EARLY MICROCIRCULATORY DYSFUNCTION FOLLOWING TRAUMATIC HAEMORRHAGE

By

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Abstract

Traumatic haemorrhagic shock (THS) is the most frequent cause of preventable death after severe injury. Shock is characterised by inadequate provision of oxygen and substrates to tissues in relation to their requirements, and it is within the microcirculation that this process is regulated. Investigation of the microcirculation is therefore key to understanding the pathological processes following THS.

In Part I, some mechanisms of microcirculatory dysfunction following trauma are presented. Endotheliopathy of trauma is associated with poor microcirculatory flow, and occurs within minutes of injury. It is also associated with higher levels of circulating cell-free DNA (cfDNA), supporting the hypothesis that cfDNA is an aetiological factor in this pathological response. Both endotheliopathy and elevated cfDNA and are related to poor clinical outcomes.

In Part II, clinical implications of microcirculatory monitoring are discussed for patients in the early phase following THS. It is safe and feasible to monitor the microcirculation following THS, and a novel point-of-care grading system has performed well, suggesting that targeted fluid resuscitation towards microcirculatory flow after THS may be possible. The optimal fluid strategy in this context is unknown, but physical properties (e.g. oncotic potential and viscosity) as well as endothelial restorative properties appear to be as important as oxygen-carrying capacity. Potential therapeutic interventions aimed at microcirculatory and endothelial resuscitation open intriguing possibilities for improving outcomes after THS.

Acknowledgments

I would like to thank my wife Laura, and my children, Felicity, Arthur, and Ralph, for supporting me through these years of research. Without their understanding, tolerance, and patience whilst I was away from them—whether at conferences, the laboratory, in the hospital, or even when staring off into the distance, thinking—this body of work would not have been possible. Thank you also to my parents, both academic theologians, for the inspiration and ambition to pursue lifelong learning, wherever that may take me.

I am very grateful indeed for the help and support of my supervisors. Professor Mark Midwinter has been a thoughtful, considerate, and supportive mentor from the outset, and guided me through the process of embarking on a PhD, and through many subsequent studies since. I'm also very grateful to Dr Paul Harrison, who has helped me with the various laboratory work, including the planning and undertaking of experiments. Our discussions about the interpretation of the data in this thesis have also been vital. I'd like to thank Surgeon Commander Sam Hutchings, the Chief Investigator of MICROSHOCK, for allowing me to join that project, training me to use the equipment and software, and for being supportive and understanding throughout the patient recruitment, data collection, interpretation of data, and writing. I'd also like to thank another mentor of mine, Colonel Doug Bowley, who was the first to encourage me to undertake this higher degree during my surgical training.

Thank you to others that have helped me along the way at the University of Birmingham, especially in the laboratory, including Dr Jon Hazeldine, Hema Chahal, and Professor Janet Lord. I'm grateful to Dr Jon Bishop for his assistance in the statistical analysis of data. Thank you also to the clinical and administrative staff at the NIHR Surgical Reconstruction and Microbiology Research Centre; I have enjoyed working there, and appreciate their assistance and generosity of time throughout my research. Thank you to the Medical Illustration department at the University Hospitals Birmingham NHS Foundation Trust for their assistance in producing the original drawings in this thesis.

I'm also very grateful to the Higher Degrees Board, the Surgeon General's Research Steering Group, and the Royal Centre for Defence Medicine for providing permission and funding for this research. Thank you to the Defence Professor of Surgery, Surgeon Captain Rory Rickard, for guidance and support throughout this period of research.

I dedicate this work to those service men and women who have been injured whilst serving in Her Majesty's Armed Forces, and those who will be injured in wars to come.

My greatest ambition is that some part of my work, whether now or in the future, might aid in the relief of their suffering.

Publications

This thesis incorporates the following 7 published papers:

1. **Naumann DN**, Hazeldine J, Midwinter MJ, Hutchings SD, Harrison P. Poor microcirculatory flow dynamics are associated with endothelial cell damage and glycocalyx shedding after traumatic hemorrhagic shock. *J Trauma Acute Care Surg*. 2018;84(1):81–88

2. **Naumann DN**, Hazeldine J, Davies DJ, Bishop J, Midwinter MJ, Belli A, Harrison P, Lord JM. Endotheliopathy of trauma is an on-scene phenomenon, and is associated with multiple organ dysfunction syndrome: a prospective observational study. *Shock*. 2018; 49(4):420-428.

3. **Naumann DN**, Hazeldine J, Dinsdale RJ, Bishop JR, Midwinter MJ, Harrison P, Hutchings SD, Lord JM. Endotheliopathy is associated with higher concentrations of cell-free DNA following major trauma: a prospective observational study. *PLoS ONE.* 2017 12(12): e0189870.

4. **Naumann DN**, Mellis C, Smith IM, Mamuza J, Skene I, Harris T, Midwinter MJ, Hutchings SD. Safety and feasibility of sublingual microcirculation assessment in the emergency department for civilian and military patients with traumatic haemorrhagic shock: a prospective cohort study. *BMJ Open*. 2016;6(12):e014162.

5. **Naumann DN**, Mellis C, Husheer SL, Hopkins P, Bishop J, Midwinter MJ, Hutchings SD. Real-time point of care microcirculatory assessment of shock: design, rationale and application of the point of care microcirculation (POEM) tool. *Crit Care*. 2016;20(1):310.

6. **Naumann DN**, Dretzke J, Hutchings S, Midwinter MJ. Protocol for a systematic review of the impact of resuscitation fluids on the microcirculation after haemorrhagic shock in animal models. *Syst Rev*. 2015;4:135.

7. **Naumann DN**, Beaven A, Dretzke J, Hutchings S, Midwinter MJ. Searching For the Optimal Fluid to Restore Microcirculatory Flow Dynamics After Haemorrhagic Shock: A Systematic Review of Preclinical Studies. *Shock.* 2016;46(6):609-22.

The following papers relate to the current work, but are not incorporated into this thesis:

8. Hutchings S, **Naumann DN**, Harris T, Wendon J, Midwinter MJ. Observational study of the effects of traumatic injury, haemorrhagic shock and resuscitation on the microcirculation: a protocol for the MICROSHOCK study. *BMJ Open*. 2016;6(3):e010893.

9. Hazeldine J, **Naumann DN**, Toman E, Davies D, Bishop JRB, Su Z, Hampson P, Dinsdale RJ, Crombie N, Duggal NA, et al. Prehospital immune responses and development of multiple organ dysfunction syndrome following traumatic injury: A prospective cohort study. *PLoS Med*. 2017;14(7):e1002338.

10. **Naumann DN**, Midwinter MJ, Hutchings S. Venous-to-arterial CO2 differences and the quest for bedside point-of-care monitoring to assess the microcirculation during shock. *Ann Transl Med*. 2016;4(2):37.

11. Lewis CT, **Naumann DN**, Crombie N, Midwinter MJ. Prehospital point-of-care lactate following trauma: A systematic review. *J Trauma Acute Care Surg*. 2016;81(4):748-55.

12. Hutchings SD, **Naumann DN**, Watts S, Wilson C, Burton C, Wendon J, Kirkman E. Microcirculatory perfusion shows wide inter-individual variation and is important in determining shock reversal during resuscitation in a porcine experimental model of complex traumatic hemorrhagic shock. *Intensive Care Med Exp*. 2016;4(1):17.

13. **Naumann DN**, Smith IM, Beaven A, Midwinter MJ. The term "prehospital" must be justified when reporting animal studies of traumatic hemorrhagic shock. *J Trauma Acute Care Surg.* 2016;81(2):394–6

14. Smith IM, Crombie N, Bishop JR*,* McLaughlin A, **Naumann DN** *et al.* RePHILL: protocol for a randomised controlled trial of pre-hospital blood product resuscitation for trauma. *Transfus Med*. 2017. doi: 10.1111/tme.12486

15. **Naumann DN**, Hancox JM, Raitt J*,* Smith IM, Crombie N, *et al.* What fluids are given during air ambulance treatment of patients with trauma in the UK, and what might this mean for the future? Results from the RESCUER observational cohort study. *BMJ open*. 2018;8(1):e019627.

Contribution Statement

Contribution statement for the 7 published papers included in this thesis:

1. **Naumann DN**, Hazeldine J, Midwinter MJ, Hutchings SD, Harrison P. Poor microcirculatory flow dynamics are associated with endothelial cell damage and glycocalyx shedding after traumatic hemorrhagic shock. *J Trauma Acute Care Surg*. 2018;84(1):81–88

This study includes the Birmingham cohort of the MICROSHOCK study. I designed this study in correspondence with MJM, SDH, and PH. I performed all of the patient recruitment, data collection, video-microscopy, and blood sample collection. I performed the analysis of video clips, and performed the ELISAs with JH, who taught me the techniques (also supervised by PH). I formulated the hypotheses and performed all data analysis and interpretation for this study. I wrote the manuscript, and the remainder of authors critically appraised the work. SDH is the Chief Investigator of the MICROSHOCK study. After the first peer review comments recommended revisions, I revised the manuscript and responded to reviewer comments.

2. **Naumann DN**, Hazeldine J, Davies DJ, Bishop J, Midwinter MJ, Belli A, Harrison P, Lord JM. Endotheliopathy of trauma is an on-scene phenomenon, and is associated with multiple organ dysfunction syndrome: a prospective observational study. *Shock*. 2018; 49(4):420-428.

In this study, I designed the methodology and formulated the hypotheses. Blood samples had previously been obtained by the West Midlands Ambulance Service, and then prepared and stored by JH. I performed all ELISAs with JH (supervised by PH and JML). I performed all data analysis and interpretation, with some assistance from JB (statistician) for the analysis featured in Figure 3.2. I wrote the manuscript, and the remainder of authors critically appraised the work. After the first peer review comments recommended revisions, I revised the manuscript and responded to reviewer comments.

3. **Naumann DN**, Hazeldine J, Dinsdale RJ, Bishop JR, Midwinter MJ, Harrison P, Hutchings SD, Lord JM. Endotheliopathy is associated with higher concentrations of cell-free DNA following major trauma: a prospective observational study. *PLoS ONE.* 2017 12(12): e0189870.

In this study, I designed the methodology and formulated the hypotheses. I performed the cell-free DNA fluorometric analysis with JH and RJD, who taught me the laboratory techniques (supervised by PH and JML). The blood samples had been obtained

from the same patients in the first two studies mentioned above. I performed all data analysis and interpretation, with some assistance from JB (statistician) for Figure 4.3 and 4.4. I wrote the manuscript, and the remainder of authors critically appraised the work. After the first peer review comments recommended revisions, I revised the manuscript and responded to reviewer comments.

4. **Naumann DN**, Mellis C, Smith IM, Mamuza J, Skene I, Harris T, Midwinter MJ, Hutchings SD. Safety and feasibility of sublingual microcirculation assessment in the emergency department for civilian and military patients with traumatic haemorrhagic shock: a prospective cohort study. *BMJ Open*. 2016;6(12):e014162.

I designed the methodology for this study (which includes a sub-group of the MICROSHOCK study, originally designed by SDH), and formulated the hypotheses. This study required protocol amendments in order to recruit patients in the Emergency Department, which I designed in correspondence with SDH and TH. Video-microscopy was performed at 4 sites: Kings College (CM and SDH); Royal London (JM and IS); Birmingham (myself); and Bastion Hospital (IMS and MJM). I collated all data, and performed the main quality assurance analysis of all video clips (the main analysis for the study). I performed all data analysis and interpretation. I wrote the manuscript, and the remainder of authors critically appraised the work. SDH is the Chief Investigator for this study. After the first peer review comments recommended revisions, I revised the manuscript and responded to reviewer comments.

5. **Naumann DN**, Mellis C, Husheer SL, Hopkins P, Bishop J, Midwinter MJ, Hutchings SD. Real-time point of care microcirculatory assessment of shock: design, rationale and application of the point of care microcirculation (POEM) tool. *Crit Care*. 2016;20(1):310.

In this study, I designed the methodology of this scoring system together with SDH. I then designed the multi-centre study, which was undertaken by me in Birmingham, and SDH in Kings College London. Video-microscopy was performed by myself, SDH, and CH. I collated all data and performed the data analysis and interpretation, with assistance from JB (statistician) for section 6.3.2 (inter-user variability). I designed the online scoring tool with SLH, who then programmed and published the online web page. I wrote the manuscript, and the remainder of authors critically appraised the work. After the first peer review comments recommended revisions, I revised the manuscript and responded to reviewer comments.

6. **Naumann DN**, Dretzke J, Hutchings S, Midwinter MJ. Protocol for a systematic review of the impact of resuscitation fluids on the microcirculation after haemorrhagic shock in animal models. *Syst Rev*. 2015;4:135.

This paper is a protocol for the next study (a systematic review). I designed and wrote this protocol (and registered it with PROSPERO), with some assistance in search criteria and advice regarding methodology from JD. I wrote the manuscript, and the remainder of the authors critically appraised the work. After the first peer review comments recommended revisions, I revised the manuscript and responded to reviewer comments.

7. **Naumann DN**, Beaven A, Dretzke J, Hutchings S, Midwinter MJ. Searching For the Optimal Fluid to Restore Microcirculatory Flow Dynamics After Haemorrhagic Shock: A Systematic Review of Preclinical Studies. *Shock.* 2016;46(6):609-22.

I designed this study based on the protocol above. I performed the literature search, and full text review. I undertook all data extraction, quality assessment, and assessments of risk of bias, and these elements were confirmed by AB. I critically appraised and formulated the narrative review and tables for the eligible studies. I wrote the manuscript, and all remaining authors critically appraised the work. After the first peer review comments recommended revisions, I revised the manuscript and responded to reviewer comments.

Presentations

The following abstracts were presented at these meetings, based on work within this thesis:

1. **European Society of Intensive Care Medicine, Milan, Italy, Oct 2016:**

The microcirculation can be assessed at the bedside: the point-of-care microcirculation (POEM) grading system.

2. **Colt Research Meeting, Royal Society of Medicine, London, UK, Dec 2016**

Assessing sublingual microcirculation in the Emergency Department for civilian and military patients with traumatic haemorrhagic shock.

3. **Military Healthcare Systems Research Symposium, Orlando, USA, Aug 2017:**

   a. Endothelial cell damage and glycocalyx shedding are associated with poor flow following traumatic haemorrhagic shock.

   b. Trauma-induced endotheliopathy at the scene of injury is associated with multiple organ dysfunction syndrome: a prospective observational study

4. **Centre for Blast Studies Meeting, Imperial College, London, UK, Nov 2017**

Endotheliopathy of trauma is likely to be a battlefield phenomenon, and is associated with multiple organ dysfunction syndrome: a prospective observational study

5. **Association of Trauma and Military Surgery, Plymouth, Nov 2017**

It's in the DNA: injury to the vascular endothelium

6. **Colt Research Meeting, Royal Society of Medicine, London, UK, Dec 2017**

From bench to bedside: understanding microcirculatory dysfunction following trauma and haemorrhagic shock

List of Abbreviations

BBATS	Brain Biomarkers After Trauma Study
CAMARADES	Collaborative approach to meta-analysis and review of animal data from experimental studies
CD	Cluster of differentiation
cfDNA	Cell free deoxyribonucleic acid
CI	Confidence Interval
CO_2	Carbon dioxide
DAMPs	Damage-associated molecular patterns
DCLHb	Diaspirin cross-linked haemoglobin
DCR	Damage control resuscitation
ED	Emergency department
ELISA	Enzyme-linked immunosorbent assays
EoT	Endotheliopathy of trauma
EPO	Erythropoietin
FAST	Focused assessment with sonography in trauma
FCD	Functional capillary density
FFP	Fresh frozen plasma
GCS	Glasgow coma scale
Hb	Haemoglobin
HBOC	Haemoglobin-based oxygen carrier
HC	Healthy control
HES	Hydroxyl-ethyl starch
HSA	Human serum albumin
HTS	Hypertonic saline
HVM	Handheld video-microscopy
ICC	Intra-class correlation coefficient
ICU	Intensive care unit
IDF	Incident dark field
IED	Improvised explosive device
IQR	Interquartile range
ISS	Injury severity score
IVM	Intravital microscopy
LDF	Laser doppler flowmetry
LR	Ringer's lactate
LSCI	Laser speckle contrast imaging
LVM	Low-viscosity high-mannuronic acid
MAP	Mean arterial pressure
MetRBC	Methaemoglobin red blood cells

MFI	Microcirculatory flow index
MHI	Microcirculatory heterogeneity index
MODS	Multiple organ dysfunction syndrome
MW	Molecular weight
NETs	Neutrophil extracellular traps
NS	Normal saline
OPS	Orthogonal polarization spectral imaging
OxyRBC	Oxygen-carrying red blood cells
PBH	Polymerized bovine haemoglobin
PEG-Alb	Polyethylene glycol-conjugated albumin
PEG-BSA	Pegylated bovine albumin
PEG-Hb	Polyethylene glycol-conjugated haemoglobin
POEM	Point-of-care microcirculation
PPV	Proportion of perfused vessels
PVD	Perfused vessel density
PRBCs	Packed red blood cells
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
RBCs	Red blood cells
REC	Research ethics committee
SBP	Systolic blood pressure
SD	Standard deviation
SDF	Sidestream dark field
SOFA	Sequential Organ Failure Assessment
SPS	Serum protein solution
STROBE	Strengthening the reporting of observational studies in epidemiology
SYRCLE	Systematic review centre for laboratory animal experimentation
THS	Traumatic haemorrhagic shock
TIC	Trauma induced coagulopathy
TVD	Total vessel density
TXA	Tranexamic acid
UPBHb	Ultrapurified polymerized bovine haemoglobin

List of Tables

List of Figures

Table of Contents

PART I

MECHANISMS OF MICROCIRCULATORY DYSFUNCTION FOLLOWING TRAUMA AND HAEMORRHAGIC SHOCK

Chapter 1

An introduction to the microcirculation

1.1. Background

The microcirculation is a vast and complex network of capillaries and their connecting arterioles and venules. Although the heart and large vessels are responsible for the pressurised transport of blood throughout the circulation, it is at this junction of the arterial and venous systems where the specialised tasks required for the diffusion of substances to and from tissues occur, in micro-vessels with an approximate diameter of 5–10 μm[1]. Arising from the embryonic mesoderm, the microcirculation has been described as an 'organ'[2], with an approximate mass of 110g and a surface area of 350m^2. Although only around 5% of the circulating volume is in the microcirculation at any one time[3, 4], it is an essential part of the circulatory system for survival of tissues because it is where gas, nutrient and metabolic product diffusion happens. Since diffusion is the main means by which movement of such substances occur, most metabolically active cells in the body are no more than 100 μm away from a micro-vessel[4]. The exact density and concentration of micro-vessels in a given region depends on the metabolic demands of the surrounding tissues.

