | 0.15 | 0.34 | 0.25 | 0.24 |
| NGAL      | 0.18 | 0.25 | 0.40 | 0.052 | 0.52 | <0.001 | 0.52 | 0.010 | 0.30 | 0.046 | 0.24 | 0.27 | 0.39 | 0.008 | 0.25 | 0.25 |
| Resistin  | 0.19 | 0.21 | 0.37 | 0.079 | 0.54 | <0.001 | 0.53 | 0.007 | 0.21 | 0.18 | 0.08 | 0.70 | 0.45 | 0.002 | 0.32 | 0.13 |
| MPO       | 0.07 | 0.64 | 0.14 | 0.50 | 0.54 | <0.001 | 0.38 | 0.067 | -0.02 | 0.92 | -0.27 | 0.20 | 0.42 | 0.005 | 0.48 | 0.019 |
| ICAM-1    | 0.18 | 0.24 | 0.20 | 0.35 | 0.21 | 0.17 | 0.32 | 0.13 | 0.09 | 0.55 | 0.01 | 0.95 | 0.27 | 0.079 | 0.09 | 0.66 |
| VCAM-1    | 0.34 | 0.025 | 0.52 | 0.009 | 0.24 | 0.13 | 0.51 | 0.011 | -0.05 | 0.72 | -0.07 | 0.75 | 0.32 | 0.034 | 0.37 | 0.076 |

Values in bold indicate significant correlations at rho>0.5

Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; MPO, myeloperoxidase; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.
Table 2. Associations [odds ratio (95% confidence interval)] between variables at Emergency Department admission (biomarker, CURB-65 score or lactate) and clinical outcomes in patients with sepsis due to pneumonia.

<table>
<thead>
<tr>
<th></th>
<th>Respiratory failure</th>
<th>Mechanical ventilation</th>
<th>30-day mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P value</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Endocan (ng/mL)</td>
<td>1.04 (0.99-1.13)</td>
<td>0.053</td>
<td>1.04 (0.97-1.10)</td>
</tr>
<tr>
<td>Syndecan-1 (ng/mL)</td>
<td>1.18 (1.05-1.33)</td>
<td>0.004</td>
<td>1.24 (1.04-1.48)</td>
</tr>
<tr>
<td>Syndecan-4 (ng/mL)</td>
<td>3.23 (1.25-20.6)</td>
<td>0.015</td>
<td>1.23 (0.42-3.57)*</td>
</tr>
<tr>
<td>Hyaluronan (µg/mL)</td>
<td>1.27 (0.96-1.69)</td>
<td>0.092</td>
<td>1.37 (0.91-2.07)</td>
</tr>
<tr>
<td>CURB-65 score</td>
<td>1.33 (0.97-1.83)</td>
<td>0.077</td>
<td>1.32 (0.72-2.43)</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>1.04 (0.92-1.16)</td>
<td>0.55</td>
<td>1.35 (1.07-1.70)</td>
</tr>
</tbody>
</table>

*Biomarker concentration log-transformed due to non-linearity

Odds ratios are for an increase of 1ng/mL for endocan, syndecan-1 and syndecan-4, and 1µg/mL for hyaluronan. Bold P values indicate a significant (P<0.05) association.

Respiratory failure was determined on days 1, 2 and 3 of hospitalization by a Pa02/Fi02 ratio<300, Sp02/Fi02<315 or Sp02<90% on >6 L/min oxygen. As there was no difference in the association when comparing days 1, 2 and 3, a single odds ratio is reported for respiratory failure across time.