Microcirculatory behaviour has received recent attention due to its apparent significance during the shocked state in sepsis[5-7], cardiogenic shock[8, 9], following major surgery[10, 11], and more recently in traumatic haemorrhagic shock[12]. There is some evidence that microcirculatory flow does not necessarily always follow macro-circulatory haemodynamics[13], and attention directed specifically towards the microcirculation may be warranted when treating patients with shock and critical illness[14]. Both flow reduction and heterogeneity of flow between regions are likely to be of clinical significance during shock[15, 16]. Since perfusion in the microcirculation is a product of the characteristics of the vessels (tone, patency) and the intraluminal contents (pressure and rheology), It is likely that

microcirculatory flow is related to the behaviour and state of the endothelial lining. Understanding the functional anatomy of the endothelial layer within the microcirculation is necessary for a fuller understanding of the mechanisms and behaviour during both the healthy and shocked states.

1.2. Functional anatomy of the microcirculation

1.2.1. Structure of the microcirculation

Although arterioles and venules have the muscle and connective tissue layers that characterise the rest of the circulation, the capillaries in between do not. Instead they consist of only the single celled inner (tunica intima) layer. Although oxygen exchange can also occur across endothelial layers of the arterioles and venules, the majority occurs at these capillaries. The endothelial layers of micro-vessels are heterogeneous depending on their location and function, and are divided into (a) continuous; (b) fenestrated; or (c) discontinuous (Figure 1.1).

1.2.1.1. **Continuous micro-vessels** (Figure 1a)

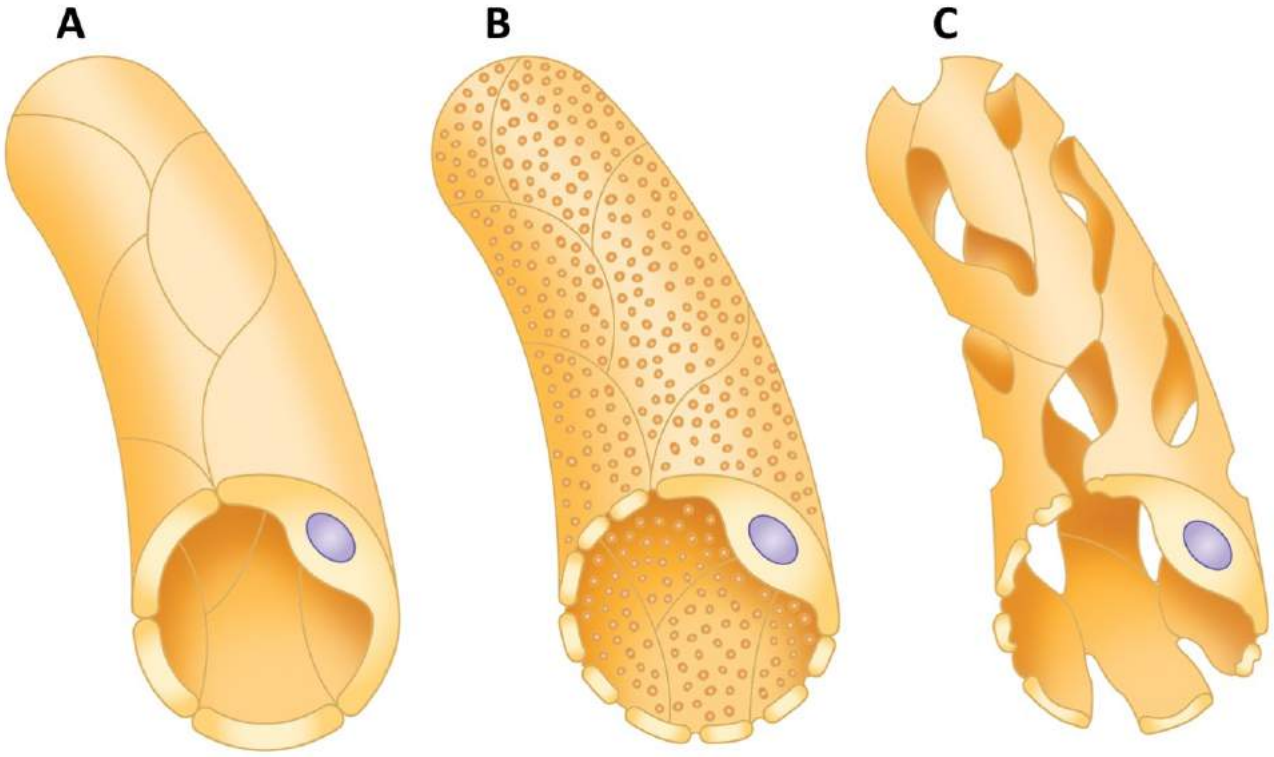
These have a basement membrane with tight junctions between endothelial cells without exposed 'windows' between them. Due to these structural constraints they are the least permeable micro-vessels and only allow the passage of water and other molecules that are less than 3nm in radius, with larger molecules only admitted to pass through the endothelial layer by selective transport[17]. Such continuous micro-vessels are found in the skin, muscle, cardiac, respiratory, and central nervous systems.

1.2.1.2. Fenestrated micro-vessels (Figure 1b)

These are characterised by small full thickness pore-like gaps in the endothelial layer (approximately 70nm in diameter)[18], which allow a higher permeability of larger molecules during filtration, secretion and absorption. These micro-vessels are mainly found in the endocrine and exocrine glands, choroid plexus, renal system and intestinal mucosa[1].

Figure 1.1. Illustration of the different types of microcirculatory vessels.

A: Continuous (e.g. skin, muscle, respiratory system)
B: Fenestrated (e.g. endocrine and exocrine glands)
C: Discontinuous (e.g. liver, spleen)



Original illustration by Andrew Dakin, Medical Illustration Department, Queen Elizabeth Hospital

1.2.1.3. Discontinuous micro-vessels (Figure 1c)

These have large fenestrations (approximately 100 – 200 nm in diameter)[18] as well as gaps between cells, and allow high permeability for exchange of particles, filtering, and haematopoiesis. Such vessels are found in organs with sinusoids such as bone marrow, liver and spleen.

Although these three types of endothelial surfaces are distinct from each other, endothelial cells are heterogeneous, dynamic, and can change in nature depending on cell signalling pathways. For example, vascular endothelial growth factor (VEGF) is able to modify micro-vessels from continuous to fenestrated[19]; conversely, lack of VEGF can cause reduction in fenestrations[20].

1.2.2. Endothelial interface

The endothelial layer components include the endothelial cells, their basal lamina and the endothelial glycocalyx that lines the luminal surface. Interactions between the endothelium and circulating cells (such as leukocytes, platelets and red cells) occurs at the endothelial surface via specialised trans-membrane cell adhesion molecules such as those in the cadherin, immunoglobulin, integrin and selectin protein families[21]. These molecules have a complex role in the interactions between the endothelium, inflammatory, and coagulation components[22].

1.2.2.1. Endothelial cells

The lining of the all blood vessels consists of a single-cell layer of flattened endothelial cells resting against the basal lamina, each approximately 3µm thick

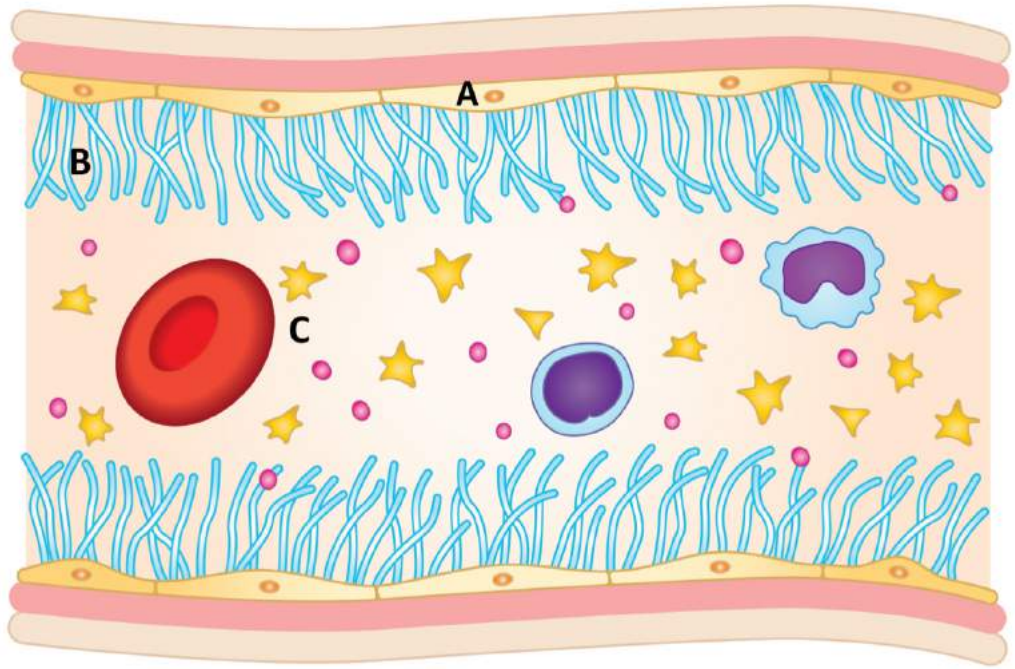
around the nucleus, but tapering on either side towards the periphery of the cell[23]. These endothelial cells arise from the embryonic mesoderm by differentiation of haemangioblasts and angioblasts[24]. Each cell is joined to surrounding cells by adhesional junctions[1]. In the rest of the circulation, the thickness of muscle and connective tissue vary depending on the location, function and size, but the endothelial lining is always present. At the smallest vessels of the microcirculation it is the only the layer of endothelial cells and basal lamina that remain, as well as some interspersed pericyte cells that wrap themselves around the micro-vessels[25].

Endothelial cells are dynamic, and there is experimental evidence that they are aligned in the direction of blood flow, and when the flow direction changes the cells also change their alignment[26]. Endothelial cells regulate the growth of new micro-vessels in tissues due to increased demand for oxygen in relation to its provision. When tissues lack oxygen there is an increase in the amount of active hypoxia-inducible factor 1 (HIF-1) within the affected cells. The increased concentration of active HIF-1 induces gene transcription of vascular endothelial growth factor (VEGF). The VEGF (and other more non-specific growth factors) acts on endothelial cells, causing them to migrate through the basal lamina and proliferate and move towards the HIF-1 signal—in effect, 'sprouting' a new branch in the micro-vessel. These cells form new micro-vessels and increase the amount of available oxygen in their new territories. Further proliferation is stopped when the HIF-1 and VEGF concentrations are restored to normal. Angiopoetin-1 also stimulates endothelial remodelling and cell migration via Tie2 receptors on the endothelial cell membrane[27].

1.2.2.2. Endothelial glycocalyx

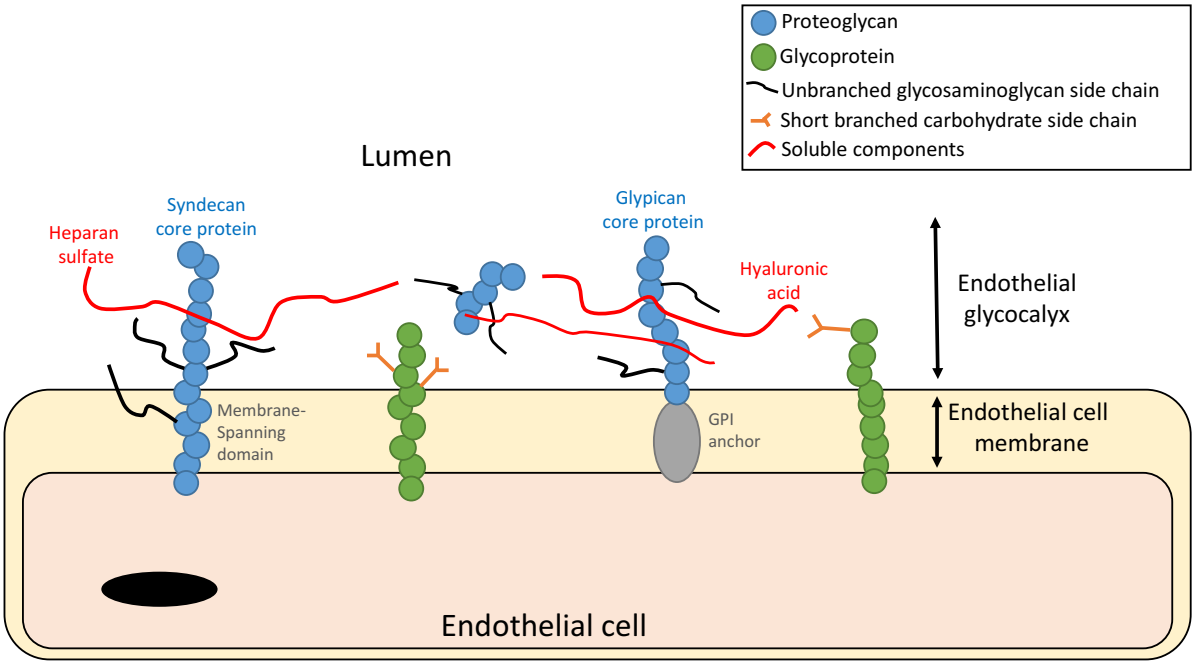
The endothelial glycocalyx is a dynamic mesh of molecules that form an exclusion zone between circulating cells and the luminal surface of the endothelium, with a thickness of approximately 2-3μm in small arteries[28] (Figure 1.2). The complex network of molecules includes some that are trans-membrane, acting as anchors for the glycocalyx to adhere to the endothelium (including proteoglycans and glycoproteins), as well as some soluble molecules that remain within the layer (including soluble glycosaminoglycans and proteoglycans) (Figure 1.3). The anchoring proteoglycans of the glycocalyx consist of core proteins with their linked glycosaminoglycan chains; the main core proteins are syndecan (4 subtypes), and glypican (6 subtypes), and the main glycosaminoglycan chains are heparan sulfate, chondroitin sulfate, keratan sulfate, dermatan sulfate, and hyaluronic acid[29].

Figure 1.2. Schematic diagram of a micro-vessel to illustrate the relationship between (A) endothelial cells, (B) the endothelial glycocalyx and the circulating contents (C)



Original illustration by Andrew Dakin, Medical Illustration Department, Queen Elizabeth Hospital

Figure 1.3. Schematic diagram of the endothelial glycocalyx, demonstrating some of the individual components of the layer.



Original illustration by the author.

The molecules that form the glycocalyx are derived from both circulating plasma components as well as the endothelial cells themselves, and there is a dynamic equilibrium between the soluble components and the circulation. The composition and thickness of the glycocalyx is affected by enzymatic action, shear forces, and contents of the circulation, so that its exact geometry and boundaries are not static, but instead represent a constantly shedding and re-assembling matrix of diverse components[29].

The endothelial glycocalyx has an important role in the mediation of vascular permeability, which may be determined by the structure, size, and charge of molecules within its layer[30]. Interactions between circulating cells and the

endothelium, as well as modulation of local rheology of the circulation are also influenced by the glycocalyx[29]. The delicate equilibrium within the glycocalyx may be lost by disruption to the normal haemostasis, leading to shedding without associated re-modelling, which may occur during critical illness, increasing vascular permeability[31]. The endothelial glycocalyx is shed following haemorrhagic shock, and restoration of this layer may be dependent on the type of fluid resuscitation, which will be discussed in Chapter 7. The importance of the stability and equilibrium of the endothelial glycocalyx has received recent attention in the study of both traumatic[32] and septic[33] shock, and this will be discussed in greater detail in the subsequent chapters in humans (Chapters 2 – 4), and animal models (Chapter 7).

1.2.2.3. Pericytes

Pericytes are a heterogeneous group of cells that wrap themselves around the endothelial layer, embedded within the basement membrane. Although their number and function varies throughout the microcirculation, their role is generally in the maintenance of homeostasis. They have an important role during angiogenesis[34]. They have regionally specific roles, such as forming part of the blood-brain barrier in the central nervous system, the blood-retinal barrier at the retina, and form an important part of the glomerular filtration system[35]. They may even have a region-dependent immune-regulatory role[36].

1.2.3. Innervation and regulation

The arterial inflow and venular outflow of the microcirculation are regulated through sympathetic innervation of the circular smooth muscle. Arterioles are the main site to

determine vascular resistance. The single cell-lined capillaries do not have a muscular layer, and therefore are not subject to neurological innervation. Instead they have their own regulatory mechanisms based on the shear stress of blood flow past the cellular surface. Endothelial cells modify their thickness and micro-vessel diameter based on localised signalling pathways and cell-cell interactions rather than neurological innervation[37]. Circulating blood components may also influence the behaviour of the endothelial cells. For example, red blood cells release adenosine triphosphate (ATP) and nitric oxide (NO) in the presence of hypoxia, causing localised vasodilatation[38, 39].

1.2.4. Flow dynamics

For a Newtonian fluid, flow through a passive tubular structure is ordinarily defined according to the Hagen–Pouiselle equation:

$$\boldsymbol{Q = \frac{\pi r^4 \Delta P}{8 \mu L}}$$

In this model, the most important physical features are the pressure gradient (ΔP), and the radius of the tube (r). Small changes in radius may have a large impact on flow. However, the passage of blood through the microcirculation is not quite as simple, since the micro-vessels are not passive, and blood is a non-Newtonian fluid, flowing through a branching vascular system[40]. Endothelial cells may swell, and the contents within the circulation vary in time and space. Additional factors such as the viscosity and shear stress (the force on the endothelium parallel to its surface) affect flow when there is interaction between blood and the endothelial surface. The magnitude of viscosity depends in a non-linear fashion on the haematocrit (volume fraction of red blood cells), and is proportional to the suspending

phase, plasma viscosity[41,42]. The apparent viscosity of the blood decreases markedly in micro-vessels with diameter <100µm due to the Fahraeus effect, whereby haematocrit decreases from the systemic values due to phase separation and central flow of the red cells; a minimum viscosity occurs in capillaries with diameter ~7µm where the red cells are aligned in a single column[43]. However, plasma viscosity continues to influence flow resistance regardless of haematocrit, and within the microcirculation the plasma viscosity may be particularly influential given the reduction in local haematocrit. In addition, the endothelial cells and the endothelial glycocalyx interact with circulating components, adding an additional 'active' factor of influences in flow resistance[44]. Flow dynamics within the microcirculation are therefore not only determined by physical factors (pressure, velocity, and diameter), but also by the characteristics of the circulation and vascular endothelium, and their influence on plasma viscosity and shear stress.

1.2.5. Regional variation in microcirculatory form and function

There are regional variations in the anatomy of the microcirculation in order to facilitate organ-specific functions. The most significant of these regional variations occur within major organs such as the lungs, heart, brain, gut and liver.

1.2.5.1. Lungs

Endothelial cells in the respiratory microcirculation are continuous. The unique configuration of surrounding microcirculatory networks to pulmonary alveoli facilitate the efficient interface between respiratory air at the alveolar epithelium and the circulation at the vascular endothelium. At this site oxygen diffuses into the micro-vessels and carbon dioxide diffuses out of the vessels. The relatively high

surface area of respiratory microcirculation in close proximity to airways allows for the additional functions of humidification and thermoregulation. These endothelial cells also have unique secretory functions, including the production of angiotensin-converting enzyme, ADPase and prostacyclins, and leukocyte-adhesion molecules in higher concentrations that the rest of the circulation[45].

1.2.5.2. Heart

Since the heart is a very metabolically active organ, its microcirculation is formed of a high concentration of continuous endothelial cells. There are many more endothelial than cardiomyocyte cells within the cardiac tissue[45,46], and there is at least one micro-vessel adjacent to every cardiomyocyte. Their interaction is essential for the control of rhythmicity, contractility and growth. The release of vasoactive and bioactive molecules causes vasoconstriction and dilatation, as well as controlling coagulation, growth and inflammatory factors[46]. The relationship between endothelial cells and cardiomyocytes is also essential for the development and repair of the heart[47]. Signalling is especially efficient due to small distances between these cells and large contact surface area.

1.2.5.3. Brain

The microcirculation in the brain is part of the blood-brain barrier, with tight junctions and adherens junctions between continuous endothelial cells, and surrounding pericytes and astrocytes that all help to reduce the permeability of the junction to larger molecules. The micro-vessels surrounding the choroid plexus are fenestrated, allowing the passage of larger molecules. Since the brain is a highly

metabolically active organ, the endothelial cells of its microcirculation have specialised glucose transporters on both apical and basal sides. There is some evidence to suggest that pericytes also regulate the microcirculatory flow[48].

1.2.5.4. Alimentary canal

The gut has a rich blood supply in order to provide for its metabolic requirements, as well as for its absorptive function. The microcirculation is fenestrated, and is densest at the site of the villi where the majority of absorption occurs, at the base of which there is diffuse arterio-venous shunting. Micro-vessels at the villi are in such close proximity that they allow for exchange of substances between themselves and the villi but also between each other.

1.2.5.5. Liver

The liver has microcirculatory vessels arising from both the hepatic artery, vein, and portal vein. These micro-vessels intermingle at the site of the hepatic sinusoids, which themselves consist of fenestrated and discontinuous endothelium without a basement membrane, in a radiating pattern that forms individual lobules. The sinusoids control the dynamic exchange between the microcirculation and the hepatocytes by modulating their size and shape due to factors such as flow or pressure. As well as the arterio-portal anastomosis and the vasa vasorum of the portal vein, there are two additional microcirculatory connections between the arterial and venous supply. These are the terminal arteriosus twigs (between hepatic arterioles and sinusoids) and the peribiliary plexus (between hepatic arterioles and portal venules around the bile ducts)[49].

1.3. Definitions

1.3.1. Trauma

This thesis discusses some of the mechanisms of microcirculatory dysfunction following trauma and haemorrhagic shock, and their clinical implications. For the purposes of the rest of the thesis, "trauma" is defined as the presence of an injury that causes direct damage to tissues, either by penetrating or blunt mechanisms. The trauma patients included in Chapters 2 – 5 have all triggered the hospital's "trauma alert" according to the pre-hospital Major Trauma Triage Tool that takes in to account the patients' vital signs and anatomical injuries. These patients have been assessed by pre-hospital personnel as having a Glasgow Coma Scale ≤13, systolic blood pressure (SBP) <90mmHg, or respiratory rate either <10 or >29 breaths per minute after being injured. Further anatomical considerations that may trigger such a "trauma alert" include penetrating injuries to the neck, head, torso, or proximal extremities, any chest deformities, the presence of 2 or more fractures, crushed or deformed extremities, proximal limb amputations, and pelvic or skull fractures.

1.3.2. Shock

"Shock" is defined as an inadequate perfusion of tissues relative to their requirements. In a clinical context, this is usually judged using clinical surrogate markers, such as SBP or mean arterial pressure (MAP), as well as arterial blood gas parameters such as base excess, lactate, and pO_2. "Haemorrhagic shock" is defined as the inadequate perfusion of tissues due to the loss of blood and subsequent hypovolaemic shock. In Chapters 2, 4, and 5, patients are defined as having haemorrhagic shock if they require blood product transfusion to restore tissue perfusion, have a plasma lactate >2mmol/l, and require transfer to the Intensive Care Unit for organ support. In Chapter 7, haemorrhagic

shock is defined for a number of different preclinical (animal) studies, and is expressed in terms of more precise volumes or percentages of blood lost, or according to measured pressures, and will be described in detail in that Chapter.

1.3.3. Microcirculatory dysfunction

The definition of "microcirculatory dysfunction" used in this thesis is the abnormal reduction in density of microcirculatory vessels, reduction in the flow of red cells through those micro-vessels, reduction in the proportion of vessels that are perfused, and an increase in heterogeneity of flow between vessels. These will be physically quantified using validated criteria in Chapters 2, 5, and 6, by using hand-held video-microscopy, as described later in this chapter. Pre-clinical studies using animal models have quantified microcirculatory dysfunction using other parameters such as red blood cell velocity, vessel diameter, functional capillary density, and shear rate, which are all reduced after haemorrhagic shock (as will be discussed in Chapter 7).

1.3.4. Endotheliopathy of trauma

"Endotheliopathy of trauma" will be defined as the activation and injury of the endothelial cells and shedding of the endothelial glycocalyx following trauma. This will be described in Chapters 2 – 4 according to the presence of circulating biomarkers of endothelial injury (thrombomodulin) or glycocalyx shedding (syndecan-1) significantly higher than the range that is present amongst healthy controls with the same demographic characteristics. These will be discussed later in this chapter. In Chapter 7, electron microscopic examination has been used for direct measurement of glycocalyx thickness for animal models of haemorrhagic shock.

1.4. Measuring microcirculatory dysfunction

1.4.1. Video-microscopy techniques

Intra-vital microscopy has been used for the visualisation of the microcirculation in pre-clinical studies of shock at various anatomical locations including the conjunctiva[50], pancreas[51], intestines and renal[52], hepatic[53], and muscle[54] tissues. The dorsal skin fold preparation has been a way of examining the microcirculation in awake hamsters[55,56]. Electron microscopy has been performed on histological samples from experimental studies to examine the microcirculation[57]. Other technologies such as laser speckle contrast imaging[58] and laser Doppler technologies[59] have also been used to visualise the microcirculation in pre-clinical studies. These imaging techniques all measure the physical parameters relating to the anatomical flow and density of the microcirculation rather than the physiological or metabolic parameters such as pO_2, lactate or base excess.

In human studies, techniques to visualise the microcirculation cannot usually depend on surgical implantation, but must instead utilise non-invasive or less-invasive technologies. Sublingual video-microscopy has been used in large animal models[60-62], and has been translated into clinical research due to its appealing non-invasive and easily accessible modality. The sublingual mucosa is supplied by the sublingual artery, which arises from the lingual artery, a branch of the external carotid artery within the carotid triangle. This suggests that the sublingual microcirculation may correspond to that within the central microcirculation, although there is currently no clinical evidence to confirm this. However, there is some evidence that sublingual microcirculatory flow reflects the flow in the splanchnic circulation, since the magnitude and time course of changes within the sublingual microcirculatory flow have been reported to reflect the same alterations within the gut[63]. These factors suggest that the sublingual microcirculation has an appealing

combination of anatomical suitability and accessibility for determining overall microcirculatory flow dynamics.

Earlier clinical investigations utilised sublingual orthogonal polarisation spectral dark-field technology, and this was soon followed by the side-stream dark field (SDF) and incident dark field camera (IDF) technology[64]. In Chapters 2, 5, and 6 of this thesis, I will describe the utilisation of an IDF device in the visualisation of human microcirculation of patients following traumatic haemorrhagic shock in greater detail. In Chapter 7, I will describe the wider breadth of techniques used in animal experiments.

1.4.2. Endothelial biomarkers

In addition to the physical flow in microcirculatory vessels, information about the state of the endothelium may be derived from the detection of circulating biomarkers. The general concept is that higher levels of biomarkers indicate pathological changes such as injury, damage or inappropriate activation of the endothelium. Molecules such as syndecan-1, thrombomodulin, sVE-cadherin, sE-selectin, Angiopoietin-2, soluble endothelial protein C receptor, and histone-complexed DNA fragments have all been proposed as markers of endothelial damage[65-67]. In Chapters 2 and 3 of this thesis I will present syndecan-1 as a biomarker of endothelial glycocalyx shedding, and thrombomodulin as a biomarker of endothelial cell injury. Syndecan-1 is one of the anchoring transmembrane proteoglycans of the glycocalyx, and thrombomodulin is a glycoprotein expressed on the endothelial surface. These will be described in more detail in the relevant chapters. Although not currently used within clinical practice, these biomarkers have potential prognostic value, since they may predict clinical outcomes; for example, increased concentrations of these biomarkers within the circulation following trauma may be associated with coagulopathy and mortality[65, 68-71].

Restoration of abnormal levels of biomarkers back to baseline has also been used to indicate a relative restoration of the endothelium following a given therapy in some experimental studies[57, 72].

1.5. Aims, hypotheses, and research questions

1.5.1. Aims

The aims of this thesis are: (a) to investigate the mechanisms of early microcirculatory dysfunction following traumatic haemorrhagic shock; and (b) to investigate the ways in which this microcirculatory dysfunction might be detected during the early emergency resuscitation of these patients, and how these data might be utilised. In order to achieve these aims, this thesis will address 12 research questions that are derived from 8 hypotheses, as listed in Table 1.1.

Table 1.1. Hypotheses and research questions

	Hypothesis	Research Question	Chapter
1	Microcirculatory flow is affected by the integrity of its endothelial lining	Are poorer microcirculatory flow dynamics associated with elevated biomarkers of endotheliopathy?	2
2	Endotheliopathy occurs very soon after injury, and is encountered during the on-scene or pre-hospital period	How soon after injury are biomarkers of endotheliopathy elevated?	3
3	Early endotheliopathy and failure to restore the endothelium are both associated with subsequent organ failure	Is early endothelial biomarker elevation associated with multiple organ dysfunction syndrome? Is the restoration of endothelial biomarker levels to "normal range" associated with better outcomes than persistently raised levels?	3
4	Tranexamic acid acts to restore the endothelium following trauma	Are biomarkers of endotheliopathy different for patients receiving pre-hospital tranexamic acid to those who did not, following trauma?	3
5	The release of cell-free DNA into the circulation after injury may be associated with endotheliopathy, and poorer outcomes	Are biomarkers of endotheliopathy correlated with levels of cell-free DNA? Are higher levels of cell-free DNA associated with poorer clinical outcomes?	4
6	Microcirculatory flow parameters can be measured early following injury, even for profoundly unwell trauma patients	Is sublingual microcirculatory video-microscopy both safe and feasible in the Emergency Department for trauma patients?	5
7	Trained professionals can assess the microcirculation at the bedside with as much accuracy as lengthy offline computer analysis	Can a microcirculatory score be assigned to patients based on a visual inspection only? Can this novel scoring system be taught to healthcare professionals? Does this scoring system correspond well to traditional offline computer analysis?	6
8	Whole blood is the optimal fluid for the restoration of the microcirculation following haemorrhagic shock	Which type of fluid is able to restore microcirculatory flow with the greatest efficacy in pre-clinical studies of haemorrhagic shock?	7

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Chapter 2

Poor microcirculatory flow dynamics are associated with endothelial cell damage and glycocalyx shedding after traumatic haemorrhagic shock

The following chapter is adapted from the published article:

Naumann DN, Hazeldine J, Midwinter MJ, Hutchings SD, Harrison P. Poor microcirculatory flow dynamics are associated with endothelial cell damage and glycocalyx shedding after traumatic hemorrhagic shock. *J Trauma Acute Care Surg*. 2018;84(1):81–88

2.1. Introduction

There has been considerable interest in the endotheliopathy of trauma, since it is associated with increased vascular permeability[1], is an independent predictor of mortality[2], and may be a key determinant in the development of inflammation and coagulopathy[3]. Endotheliopathy of trauma is distinct from normal physiological activation of the endothelium, since the endothelium is not only activated, but also becomes physically damaged, with shedding of the glycocalyx and disruption of tight junctions, with resultant capillary leakage[4]. In particular, endothelial glycocalyx shedding may have a role in microcirculatory dysfunction due to its essential role in the integrity and function of the oxygen exchange surface of all micro-vessels[5, 6]. Endotheliopathy of trauma is associated with sympathoadrenal activation[7, 8], and may be induced by the action of damage-associated molecular patterns (DAMPs) that are released into the circulation following tissue injury[9, 10], as well as raised levels of pro-inflammatory cytokines responsible for disruption of endothelial integrity[11]. Aside from the inflammatory and coagulopathic consequences of endotheliopathy, there may also be derangements in flow dynamics within the micro-vessels so that oxygen perfusion is inadequate, leading to hypoxia and acidosis. Prior to the current study, the relationship between endotheliopathy and microcirculatory flow dynamics following traumatic haemorrhagic shock (THS) has yet to be investigated.

Away from the patient, enzyme-linked immunosorbent assays (ELISA) can be performed for surrogate markers of endotheliopathy, such as thrombomodulin (CD141) and syndecan-1 (CD138). Thrombomodulin is a transmembrane glycoprotein in endothelial cells that acts as a cofactor during activation of Protein C by thrombin[7, 12]. It is not normally secreted by endothelial cells, but is present in the circulation due to endothelial cell damage[13]. This makes it a useful biomarker of endothelial injury, and has been used in this

role for patients following sepsis[7, 14], myocardial infarction[15], cardiac arrest[16], and trauma[2, 8, 17]. Syndecan-1 is a transmembrane heparan sulfate proteoglycan whose extracellular domain forms part of the endothelial glycocalyx. It has been used as a biomarker for glycocalyx shedding for patients following trauma[2, 11, 18].

At the bedside, non-invasive video-microscopy can be used to visualise the flow of red blood cells in the sublingual microcirculation. The latest Incident Dark Field (IDF) technology allows for the acquisition of high definition views of the flow and perfusion of these micro-vessels whose primary function is oxygen and substrate exchange. This technique has been used to examine the response of the microcirculation to critical illness, sepsis and shock[19]. More recently, investigators have demonstrated a prognostic value in this technology for haemorrhagic trauma[20], in particular in the detection of loss of haemodynamic coherence[21]. There have been no clinical studies that have tested whether glycocalyx shedding and endothelial cell damage are associated with poorer flow dynamics in the microcirculation.

The aim of the current study was to test whether endotheliopathy following traumatic haemorrhagic shock was associated with physical changes in the flow, density and perfusion of vessels in the microcirculation, from initial resuscitation to stabilisation in the Intensive Care Unit (ICU). It was hypothesised that there would be an association between endotheliopathy and poor flow dynamics of the microcirculation (Hypothesis 1; Table 1.1).

2.2. Methods

2.2.1. Study design

A prospective longitudinal observational study was conducted in order to compare flow dynamic parameters of the microcirculation with serum markers of endothelial damage and glycocalyx shedding. All data are from a single site in the MICROSHOCK study[22]. This study had been granted prior ethical approval (Research Ethics Committee reference: 14/YH/0078; Yorkshire and the Humber Leeds West), and a protocol was published in advance[22].

2.2.2. Patient selection

Patients who were potentially eligible for inclusion at a single Major Trauma Centre (University Hospitals Birmingham, UK) were identified through the screening of trauma team activations. Trauma patients were eligible for inclusion if they required blood products, had been intubated, required admission to ICU, and had a point-of-care lactate of greater than 2mmol/l. They were enrolled into the study either in the Emergency Department (ED) or ICU depending on suitability for study observations. Patients were excluded if they were under the age of 16, prisoners, or had extensive facial injuries that would make access to the sublingual area problematic. Patients that were expected to die due to the extensive nature of their injuries, and were being treated palliatively, were also excluded. Selection bias was minimised by ensuring that there were no significant differences between patients enrolled in the study and those that were eligible but not included.

2.2.3. Capacity and consent

All patients were expected to lack capacity to consent for study participation at the time of study enrolment due to the nature of their injuries, and a requirement for recruitment as soon as possible after arrival in the hospital. Study subjects were therefore enrolled under the guidance of the Mental Health Act 2005 and the Declaration of Helsinki. Approval for subjects to participate in this study was obtained from the physician in charge of the care of the patient (designated as the "Professional Consultee"), who was not a study investigator. If appropriate, agreement for study participation could also be requested after discussion with a member of the patient's family, or a close friend (designated as a "Personal Consultee"). Ultimately, if the patient regained capacity after data had already been obtained, the study was discussed with them and they were asked for consent for all data to be retained. If they did not regain capacity, then the previous permissions from their Professional or Personal Consultee remained extant.

2.2.4. Data collection

Patient demographics (age, gender), injury details (including mechanism and timings), physiological parameters (heart rate and systolic blood pressure), and Glasgow Coma Scale (GCS) were recorded prospectively, and then corroborated using a combination of electronic and paper medical records. The most recent point-of-care lactate reading to each time point was also recorded, as well as the number and type of blood products and volumes of crystalloids used during the study period. Injury Severity Scores (ISS) were obtained from the Trauma Audit and Research Network, a central validated resource in the UK. Outcomes recorded for patients included ICU-free and hospital-free days (calculated by recording the number of days that the patient was present in ICU and hospital respectively

during a period of 30 days), mortality within 30 days, and Sequential Organ Failure Assessment (SOFA) scores at days 3, 6, and 10.

2.2.5. Sublingual video-microscopy

A single operator (D.N.N.) conducted IDF video-microscopy in order to acquire non-invasive video clips of the sublingual microcirculation (Cytocam, Braedius Medical B.V., Huizen, The Netherlands). Briefly, the camera was placed gently under the tongue until a clear view of the mucosal microcirculation was acquired, without blood or saliva artefact, with minimal pressure, and optimal focus and illumination according to consensus quality requirements for this technology[23]. Multiple clips of 100 frames each were recorded and stored on a computer for analysis. At least 5 good quality clips were recorded at each time point in order to facilitate optimal analysis[24].

2.2.6. Microcirculatory analysis

Videos that had been acquired by IDF video-microscopy were exported for analysis and were individually graded for quality according to the most commonly reported domains (illumination, focus, content, duration, pressure, and stability)[25]. Of the highest quality clips, 3-5 from each patient time point were kept for analysis. These clips were allocated random numbers to ensure that their analysis was blinded to patient, time point, and clinical status. Semi-automated analysis was undertaken for each clip using dedicated computer software (Automated Vascular Analysis V.3.2, Microvision Medical, The Netherlands). All videos were then un-blinded, and the time points assigned average values for total vessel density (TVD, mm/mm^2), perfused vessel density (PVD, mm/mm^2), proportion of perfused vessels (PPV, %), microcirculatory flow index (MFI), and microcirculatory heterogeneity index (MHI)

according to consensus guidelines for reporting sublingual microcirculation[24]. In addition, point-of-care microcirculation (POEM) scores (a composite score for flow and heterogeneity)[26] were allocated for each time point. The methodology for the allocation of POEM scores will be described in greater detail in Chapter 6 of this thesis. In summary, flow is defined as "normal", "impaired", or "critical" for a video clip if <25%, 25–50%, or >50% of the vessels in the visual field respectively are sluggish or stopped. In addition, heterogeneity is either "present" or "absent" if >5 or <5 vessel segments have different flow to the remainder. This is repeated 4 times, and an algorithm calculates the overall score based on the majority of responses. A score is allocated from 1 (worst) to 5 (best).

2.2.7. Serum sampling

At the same time point at which video-microscopy was performed, a 6 ml sample of peripheral blood was taken from an available venous access point. After a 30-minute incubation at room temperature, samples were centrifuged at 1,620 x g for 10 minutes at 4^{o}C, after which aliquots of serum were stored in cryotubes at -80^{o}C. Serum samples were collected and stored in the same manner for 17 healthy volunteers, matched for age and sex to the patient cohort.

2.2.8. Enzyme-linked immunosorbent assays

Commercially available ELISAs were used according to their relevant protocols for the quantitative measurement of serum concentrations of syndecan-1 and thrombomodulin. Syndecan-1 (CD 138; Abcam; product code ab46506, Cambridge, MA) was used as a surrogate marker of glycocalyx shedding, because it is an anchoring proteoglycan within the endothelial glycocalyx, and its presence within the circulation suggests

breakdown of the normal architecture of the glycocalyx. Thrombomodulin (CD 141; Abcam; product code ab46508, Cambridge, MA) was used as a surrogate marker of endothelial cell damage, since it is soluble and present within the circulation following direct injury to endothelial cells[13]. Although glycocalyx shedding and endothelial cell injury are unlikely to be mutually exclusive, these biomarkers have been reported in these terms in previous investigations of endotheliopathy[5-8]. For the thrombomodulin assay, the intra- and inter-assay coefficients of variation were reported as 3.9% and 9.8% by the manufacturer. For the syndecan-1 assay, these were 6.2% and 10.2% respectively.

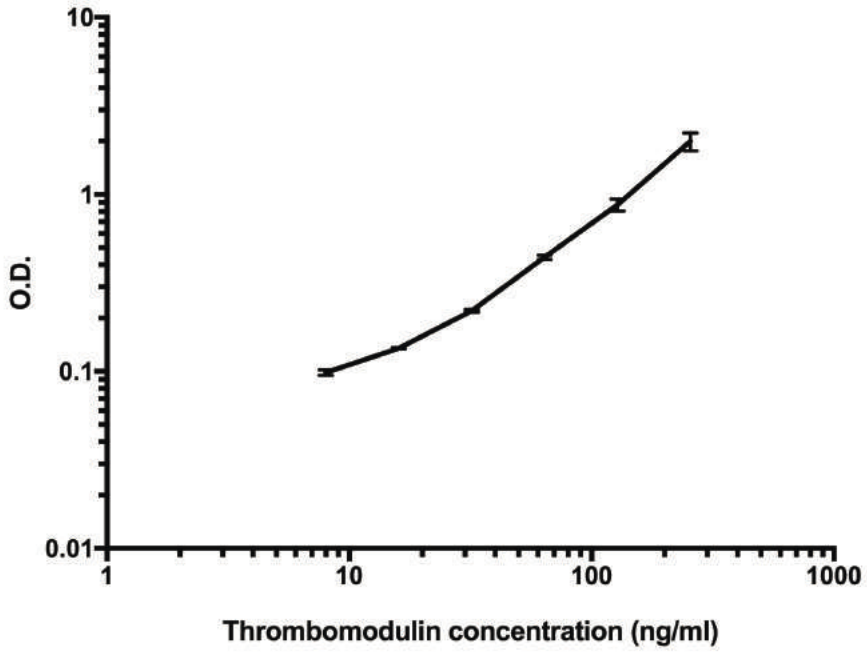
For each ELISA, a 96-well plate was used, containing wells that had been coated with monoclonal antibodies by the manufacturer for the biomarker of interest. Standards were prepared according to the manufacturer's protocol, by serial dilution using the standard diluent buffer. For the thrombomodulin ELISA, the standard concentrations were 20ng/ml, 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, and 0.625ng/ml. For the syndecan-1 ELISA, the standard concentrations were 256ng/ml, 128ng/ml, 64ng/ml, 32ng/ml, 16ng/ml, and 8ng/ml. These were prepared in duplicate. Blank controls were used for all ELISAs. Control solutions were provided by the manufacturer.

For the assays, 100μl was added to wells for each standard, blank controls, control solutions, and patient samples. Then 50μl of 1X Biotinylated anti-syndecan-1 or anti-thrombomodulin was added to all wells for the syndecan-1 and thrombomodulin assays respectively. The plates were then covered and incubated for 1 hour at room temperature (18-25^{o}C). After this period, the plates were washed three times according to the manufacturer's protocol, using 300μl of 1X Wash Buffer. Then 100μl of Streptavidin-HRP solution was added to all wells, and the plates were incubated again for 30 minutes. After this period, the plates were washed in the same manner as previously, and then 100μl of

Chromogen TMB substrate solution was added to each well. After incubation in the dark for 15 minutes, 100µl of Stop Reagent was added to each well. Plates were then immediately taken to the spectrophotometer, where readings were taken at 450nm as the primary wavelength, and 620nm as a reference.

The absorbance was calculated for each well by subtracting the optical density of the average of blank controls. The standard curve was then calculated using the mean absorbance for each duplicate standard concentration, and a graph plotted with standard concentration on the x-axis and absorbance on the y-axis (Figure 2.1). The concentration of syndecan-1 or thrombomodulin in each sample well was then interpolated from the standard curve. This was performed using GraphPad Prism version 7.0 (GraphPad Software, California, USA). The concentrations of the control wells were checked against the expected concentrations in order to ensure that the assay had been performed correctly.

Figure 2.1. Example of a standard curve derived from thrombomodulin concentration



2.2.9. Data analysis

Continuous data were tested for normality using the Shapiro-Wilk test. Normal data are presented as mean and standard deviation (SD), and non-normal data are presented as median and interquartile range (IQR). Spearman's rank correlation coefficient was used to determine the correlation between thrombomodulin and syndecan-1 concentrations for all patients, as well as the correlation between each of the biomarker concentrations and flow dynamics with lactate. Patient time points were calculated from time of injury, and were divided into three groups: (i) <10 hours; (ii) 10 – 30 hours; and (iii) 30 – 50 hours since injury.

It is well established in previous studies of microcirculatory flow dynamics that healthy volunteers score near-maximum values of the traditional parameters of choice (such as PPV close to 100%, MFI equal to or close to 3.0, and heterogeneity near to 0)[27-29]. Comparison of flow dynamics between healthy controls and critically unwell patients is therefore unlikely to yield surprising or meaningful results. Of greater interest would be the difference between patients within the critically unwell cohort. In order to test the association of flow dynamics and endothelial cell damage and glycocalyx shedding amongst the injured cohort in the current study, flow dynamic parameters were dichotomised above and below the average value at each time point (the median for non-normal data and the mean for normal data) in a manner similar to that previously reported for microcirculatory parameters[30]. The “worse” group were considered to have poor microcirculatory flow dynamics. Concentrations of syndecan-1 and thrombomodulin were compared between these “above” and “below” average groups. Multiple pairwise comparisons of biomarkers between patients and healthy controls were made using Dunn’s multiple comparisons tests. Comparison of flow dynamics between time points was made using the Skillings-Mack test for repeated measures of non-normal data that allows for some missing values. A *p*-value of

< 0.05 was considered statistically significant. Box and whisker graphs are presented according to the Tukey method, with horizontal bars as the median value, boxes as the interquartile range, and the upper and lower whiskers being 1.5 times the interquartile range above and below the 75th and 25th percentile respectively.

2.3. Results

2.3.1. Patient characteristics

Analysis was performed for 155 sublingual video-microscopy clips that corresponded to 39 time points from 17 trauma patients. Six patients had data from all three time points, 10 had data from two time points, and one patient died after the first time point. Patient characteristics are shown in Table 2.1.

2.3.2. Endothelial cell damage and glycocalyx shedding

When pairs of thrombomodulin and syndecan-1 concentrations for all healthy controls and patients were compared, there was a significant correlation between them (r = 0.634; p < 0.001) (Figure 2.2), indicating an association between glycocalyx shedding and endothelial cell damage.

2.3.3. Endotheliopathy, flow disruption and perfusion

Thrombomodulin and syndecan-1 were both significantly correlated with lactate concentrations (Figure 2.3) (r = 0.331; p < 0.05; and r = 0.440; p < 0.01 respectively), suggesting that endotheliopathy is associated with perfusion mismatch. When flow dynamics were compared to lactate concentrations, there were significant correlations with PVD (r = -0.416; p < 0.01); PPV (r = -0.440; p < 0.01); MFI (r = -0.464; p < 0.01); MHI (r =

0.363; $p < 0.05$); and POEM score ($r = -0.440$; $p < 0.01$). There was no significant correlation with TVD ($r = -0.312$; $p = 0.053$).

Table 2.1. Study patient characteristics

Patient characteristic	All
Age, years	35 (25–52)
Sex, male:female	16:1
Mechanism of injury	
Road traffic accident	10 (59)
Stabbing	4 (24)
Fall	2 (11)
Crush	1 (6)
Injury Severity Score	27 (23–34)
Physiological parameters on arrival	
Heart rate, min^{-1}	108 (98–118)
Systolic blood pressure, mmHg	91 (61–108)
Glasgow Coma Scale	9 (3–14)
Plasma lactate, mmol/l	6.0 (3.6–10.2)
Blood products required in ED	
Packed red cells, units	3 (2–4)
Fresh frozen plasma, units	2 (0–4)
Crystalloid fluid, ml	
Injury to T1	1000 (500 – 1475)
Between T1 and T2	3000 (2000 – 4000)
Between T2 and T3	3000 (1250 – 4000)
Outcomes	
ICU-free days	11 (0–19)
Hospital-free days	0 (0–5)
SOFA score, day 3	6 (8 – 9)
SOFA score, day 6	4 (3 – 6)
SOFA score, day 10	3 (3 – 4)
30 day mortality	3 (18)

Summary data are presented as median and interquartile range in parentheses; categorical data are reported as N (%). ICU- and Hospital-free days are calculated for a 30-day period.
ED: Emergency Department; ICU: Intensive Care Unit; SOFA: Sequential Organ Failure Assessment
T1: first time point (<10h); T2: second time point (10-30h); T3: third time point (30-50h)

Figure 2.2. The relationship between thrombomodulin and syndecan-1 concentrations for all patients and healthy controls.

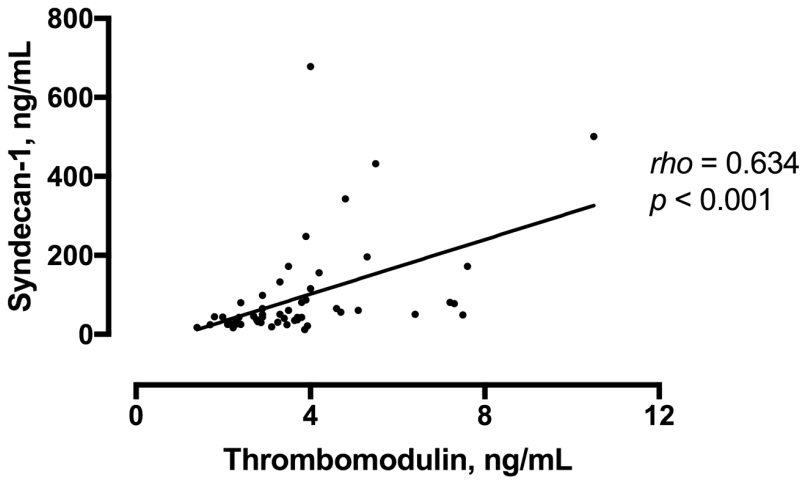
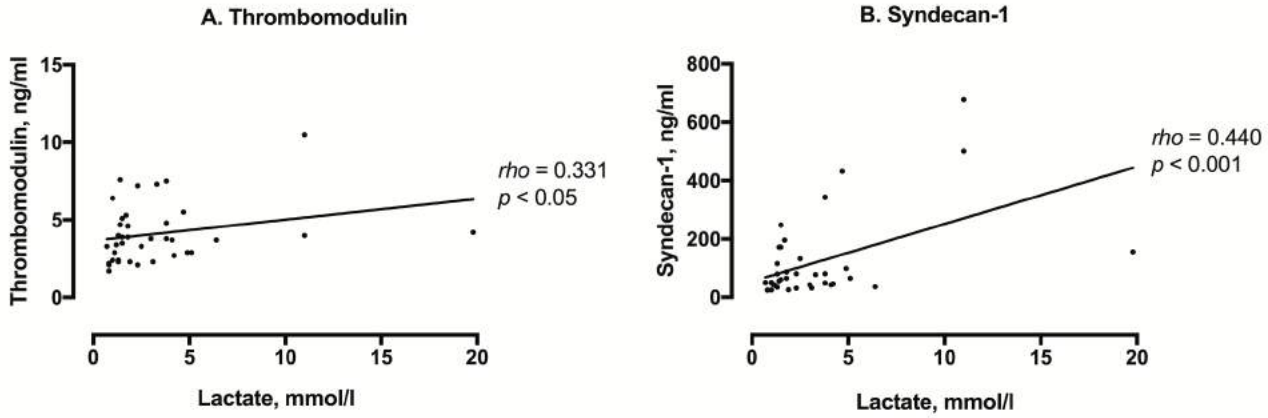


Figure 2.3. The relationship between (a) thrombomodulin and (b) syndecan-1 concentrations and lactate for all patients.

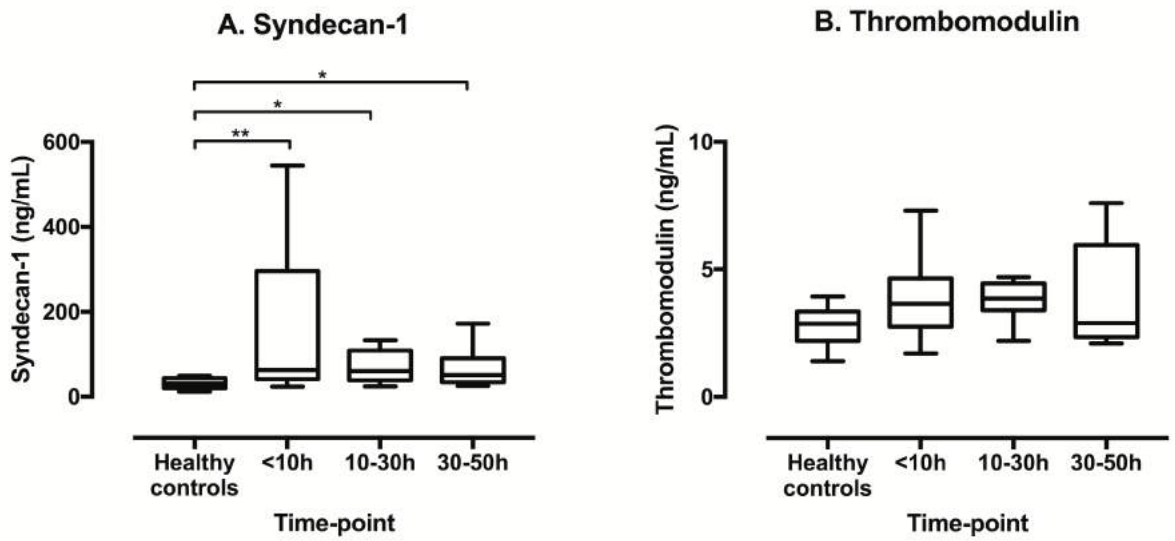


2.3.4. Glycocalyx shedding and endothelial cell damage over time

Figure 2.4 illustrates the concentrations of syndecan-1 and thrombomodulin for all patients over time. When syndecan-1 concentrations were compared with healthy controls (30 (IQR 20 – 44) ng/ml), they were significantly higher at the 10h (63 (IQR 41 – 296) ng/ml), 10-30h (61 (IQR 38 – 109) ng/ml) and 30-50h (51 (IQR 34 – 90) ng/ml) time points. In contrast, there were no significant differences between thrombomodulin concentrations for healthy controls (2.9 (IQR 2.2 – 3.4) ng/ml) and patients at the <10h (3.7 (IQR 2.8 – 4.7) ng/mL), 10-30h (3.9 (IQR 3.4 – 4.5) ng/ml), or 30-50h (2.9 (IQR 2.4 – 6.0) ng/ml) time points.

Figure 2.4. Concentrations of (a) syndecan-1 and (b) thrombomodulin over the study time points for all patients compared to healthy controls (HCs).

**p* < 0.05; ***p* < 0.01 *vs.* HCs using Dunn's multiple comparisons test

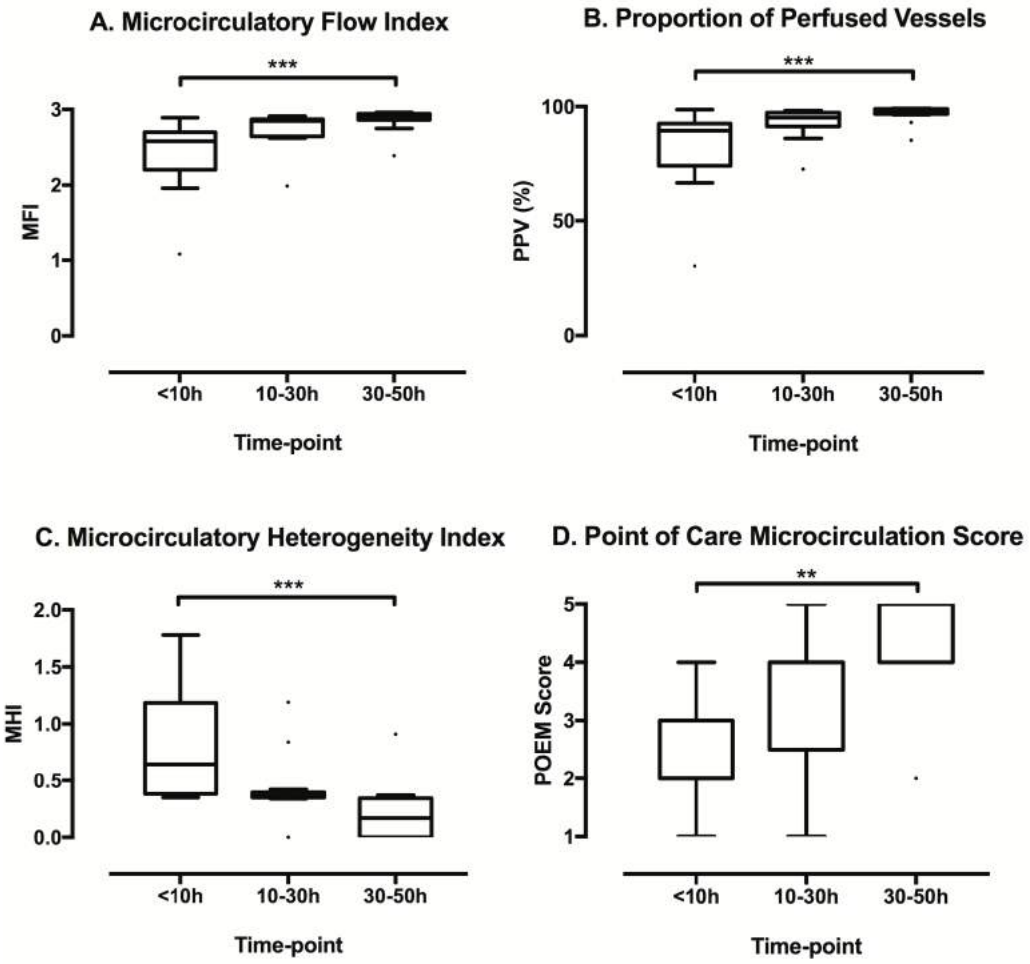


2.3.5. Flow parameters improve over time

Flow dynamics for all three time points are illustrated in Figure 2.5. There was a significant improvement over time for measures of flow (MFI; $p < 0.001$), perfusion (PPV; $p < 0.001$), heterogeneity (MHI; $p < 0.001$), and combined flow and heterogeneity (POEM score; $p < 0.01$). There were no significant differences over time for measures of total or perfused vessel density (TVD ($p = 0.276$) and PVD ($p = 0.389$) respectively).

Figure 2.5. Flow dynamics for all patients over time, including (a) Microcirculatory Flow Index; (b) Proportion of Perfused Vessels; (c) Microcirculatory Heterogeneity Index; and (d) Point-of-Care Microcirculation score.

**$p < 0.01$; ***$p < 0.001$ using the Skillings-Mack test



2.3.6. Glycocalyx shedding and flow dynamics

Figure 2.6 illustrates the concentrations of syndecan-1 at each time point relative to flow dynamic parameters. Higher heterogeneity of flow (MHI), as well as worse perfusion (PPV) and flow (MFI) were associated with glycocalyx shedding at all three time points. The most significant differences were observed within 10h of injury; at this time point, syndecan-1 concentrations were significantly higher than healthy controls (30 (IQR 20 – 44) ng/ml) when there was worse TVD (78 (IQR 63 – 417) ng/ml); PVD (156 (IQR 63 – 590) ng/ml); PPV (249 (IQR 64 – 578) ng/ml); MFI (249 (IQR 64 – 578) ng/ml); MHI (45 (IQR 38 – 68) ng/ml); and POEM scores (108 (IQR 44 – 462) ng/ml) (all $p < 0.01$). Below average TVD and PVD were associated with glycocalyx shedding at <10h and 10-30h (all $p < 0.05$), but not at the 30-50h time points.

2.3.7. Endothelial cell damage and flow dynamics

Figure 2.7 illustrates the concentration of thrombomodulin at each time point relative to flow dynamic parameters. In contrast with syndecan-1, thrombomodulin was only raised within 10 hours of injury when compared to healthy controls (2.9 (IQR 2.2 – 3.4) ng/ml) for worse PPV (4.1 (IQR 3.4 – 6.2) ng/ml) and MFI (4.1 (IQR 3.4 – 6.2) ng/ml) (both $p < 0.05$). Below average density (TVD) and functional density (PVD) were associated with endothelial damage only at the 10-30h time point (3.7 (IQR 3.3 – 3.9) ng/ml and 3.7 (IQR 3.3 – 3.9) ng/ml respectively), but not at the <10h or 30-50h time points. No associations were found between endothelial cell damage and heterogeneity of flow (MHI) or POEM scores (*p* values all non-significant).

Figure 2.6. Concentrations of syndecan-1 at each time point, dichotomised to "above" and "below" the average values of (a) Microcirculatory Heterogeneity Index; (b) Proportion of Perfused Vessels; and (c) Point-of-Care Microcirculation score; and (d) Microcirculatory Flow Index compared to healthy controls (HCs).

**p* < 0.05, ***p* < 0.01; ****p* < 0.001 *vs.* HCs using Dunn's multiple comparisons test

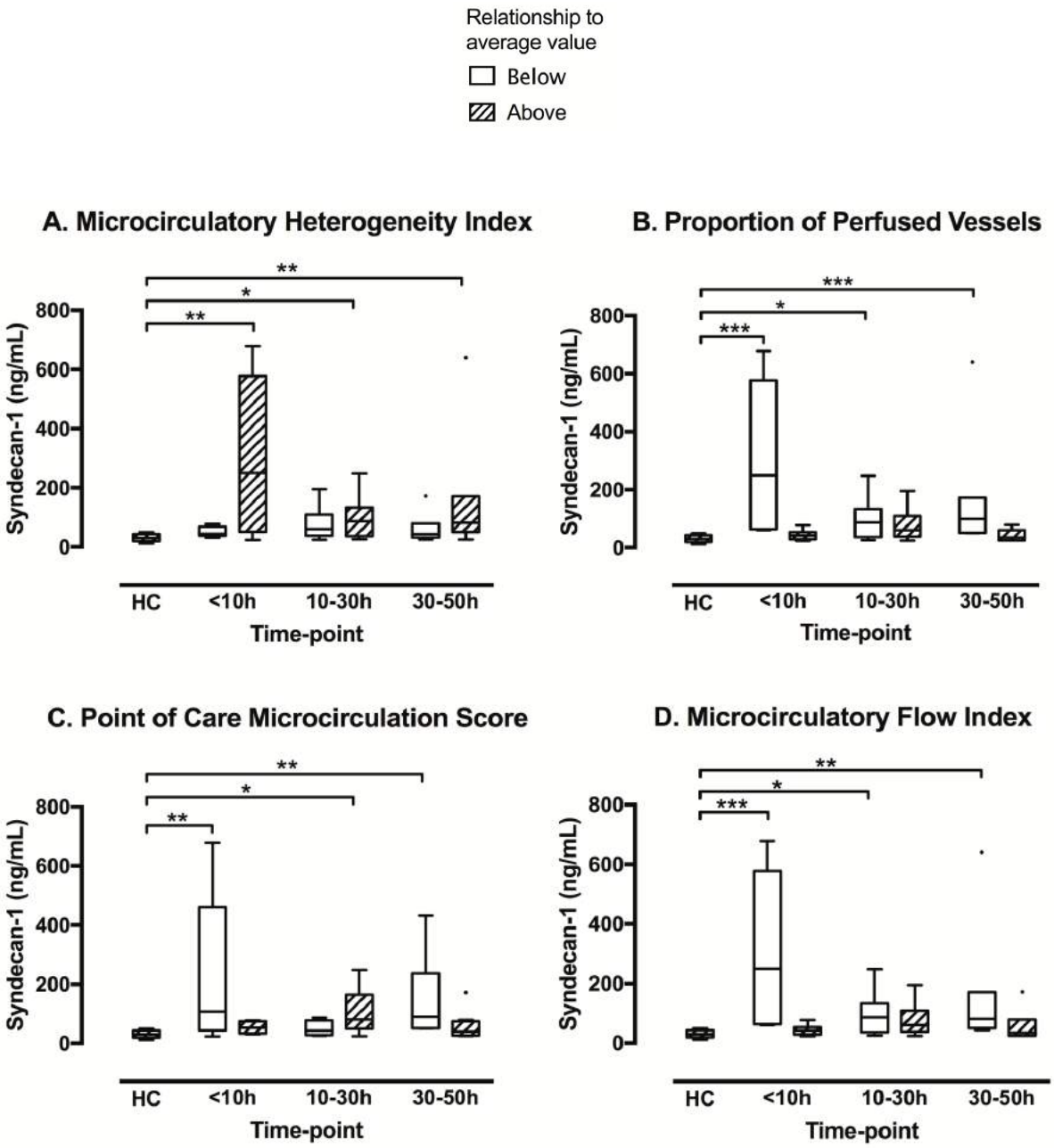
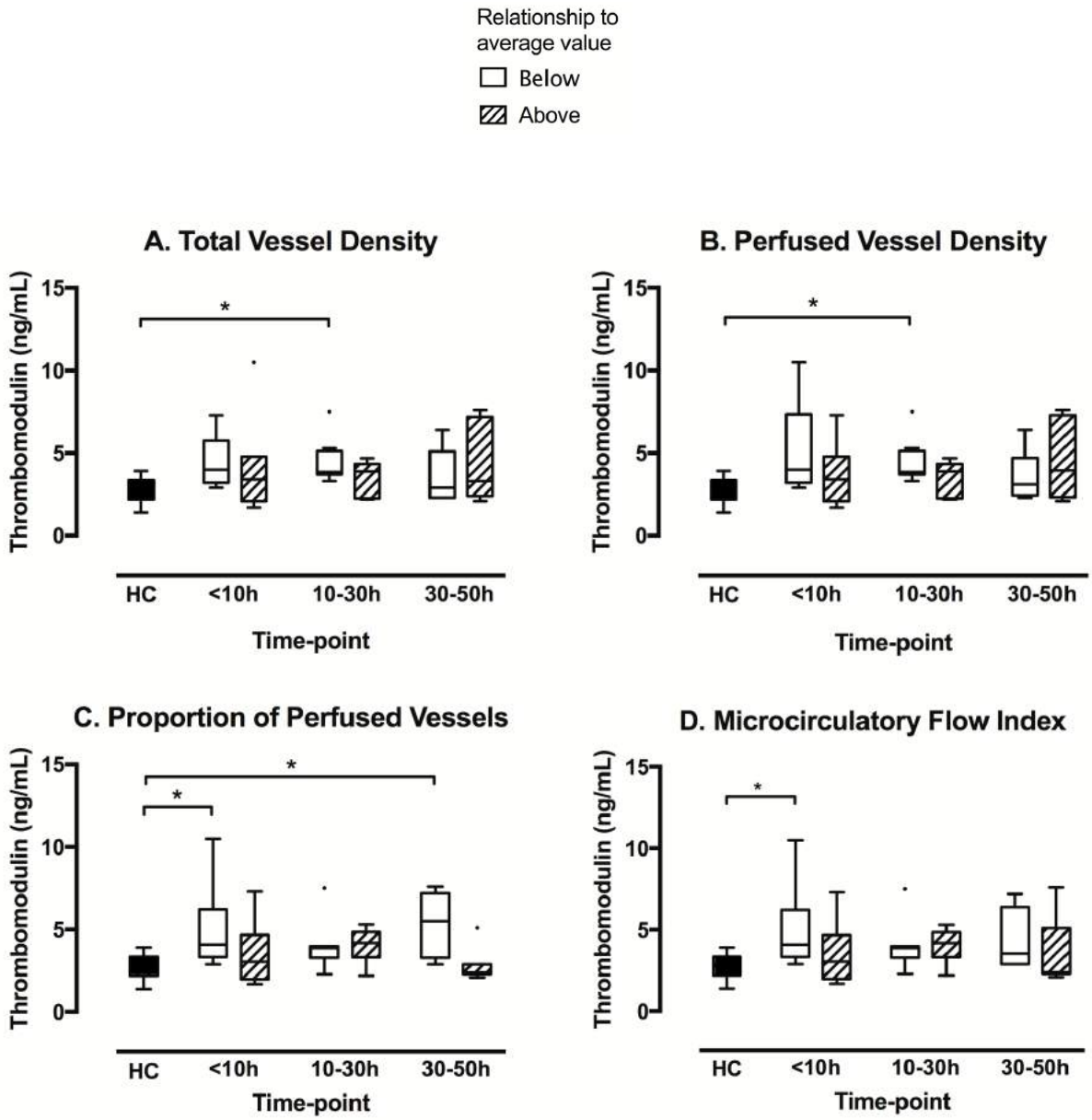


Figure 2.7. Concentrations of thrombomodulin at each time point, dichotomised to "above" and "below" the average values of (a) Total Vessel Density; (b) Perfused Vessel Density; (c) Proportion of Perfused Vessels; and (d) Microcirculatory Flow Index compared to healthy controls (HCs).

*$p < 0.05$ *vs.* HCs using Dunn's multiple comparisons test



2.4. Discussion

The main finding from this study is that there is an association between endotheliopathy and impaired microcirculatory perfusion following traumatic haemorrhagic shock, the effects of which were most striking within 10 hours of injury. The correlation between plasma lactate readings and both endothelial biomarkers and microcirculatory flow dynamics may suggest a concurrent oxygen perfusion deficit associated with endotheliopathy and microcirculatory flow disruption, although these correlations were not strong. Deterioration in flow may represent one of the mechanisms of microcirculatory dysfunction attributable to endotheliopathy, and poor flow dynamics following shock may be responsible for worsening endotheliopathy. Although the current study does not attribute causality, it is likely that poor flow, glycocalyx shedding and endothelial cell disruption are mutually detrimental, and together may contribute to derangement in oxygen exchange, inflammatory dysregulation and coagulopathy following trauma and haemorrhagic shock. In terms of the exact sequence of events, further investigations are required to determine whether it is the endotheliopathy that causes flow disruption due to alterations in vessel diameter, viscosity, and shear forces, or whether it is reduced flow that causes endotheliopathy, perhaps due to tissue ischaemia and sympathoadrenal activation[7,8].

Other investigators have reported an association between injury severity, catecholamine-driven sympathoadrenal response, hypocoagulability, and raised levels of syndecan-1 and thrombomodulin as markers of glycocalyx shedding and endothelial cell injury[2, 31]. Trauma-induced coagulopathy has been observed in patients with glycocalyx shedding, and it has been postulated that endogenous auto-heparinisation may be responsible for this[32], as well as the generation of thrombin and activation of protein C[33].

The term "shock-induced endotheliopathy" (SHINE) has been used to describe the same phenotype observed in several different critical illnesses characterised by shock[4]. These observed effects of endotheliopathy may be considered in the context of the microcirculatory flow disruption observed in the current study, which are likely to exacerbate the pathologic process by reducing the efficient flow of red cells and oxygen delivery to tissues.

It is notable that most of the flow parameters showed dramatic improvement over the three time points (Figure 2.5), and yet the glycocalyx does not appear to have been restored to normality according to the levels of syndecan-1 (Figure 2.4). Although the median levels of syndecan-1 improved over time, they were still raised above that of healthy controls, a finding in keeping with a previous study[11]. Restoration of flow parameters may be a reflection of a pressure-based resuscitation strategy and goal-directed therapy aimed at restoring oxygen perfusion and reducing acidosis, rather than any therapy deliberately targeted at repairing the endothelium. The exact consequences of incomplete endothelial restoration are unknown, but it seems likely that any inflammatory and coagulopathic processes caused by the injured endothelium would continue to have an effect on subsequent clinical progression until the endothelium was returned to its normal state. Furthermore, it is unknown what degree of "useful" endothelial activation following trauma there may be. It is a possibility that there is a level of endothelial activation that might cause levels of syndecan-1 and thrombomodulin to increase significantly above those of healthy controls, but still not be pathological. Others have recently proposed threshold values for syndecan-1 above which there was a significant association with mortality[34], but further investigations of the precise role of endothelial activation following trauma are justified.

The flow parameters observed in this study included those used for research but also a point-of-care tool that may be used at the bedside (which will be described in greater detail in Chapter 6)[26]. Other investigators have also proposed bedside utilisation of more traditional research parameters[35, 36]. Sublingual video-microscopy is safe to be performed for patients following injury, even in the ED for haemodynamically unstable patients (as will be discussed in greater detail in Chapter 5)[37]. If conducted in real time, these parameters may be able to aid in the identification of patients likely to suffer from ongoing consequences of endotheliopathy by directly visualising the dynamic changes from initial resuscitation through to ICU. Since endotheliopathy may predict poor patient outcome[2, 14], this information is of potential value in the clinical context.

A recent experimental swine model of trauma and haemorrhagic shock showed that even with identical injury and haemorrhage, there was a wide variation in microcirculatory dysfunction between animals from the outset[30], confirming the findings of an earlier small animal model[38]. The level of microcirculatory dysfunction was not predictable based on injury-specific details, but instead may be subject to genetic susceptibility. The same may also be true for injured humans; for example, age is associated with a different biomarker profile related to circulating inflammatory and sympathoadrenal mediators[39]. Diagnostic techniques may be desirable if they can distinguish between patients at risk of endotheliopathy and those who are not. In the current study there was a wide variation in biomarker levels and flow dynamics parameters, with some overlap in values between healthy volunteers and injured patients. Furthermore, although statistically significant, the correlations between the lactate and the study parameters of interest are not perfect. In addition, there may be time lag effects between these events. These are findings that might be anticipated in a trauma population due to the heterogeneity of injury patterns and

severity along with differences in genetic susceptibility between patients. Biomarkers and flow dynamics alone may not be sensitive or specific enough to determine clinical decisions during resuscitation, but may have a role in the overall assessment of patients in the context of their other physiological and biochemical parameters. This may be especially relevant if endothelial and microcirculatory behaviour is subject to genetic variation between individuals in the presence of the same injury patterns.

The current management strategy for traumatic haemorrhagic shock involves an early damage control resuscitation (DCR) phase that begins during the pre-hospital evacuation of the casualty, followed by fluid resuscitation with empirical ratios of packed red cells, plasma, and platelets. Following empirical resuscitation, a more bespoke, individualised approach to ongoing resuscitation is favourable, using additional diagnostic modalities such as thromboelastography[40]. Currently there is no point-of-care test for endotheliopathy of trauma, but it appears that sublingual video-microscopy has the potential to provide rapid point-of-care information relating to this important pathology during individualised resuscitation. Previous investigators have described a restoration of the endothelial glycocalyx with plasma-based fluid resuscitation[41-44], and the ideal resuscitation fluid for restoring microcirculatory flow dynamics should act to restore the endothelial glycocalyx (as will be described in greater detail in Chapter 7)[45]. Whether additional microcirculatory data might direct fluid resuscitation towards a plasma-based strategy in the clinical situation is yet to be determined, and further clinical investigation of this is warranted.

2.4.1. Limitations

This study included a relatively small number of patients, with all of the associated statistical limits. Analysis was performed on an individual time point (rather than individual patient) level in order to increase the number of data points to address our research question. The patients included in the current study were profoundly unwell, with high injury severity scores and poor physiological parameters on admission. The data from the current study may not necessarily be translatable for patients with less severe injuries and physiological burden. Sublingual video-microscopy is not currently in widespread clinical use, and the utility of these data in the clinical context are yet to be examined in prospective clinical trials.

All patients in this cohort received crystalloid fluids during their pre-hospital and in-hospital resuscitation, and it is not known to what extent these may have affected the microcirculatory flow. It is also unknown whether the delivery of crystalloid fluids may have an influence on the concentrations of biomarkers (such as a dilutional affect). For example, it is not known whether a reduction in biomarker level to normal might be due to haemodilution or a genuine restoration of the endothelium. Further investigations of microcirculatory flow in relation to the volumes and types of fluids in a larger number of patients would be warranted.

2.5. Conclusion

There is an association between endotheliopathy (including endothelial cell damage and glycocalyx shedding) and alterations in flow, density, functional density, proportion of perfused vessels, and micro-vessel heterogeneity. This microcirculatory dysfunction may explain in part some of the mechanisms of the oxygen perfusion deficit that is attributable

to endotheliopathy. The clinical utility of these flow parameters at the bedside is yet to be elucidated.

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Chapter 3

Endotheliopathy of trauma is an on-scene phenomenon, and is associated with poor clinical outcomes

The following chapter is adapted from the published article:

Naumann DN, Hazeldine J, Davies DJ, Bishop J, Midwinter MJ, Belli A, Harrison P, Lord JM. Endotheliopathy of trauma is an on-scene phenomenon, and is associated with multiple organ dysfunction syndrome: a prospective observational study. *Shock*. 2018;49(4):420-428.

3.1. Introduction

In the previous chapter, we discussed the relationship between endotheliopathy of trauma and microcirculatory flow disruption. These pathological processes are associated with each other, and were most profound at the earliest time points. It would therefore be of some value to describe the time course over which these processes might occur following injury, whether they affect patient outcomes, and whether there is a treatment that might be given to mitigate them. This chapter will address these questions.

Trauma induced coagulopathy (TIC) has been observed in injured patients on arrival in hospital[1], and within the pre-hospital environment[2, 3], suggesting that it may occur soon after injury. Abnormal inflammatory function following trauma has also been observed on admission to hospital[4], and we have recently reported that this occurs within the first hour following injury[5]. Activation of the interconnected inflammatory and coagulation pathways are influenced by a "genomic storm" that occurs following injury, mimicking the response to critical inflammatory stress[6]. This may include the disruption of the endothelial barrier, which has been proposed as a unifying mechanism for these processes following trauma[7]. There have been no investigations of this specific process in the pre-hospital setting. Data regarding the timing and nature of endotheliopathy following injury and before arrival in hospital may facilitate a greater understanding of the mechanisms of coagulopathy and inflammatory disorder following trauma.

The endothelial layer consists of endothelial cells, their basal lamina and the endothelial glycocalyx that lines the luminal surface. Endothelial injury and glycocalyx shedding may occur following trauma as a consequence of its exposure to circulating damage-associated molecular patterns (DAMPs)[8], neutrophil extracellular traps[9], sympathoadrenal activation[10], hypovolaemia[11], and ischemia[12]. In injured patients, soluble

thrombomodulin and syndecan-1 have been used as surrogate markers of endothelial cell injury and glycocalyx shedding respectively[10, 13], as described in the previous chapter. These biomarkers have been observed in abnormal concentrations in blood samples acquired from patients at hospital admission[13], but it is unknown exactly when endotheliopathy occurs following a traumatic insult. If endothelial activation and microcirculatory dysfunction are to be confirmed as a unifying mechanism for the pathological inflammatory processes following trauma[7], then it would be expected that endotheliopathy precedes, or concurrently occurs, with coagulopathic and inflammatory changes following injury. We sought to investigate endotheliopathy of trauma (EoT) within a cohort of trauma patients that we recently observed to have early inflammatory and immune cell dysregulation[5].

A recent *in vitro* study of the EoT demonstrated that when injured human umbilical vein endothelial cells were exposed to tranexamic acid (TXA), concentrations of both thrombomodulin and syndecan-1 (used as markers of endotheliopathy) were reduced significantly more than for those cells without TXA[14]. This study generated the hypothesis that TXA may play some role in the amelioration of the EoT, but this has not been tested in the clinical setting.

The current study measured biomarkers of endothelial cell injury (thrombomodulin) and glycocalyx shedding (syndecan-1) as surrogate markers of EoT. The aim of the study was to provide an estimation of the time of onset of EoT post-injury, and to investigate whether the evolution of EoT between the scene of injury and hospital admission is associated with subsequent organ failure. Furthermore, since these two time points represent “before” and “after” treatment with TXA, we tested the hypothesis that TXA might reduce biomarkers of EoT as has been reported *in vitro*[14].

It was hypothesised that there would be an early rise in biomarkers of EoT following trauma, which would be associated with poorer outcomes, and that patients who received TXA would have a greater decrease in biomarker levels than those who did not receive TXA (Hypotheses 2–4; Table 1.1).

3.2. Methods

3.2.1. Study design and setting

A prospective longitudinal observational study was undertaken using a pre-hospital design described previously[5]; trauma patients were enrolled during pre-hospital evacuation, as soon after injury as possible by paramedic personnel across a large UK major trauma network. After being conveyed to the regional Major (Level 1) Trauma Centre (University Hospitals Birmingham NHS Foundation Trust), study participants were followed up by embedded trauma research personnel. The current study includes patients from the Brain Biomarkers After Trauma Study (BBATS); Research Ethics Committee (REC) approval was granted prior to the start of the study (REC 5, Wales, Ref. 13/WA/0399). This study is reported according to the STROBE guidance for observational studies. Patients were enrolled from May 2014 to February 2017.

Pre-hospital blood products were not available in this trauma network during the study period. Pre-hospital TXA delivery was given according to a specific protocol that included injured patients with any of the following: systolic blood pressure <90 mmHg; heart rate >100 bpm; risk of significant haemorrhage; or who required intravenous fluid therapy. Patients were not given TXA if more than 3 hours had passed since injury, if they had an isolated head injury, a known history of convulsions, or hypersensitivity to TXA.

3.2.2. Study participants

Trauma patients with the likelihood of an Injury Severity Score (ISS) of 8 or higher were eligible for inclusion, regardless of type of injury. Since ISS is not usually calculated until after the patient has left hospital, specific training was delivered to the pre-hospital practitioners within the study region before the start of the study, so that prediction of likely ISS could be made as appropriate. Patients were excluded if they were pregnant, prisoners, or under the age of 16. Patients could only be enrolled if there was a peripheral venous sample of blood taken within 60 minutes from time of injury. All eligible patients were screened by dedicated research staff on arrival in ED to minimise the risk of selection bias. For the purposes of the current study, patients with isolated traumatic brain injury were excluded. Time of injury was defined as the "call time" (i.e. the time that an emergency call was received by the ambulance control room).

3.2.3. Capacity and consent

Due to the injuries sustained and requirement for swift emergency evacuation, it was considered that patients would not have capacity to consent. The REC-approved protocol was undertaken according to the World Medical Association Declaration of Helsinki, and the Mental Capacity Act 2005. As described in Chapter 2, a Professional Consultee may agree to patient enrolment, as well as a close relative or friend (Personal Consultee). Once the patient regained capacity, they were approached in order to gain consent for the retention of data already obtained, and for continued follow up. If a patient did not regain capacity, the permission from their Professional or Personal Consultee to participate in the study remained extant.

3.2.4. Blood sampling and storage

Peripheral blood samples were taken during the pre-hospital evacuation of all patients within 60 minutes of injury. The timing of this sample was meticulously recorded in contemporaneous records by pre-hospital personnel (designated as the "pre-hospital" time point). A further peripheral blood sample was taken between 4 and 12 hours after injury (designated the "in-hospital" time point). Blood samples were collected into BD Vacutainers (Becton Dickinson, Oxford, UK) containing z-serum clotting activator. Following a 30-minute incubation at room temperature, samples were centrifuged at 4^{o}C for 10 minutes at 1,620 x g. Aliquots were stored at -80^{o}C until analysed. Serum samples were acquired and stored for 19 "healthy control" (HC) volunteers, who were matched as a group for sex and age to the study cohort. All HCs had declared themselves to be in good health, and not currently under any medical investigations or treatment, and had no chronic diseases.

3.2.5. Enzyme-linked immunosorbent assays

Commercially available ELISAs were used to measure concentrations of syndecan-1 (CD 138) (Abcam, ab46506, Cambridge, MA) and thrombomodulin (CD 141) (Abcam, ab46508, Cambridge, MA) in the serum samples (as described in detail in Chapter 2). Analysis was undertaken in accordance with the relevant protocols, including the use of control and blank wells in order to validate the results.

3.2.6. Data collection

Data were recorded prospectively for all patients, and then confirmed using electronic and paper records. Data included patient demographics (age, gender), and

mechanism of injury. Injury severity scores (ISS) were acquired from the centralised UK Trauma Audit and Research Network after discharge from hospital. Physiological parameters in the pre-hospital period included systolic blood pressure (SBP), heart rate (HR), and respiratory rate. Those in ED included lowest SBP and associated HR, and Glasgow Coma Scale. Plasma lactate and base deficit in ED were also recorded. Timings of blood samples were collected contemporaneously by pre-hospital practitioners. Delivery of TXA between the first and second time points was recorded in order to dichotomise patients into those who had received TXA and those who had not.

3.2.7. Outcomes

The primary outcome for this study was development of multiple organ dysfunction syndrome (MODS), defined as a sequential organ failure assessment (SOFA) score of 6 or higher on 2 or more consecutive days during their admission in hospital after the first 48h following injury[4, 5]. Mortality within 90 days was also recorded for all patients.

3.2.8. Data analysis

A Shapiro-Wilk test was used to determine the normality of continuous data. Non-normal data are presented as median and interquartile range (IQR). Syndecan-1 and thrombomodulin concentrations were considered abnormal if they were above the 97.5th percentile of HC levels; patients were considered to have endotheliopathy if they had abnormal values for either biomarker (syndecan-1, thrombomodulin, or both). Correlation between non-normal continuous variables was undertaken using Spearman's rank correlation coefficient. Non-normal continuous data were compared between groups using a Kruskal-Wallis test, followed by pairwise comparisons between groups using Dunn's

multiple comparisons test. Categorical data were compared using Fisher's exact test for pairwise comparisons, and chi-squared analysis for trends between groups, and reported as n/denominator. A *p*-value of < 0.05 was considered significant. Where appropriate, further designation of significance was made by indicating when *p*-values were < 0.01, < 0.001 and < 0.0001.

3.2.8.1. Estimation of the time of onset of endotheliopathy

It is highly unlikely that any study of humans will be able to determine the concentrations of biomarkers at the point of wounding, and therefore statistical modelling was considered the best opportunity to estimate the timing of biomarkers following injury. In order to determine the most likely time at which endothelial injury and glycocalyx shedding occur following injury, the concentrations of thrombomodulin and syndecan-1 for all patients were plotted against time from injury. Syndecan-1 and thrombomodulin concentrations derived from HCs were plotted at the origin, on the assumption that trauma patients would have been within the same range prior to injury. Analysis was then performed by fitting generalised additive models to the data using penalised regression splines with smoothing parameters selected by residual maximum likelihood. This modelling approach accounts for the distinctly non-linear pattern of the data that cannot be adequately modelled with standard parametric regression approaches. For each biomarker, two curves were plotted: the group of "abnormal" values (higher than the 97.5^{th} percentile of HCs) and the group of "normal" values which were between the 2.5^{th} and 97.5^{th} percentiles of HCs. The point at which the abnormal curve crossed the line of significance was considered to be an estimation for the point at which endotheliopathy occurred following injury.

3.2.8.2. Measuring the effects of TXA

Patients who had pre-hospital endotheliopathy were dichotomised into those who received TXA within the pre-hospital period (between time points) and those that did not. Biomarkers were then compared before and after treatment in order to determine whether there were any differences between the two groups.

3.2.8.3. Investigating the association between endotheliopathy and MODS

Patients with both pre- and in-hospital serum samples were categorised according to their biomarkers over the two time points: (a) those with persistent abnormal biomarkers; (b) those that had abnormal biomarkers that reduced to normal range; and (c) those with normal biomarkers throughout. The numbers of patients who developed MODS were compared between these three groups. Furthermore, individual biomarkers were compared between those who developed MODS and those who did not.

3.3. Results

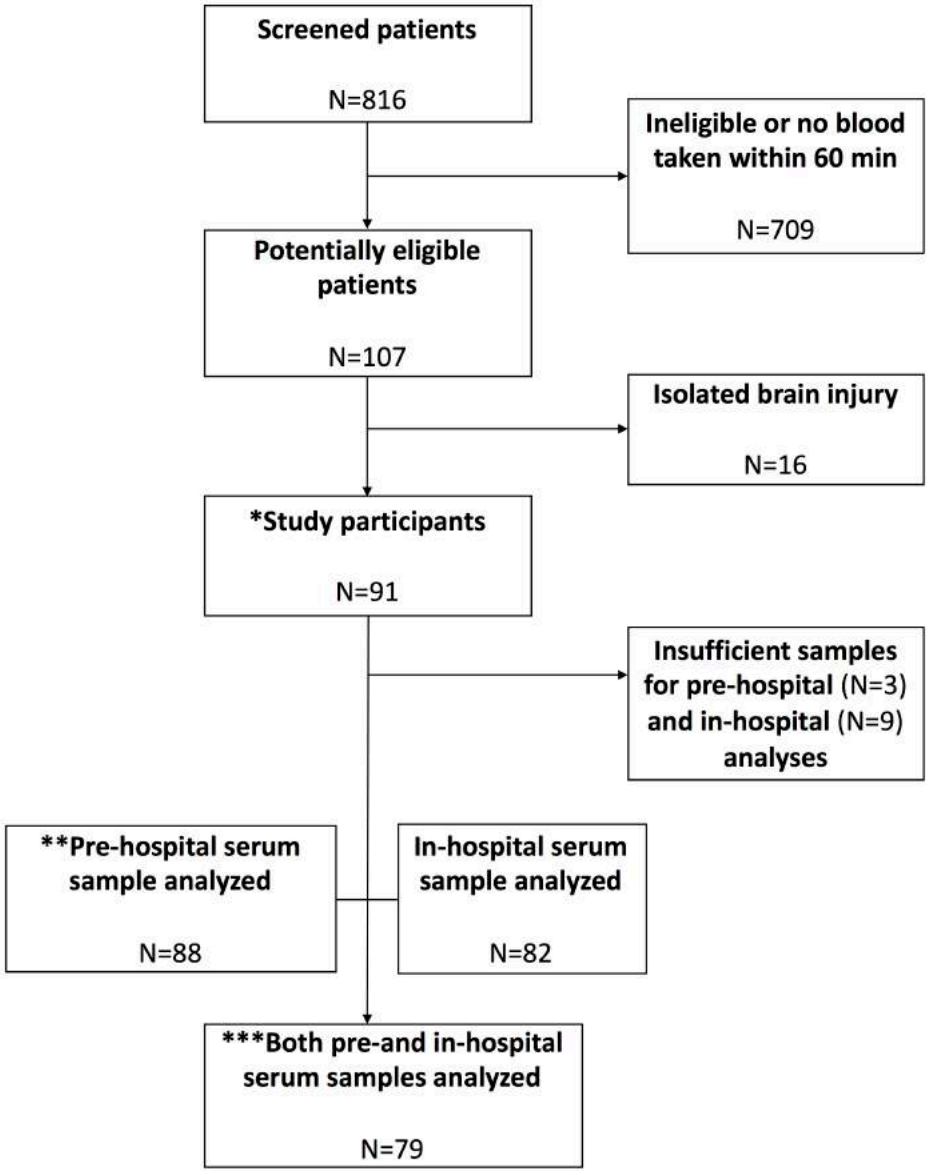
3.3.1. Study participation

A total of 816 patients were screened for eligibility during the study period, of which 107 were potentially eligible patients who had blood samples taken within 60 minutes of injury. After 16 were excluded due to isolated traumatic brain injury, there were 91 patients included. The median time of blood sampling for the pre-hospital time point was 45 (IQR 33–55) minutes after injury. The median time of blood sampling for the second (in-hospital) time point was 5.5h (IQR 4.2h – 10.5h).

Enrolment and participation at each stage are illustrated in a flow diagram (Figure 3.1), with asterisks to indicate which denominator is used for each stage of data analysis.

The pre-hospital blood samples were insufficient for analysis for 3 patients, and the in-hospital blood samples were insufficient for a further 9 patients, so that 79/91 patients had both pre- and in-hospital samples suitable for analysis.

Figure 3.1. Flow diagram of patient enrolment and participation at each stage of analysis. The denominators for analysis are: *N=91 for analysis of biomarkers according to multiple organ dysfunction syndrome (MODS) and mortality, and comparison of tranexamic acid (TXA) and non-TXA; **N=88 for analysis of timing of endotheliopathy; and ***N=79 for analysis of dynamic changes in endotheliopathy between time points



The denominators used for the different analyses differ accordingly:

(a) Analysis of the timing of EoT after injury: N=88 (all pre-hospital samples)

(b) Comparison of biomarkers according to outcomes (MODS and mortality); and comparison between TXA and non-TXA: N=91 (all patients).

(c) Comparison of patient groups according to dynamic changes in endotheliopathy between pre- and in-hospital time points: N=79 (all patients with both pre- and in-hospital samples)

3.3.2. Patient characteristics

The median age of all patients was 38 (IQR 24–55) years, and 78/91 were male. Patient characteristics are illustrated in Table 3.1, with comparison between patients with pre-hospital EoT and those without. There was a higher proportion of patients with blunt injury in the pre-hospital endotheliopathy group than those without pre-hospital endotheliopathy (60/71 *versus* 9/17; $p < 0.01$). All other characteristics were not significantly different between the groups.

3.3.3. Presence of endotheliopathy

According to serum concentrations of thrombomodulin and syndecan-1 for HCs and patients, there was a significant difference between them at both time points, for both biomarkers, as illustrated in Table 3.2. According to the presence of abnormal syndecan-1 or thrombomodulin concentrations, pre-hospital and in-hospital endotheliopathy were present in 71/88 (81%) and 51/82 (62%) patients respectively.

Table 3.1. Study participant characteristics according to presence of endotheliopathy at the pre-hospital time point

Patient characteristic	All (N=88)	Pre-hospital Endotheliopathy (N=71)	No pre-hospital endotheliopathy (N=17)	*p*-value
Age, years	33 (24–53)	38 (24–56)	25 (23 – 44)	0.343
Sex, male	75 (85)	61 (86)	14 (82)	0.993
Mechanism of injury				
Blunt	69 (78)	60 (85)	9 (53)	0.008*
Penetrating	19 (22)	11 (15)	8 (47)	
Injury Severity Score	23 (12–36)	24 (14–36)	16 (9–29)	0.267
Pre-hospital parameters				
Heart rate, min^{-1}	100 (84–110)	100 (80–118)	99 (85–108)	0.686
SBP, mmHg	121 (96–141)	118 (96–140)	127 (118–143)	0.364
Respiratory rate, min^{-1}	20 (16–25)	20 (16–25)	18 (13–20)	0.115
Physiological parameters in ED				
Lowest SBP, mmHg	115 (99–124)	114 (99–124)	119 (102–124)	0.666
Heart rate, min^{-1}	88 (76–99)	87 (75–99)	89 (78–103)	0.629
Glasgow Coma Scale	13 (5–15)	13 (4–15)	15 (10–15)	0.150
Plasma lactate, mmol/l	4.1 (2.5–6.5)	4.2 (2.7–6.7)	3.5 (2.0–5.5)	0.259
Base deficit, mmol/l	-3 (-6 – -1)	-3 (-6 – -1)	-3 (-6 – -1)	0.438

Summary data are presented as median (interquartile range); categorical data are reported as N (%)
ED: Emergency Department; SBP: systolic blood pressure
*Significant according to Fisher's exact test

Table 3.2. Serum syndecan-1 and thrombomodulin concentrations for healthy controls and patients according to time point.

Biomarker, ng/ml	Healthy controls (N=19)	Pre-hospital (N=88)	In-hospital (N=82)
Syndecan-1	30 (20–44)	59 (39 – 140)***	53 (28 – 150)*
Thrombomodulin	2.9 (2.2 – 3.4)	4.9 (3.8 – 6.4)***	4 (3.4 – 5.1)**

Data are presented as median (interquartile range), ng/ml
* $p < 0.01$, ** $p < 0.001$, and *** $p < 0.0001$ when compared to healthy controls using Dunn's multiple comparisons test

For patients with samples available at both time points (N=79) (Figure 3.1), 50/79 (63%) had persistently abnormal biomarkers (≥1 biomarker was abnormal at both time points), 18/79 (23%) had abnormal biomarkers that returned to normal (≥1 biomarker was abnormal at the first time point, but both were normal at the second time point), and 11/79 (14%) had both biomarkers normal throughout; their characteristics are illustrated in Table 3.3. There was a significant difference across groups for plasma lactate, base deficit, and SBP (all $p < 0.05$). Of all patients with biomarkers at both time points, there were 20/79 (25%) who received blood products between the time points. There was a significant difference in transfusion requirement between these groups according to biomarker dynamics; almost all of those requiring transfusion were in the group that had persistently abnormal biomarkers ($p < 0.01$) (Table 3.3). Multivariable analysis to examine the association between blood product requirement and group adjusted for ISS could not be performed due to the low number of transfusions in the "abnormal-normal" and "normal-normal" groups, which would risk over-fitting the data.

3.3.4. Endotheliopathy occurs within minutes of injury

There was evidence of glycocalyx shedding and endothelial cell damage within the pre-hospital (<60 min) phase amongst this cohort of patients. Before statistical modelling, the first recorded abnormal syndecan-1 and thrombomodulin concentrations amongst the patient cohort were observed at 17 and 18 minutes following injury respectively (Figure 3.2). When the statistical model was used, glycocalyx shedding and endothelial cell injury were estimated to occur at approximately 5 and 8 minutes following injury respectively (Figure 3.2).

Table 3.3. Patient characteristics according to change in serum syndecan-1 and thrombomodulin concentrations between the pre-hospital and in-hospital time points

Patient characteristic	Abnormal-abnormal (N=50)	Abnormal-normal (N=18)	Normal-normal (N=11)	*p*-value
Age, years	39 (25–63)	36 (24–45)	25 (23–48)	0.417
Sex, male	42 (84)	16 (89)	9 (82)	0.974
Mechanism of injury				
Blunt	39 (78)	17 (94)	7 (64)	0.729
Penetrating	11 (22)	1 (6)	4 (36)	
Injury Severity Score	25 (15–38)	29 (16–38)	17 (9–29)	0.230
Physiological parameters in ED				
Lowest SBP, mmHg	113 (95–122)	119 (105–129)	122 (106–134)	0.030*
Heart rate, min^{-1}	87 (80–99)	93 (81–104)	88 (76–104)	0.627
Glasgow Coma Scale	13 (3–14)	12 (6–14)	15 (11–15)	0.083
Plasma lactate, mmol/l	4.8 (3.0–7.2)	3.7 (2.5–6.4)	2.4 (1.3–4.6)	0.037*
Base deficit, mmol/l	-4 (-8 – -2)	-3 (-6 – -1)	-2 (-3 – 0)	0.023*
Blood product requirement				
All patients transfused	19 (38)	0 (0)	1 (10)	0.003**
Patients given RBCs	19 (38)	0 (0)	1 (10)	0.003**
Patients given FFP	12 (24)	0 (0)	0 (0)	0.017**

Summary data are presented as median (interquartile range); categorical data are reported as N (%)
ED: Emergency Department; SBP: systolic blood pressure; RBC: red blood cells; FFP: fresh frozen plasma
*Significant according to Kruskal-Wallis test
**Significant according to chi-squared test

3.3.5. Endotheliopathy is associated with poor perfusion

When pre-hospital and in-hospital concentrations of syndecan-1 and thrombomodulin were compared to the plasma lactate during ED resuscitation (as a surrogate marker of microcirculatory perfusion), there was a significant correlation with both pre-hospital and in-hospital syndecan-1 (Figures 3.3a and 3.3b respectively), and in-hospital thrombomodulin (Figure 3.3d); all $p < 0.05$. However, there was no significant correlation between lactate and pre-hospital thrombomodulin concentration (Figure 3.3c).

Figure 3.2. Concentrations of (a) syndecan-1 and (b) thrombomodulin in relation to time of injury. Healthy controls are plotted at time = 0 min. Patients are divided into those with elevated concentrations of the biomarker (>97.5th percentile of healthy controls), and those with normal concentrations (2.5th – 97.5th percentiles of healthy controls). For each curve the central trend line represents the average values, with the upper and lower trend lines representing the 2.5th and 97.5th percentiles of observed values. The vertical interrupted red line indicates the time at which biomarkers are likely to be first elevated.

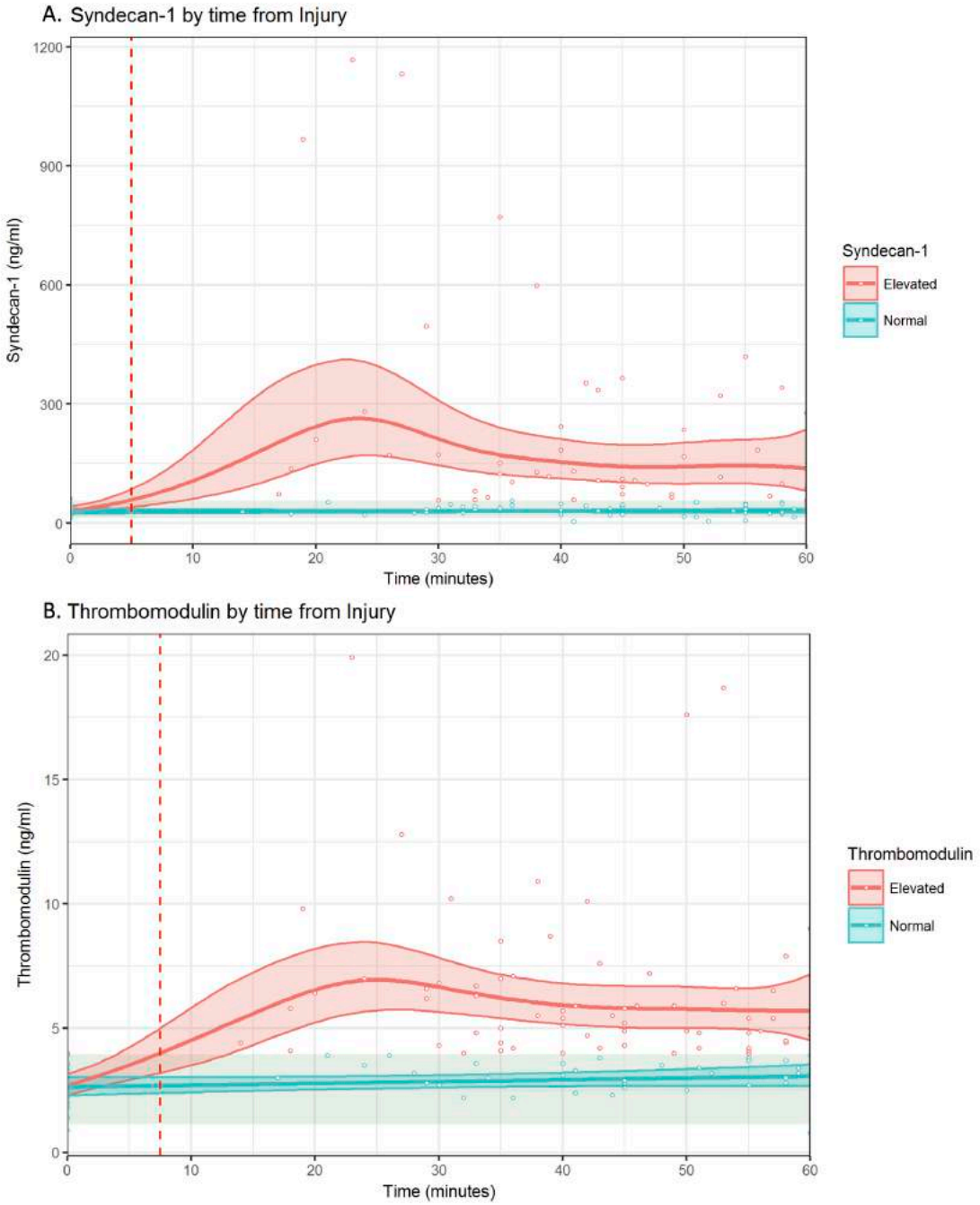
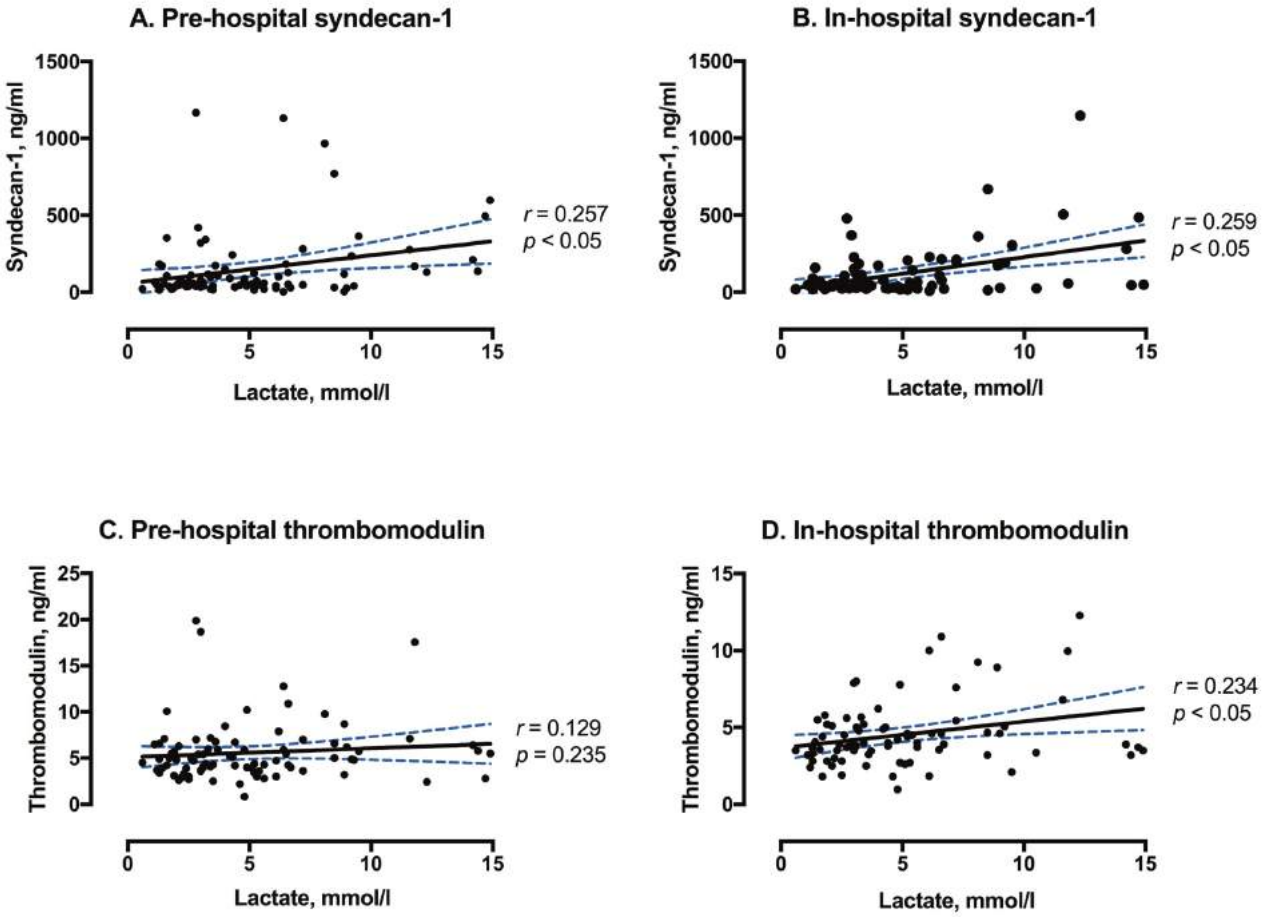


Figure 3.3. Concentrations of Syndecan-1 and thrombomodulin in relation to lactate concentration during ED resuscitation; including (a) pre-hospital syndecan-1; (b) in-hospital syndecan-1; (c) pre-hospital thrombomodulin; and (d) in-hospital thrombomodulin.

Blue dashed lines represent 95% confidence intervals.



3.3.6. Endotheliopathy is associated with MODS

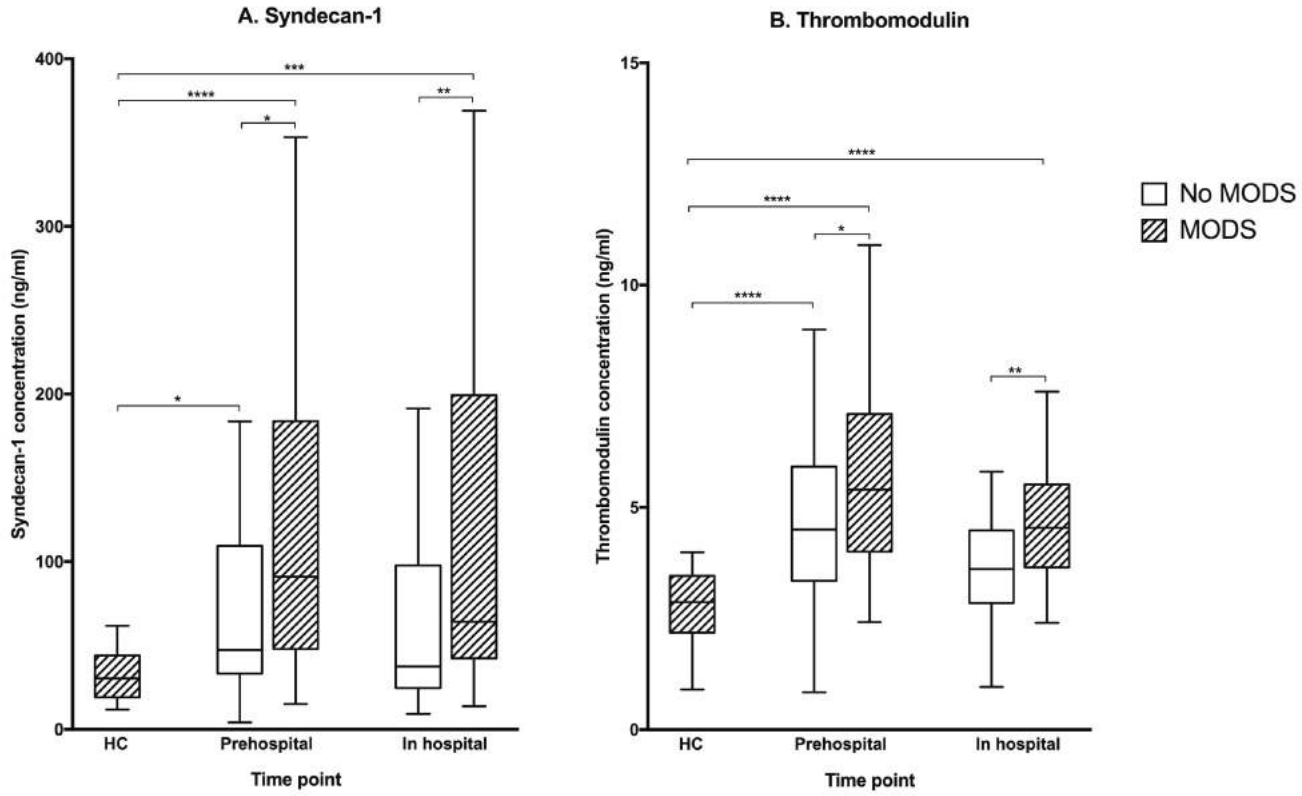
Figure 3.4 illustrates the biomarker levels of patients according to whether they developed MODS. When patients who developed MODS were compared to those without MODS, the former had significantly higher syndecan-1 concentrations at the pre-hospital (91 (IQR 48–184) ng/ml *vs.* 47 (IQR 33–109) ng/ml; $p < 0.05$) and in-hospital (64 (IQR 42–

199) ng/ml *vs.* 38 (IQR 25–98) ng/ml; $p < 0.01$) time points. For patients with MODS, the pre- and in-hospital syndecan-1 and thrombomodulin levels were both significantly higher than those of HCs (30 (IQR 19–44) ng/ml); both $p < 0.0001$ and $p < 0.001$ respectively. Patients without MODS also had significantly higher syndecan-1 concentrations in the pre-hospital samples ($p < 0.05$) but not in the in-hospital samples ($p = 0.216$) (Figure 3.4a).

Figure 3.4. Concentrations of (a) syndecan-1 and (b) thrombomodulin according to time point and development of multiple organ dysfunction syndrome (MODS).

Healthy controls (HC) are displayed for comparison.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ *vs.* HCs



Patients with MODS had significantly higher thrombomodulin concentrations at the pre-hospital (5.4 (IQR 4.0–7.1) ng/ml *vs.* 4.5 (IQR 3.4–5.9) ng/ml; $p < 0.05$) and in-hospital (4.5 (IQR 3.6–5.5) ng/ml *vs.* 3.6 (IQR 2.9–4.5) ng/ml; $p < 0.01$) time points. For patients with MODS, the pre- and in-hospital biomarker levels were both significantly higher than those of HCs (2.9 (IQR 2.2–3.5) ng/ml); $p < 0.0001$ for both. Patients without MODS had significantly higher thrombomodulin concentrations in the pre-hospital samples ($p < 0.0001$) but not in the in-hospital samples ($p = 0.065$) (Figure 3.4b)

3.3.7. Changes in biomarkers are associated with MODS

For those patients with both pre-hospital and in-hospital samples (n=79), 42/79 patients developed MODS. These included 31/42 (74%) who had persistently abnormal biomarkers, 8/42 (19%) had abnormal pre-hospital biomarkers that normalised, and 3/42 (7%) had normal biomarkers at both time points; $p < 0.05$.

When patients were compared according to dynamic change in biomarkers, MODS developed in 31/50 (62%) in the group that had persistently abnormal biomarkers, 8/18 (44%) in the group that had abnormal biomarkers that returned to normal range, and 3/11 (27%) in the group that had normal biomarkers throughout; $p < 0.05$ (Figure 3.5a). When syndecan-1 and thrombomodulin were examined separately, there was a similarly significant trend for MODS according to both biomarkers; $p < 0.05$ and $p < 0.01$ respectively (Figure 3.6).

There were 12 patients who died. There was no significant difference in mortality within groups according to dynamic changes in biomarkers; 9/50 with persistently abnormal biomarkers, 2/18 of those with abnormal biomarkers that returned to normal range, and 1/11 of those with normal biomarkers throughout died; $p = 0.652$ (Figure 3.5b).

Figure 3.5. Presence of (a) multiple organ dysfunction syndrome (MODS) and (b) 90-day mortality amongst patients according to dynamic changes in levels of both syndecan-1 and thrombomodulin between pre-hospital and in-hospital time points.

Significance is indicated according to chi-squared test for trend.

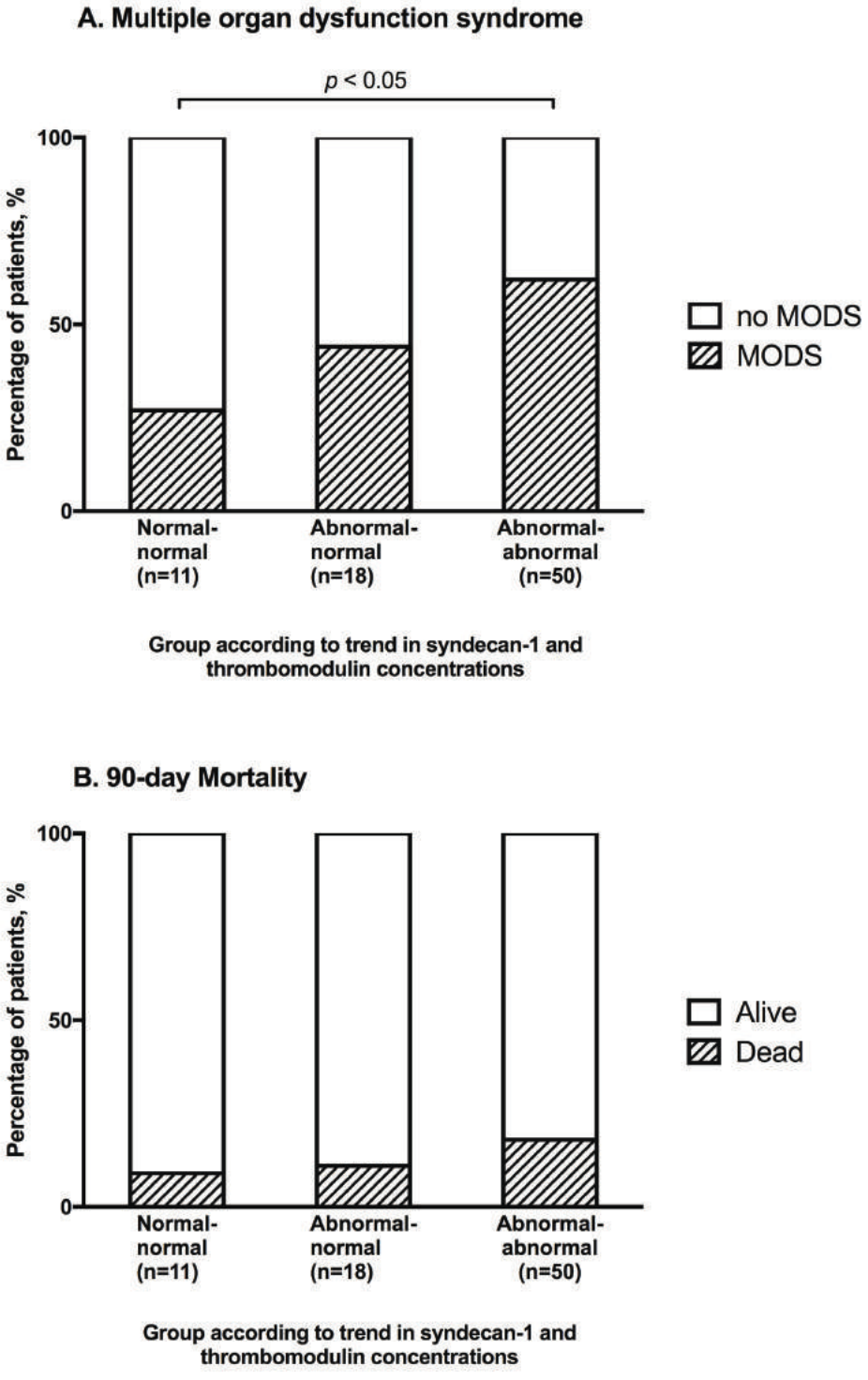
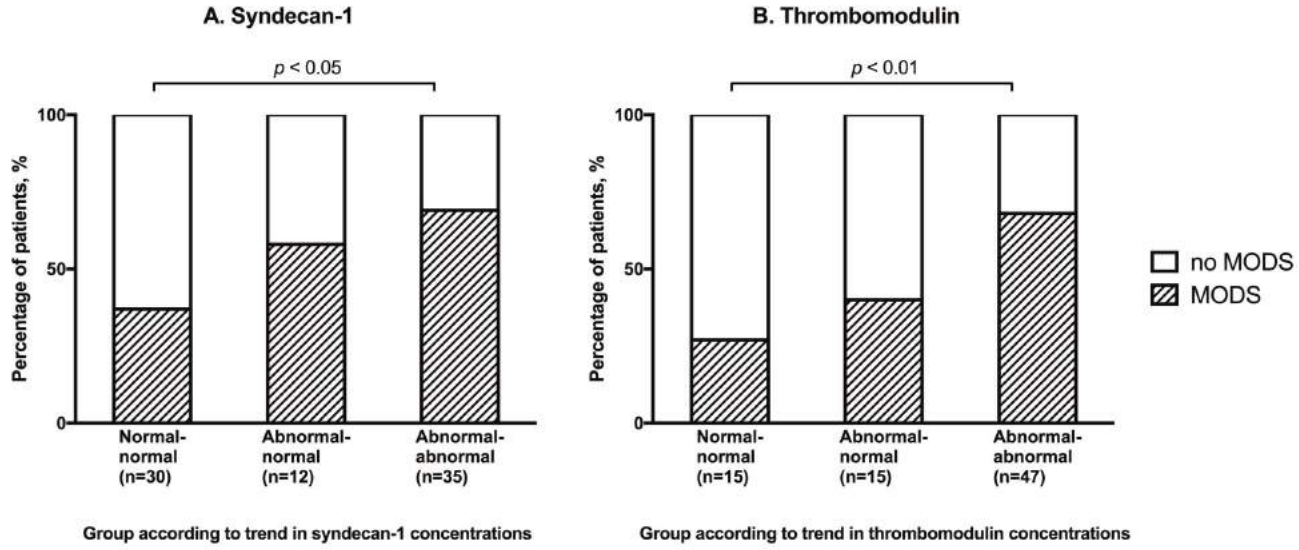


Figure 3.6. Presence of multiple organ dysfunction syndrome (MODS) according to dynamic changes in individual biomarker levels (a) syndecan-1 and (b) thrombomodulin between pre-hospital and in-hospital time points.

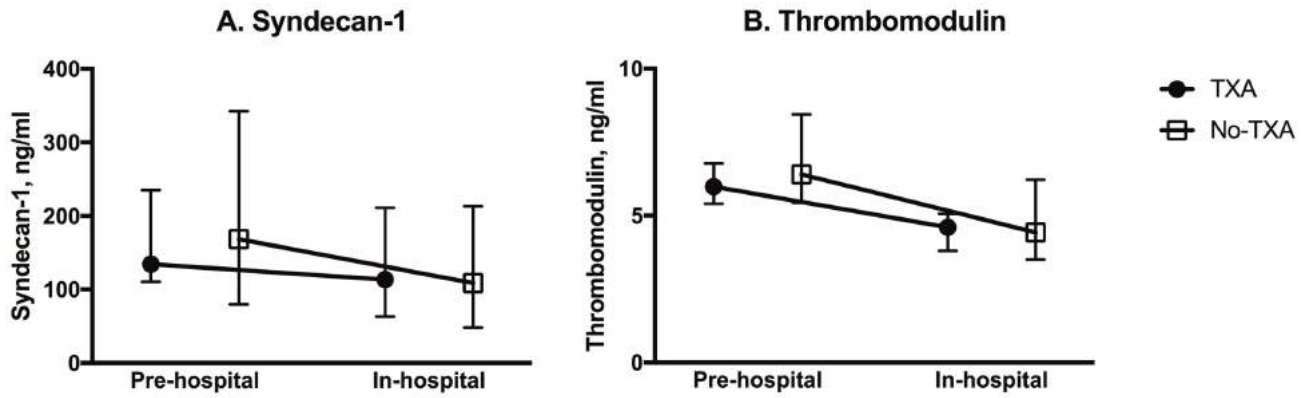
Significance is indicated according chi-squared test for trend.



3.3.8. TXA did not influence biomarkers of endotheliopathy

Of all patients, there were 55/91 who received pre-hospital TXA, 35/91 did not, and one patient was randomised to either TXA or placebo as part of the CRASH-3 study[15]. There were 38 patients with abnormal pre-hospital syndecan-1, of which 23/38 received TXA. There were no differences in syndecan-1 concentration at either time point according to TXA group (Figure 3.7a). There were 43 patients with abnormal pre-hospital thrombomodulin, of which 25/43 received TXA. There were similarly no differences in thrombomodulin concentrations at either time point according to TXA group (Figure 3.7b).

Figure 3.7. Pre-hospital and in-hospital levels of syndecan-1 and thrombomodulin according to whether TXA was given to the patient during the pre-hospital period. Vertical bars represent 95% confidence intervals around the median.



3.4. Discussion

The main finding of this study is that trauma-induced endotheliopathy occurred within minutes of injury, and failure of abnormal biomarkers to return to normal was associated with a higher burden of organ dysfunction. MODS was associated with higher syndecan-1 and thrombomodulin at both pre-hospital and in-hospital time points, but there was no association between delivery of TXA and biomarker levels. Endotheliopathy is likely to occur rapidly following injury, with observed biomarker levels raised at 17 minutes within the patient cohort, and statistical modeling of data predicting that this occurs very rapidly within 5 – 8 minutes of injury. These data are in keeping with the hypothesis that endotheliopathy is a common mechanism[7, 16], and may partly explain why phenomena such as TIC and inflammation have been recorded in the pre-hospital environment and on arrival of patients in hospital. These data are also in keeping with previous evidence that high

syndecan-1 is associated with poorer outcomes amongst trauma patients, even when taken very early after patient admission to hospital[17].

These observations of early endotheliopathy following injury are in keeping with the current understanding of the mechanisms of early coagulopathy, since glycocalyx shedding and endothelial cell injury stimulate the generation of thrombin and activation of protein C, with subsequent hyperfibrinolysis[18]. Since inflammatory derangement following cellular injury may also be associated with subsequent sepsis[19] and organ failure[4], early reversal of these interconnected processes has the potential to improve outcomes. The patients that developed MODS in this study came from the same cohort that showed inflammatory and immune cell dysregulation in our previous detailed study of immune function, with raised levels of IL-6, IL-8, and TNFα at both pre-hospital and in-hospital time points when compared to healthy controls[5]. Together, these data support the hypothesis that there is an early and important relationship between endothelial injury and altered immune function following trauma, and that these derangements occur within minutes of injury. Multiple studies have reported independent associations between high injury severity, high plasma adrenalin level, and high circulating syndecan-1 and thrombomodulin levels, suggesting that sympathoadrenal hyper-activation following shock may be a key driving factor in this process[5].

During the resuscitation of critically unwell trauma patients, lactate has been shown to be a useful biomarker of perfusion as a one-off reading and also in terms of progression between values (as lactate clearance)[20]. In a similar manner, one-off individual biomarkers of endotheliopathy are presented here, but also progression of biomarkers during resuscitation, where the normalisation of biomarkers are taken as a surrogate marker of endothelial restoration. In the current study, MODS was associated with persistent

endotheliopathy, and less common amongst those with endothelial biomarkers that returned to normal range. Restoration of the injured endothelium may therefore represent a meaningful clinical target for goal-directed therapy during pre-hospital evacuation. Deliberate targeting of endothelial integrity is not currently undertaken in clinical practice. Plasma-based fluid resuscitation represents one possible treatment of choice for patients in this context, since both pre-clinical[21] (discussed later in Chapter 7) and clinical[22] investigations have reported restoration of the endothelial glycocalyx after its delivery. The current study was non-randomised, and patients who had received plasma were more likely to be in the group that had persistently abnormal biomarkers. Although there were no significant differences in ISS between groups, a greater requirement for blood product transfusion in this group suggests a higher number of patients with haemorrhagic trauma. A similar selection bias has also been reported in observational studies of pre-hospital blood products, limiting meaningful clinical interpretation[23]. These data cannot therefore be used to determine the efficacy of plasma-based resuscitation in the mitigation of endotheliopathy of trauma; further clinical investigations are required to test the hypothesis that plasma may allow endothelial restoration, especially if randomised controlled trials of pre-hospital plasma-based fluid resuscitation report improved clinical outcomes.

The need for pre-hospital research has been emphasised by the World Health Organisation[24] and the Institute of Medicine of the National Academies[25], and may enable questions to be addressed that are not necessarily answered by pre-clinical studies[26]. Although the old concept of the "golden hour" may not have as much of a bearing on outcomes as it was first supposed[27], it appears to represent a time during which a cascade of important pathological processes occur following injury, very close to the point of wounding. The data from this study have been obtained within the pre-hospital period,

within the first hour following injury. Rather than waiting until arrival in hospital to attempt to address pathological processes, there may be some justification for working towards earlier, and more bespoke, intervention for critically unwell patients[28]. Such an approach has already been advocated in terms of remote damage control resuscitation[29], and the consideration of the endothelium and blood together as an "organ" during resuscitation following trauma[30].

The derangement of fibrinolysis has been considered as a target for trauma resuscitation in bleeding patients, such as with tranexamic acid[31] and fibrinogen concentrate[32], but there are no clinical therapies directly targeting the restoration of the glycocalyx or endothelial integrity. We were unable to confirm recent *in vitro* findings that early delivery of TXA can reduce the burden of endotheliopathy following injury[14, 33]. This may be due to the observational study design, and the heterogeneity in patient injury patterns and severity, as shown by the large confidence intervals in the data. Further investigation may be warranted on a larger scale, with a more homogenous population, using randomisation to reduce the risk of selection bias.

More than half of patients who did not reverse their endotheliopathy went on to develop multiple organ failure. Although causality cannot be demonstrated in the current study, the relationship is in keeping with previous descriptions of early inflammatory dysfunction and MODS[4, 5]. The reason that some patients have biomarkers that are restored to normal range but others do not (or indeed why some have normal biomarkers throughout) is unknown. There was evidence that plasma lactate, base deficit and SBP were significantly different between these groups, which suggests that the degree of shock and perfusion may be related to endothelial integrity – a finding supported by the relationship between biomarkers and plasma lactate in the current study. It is notable that although this

association was statistically significant, the correlation was not perfect, suggesting that there is a complex relationship between perfusion and endothelial integrity, with other confounding factors in a heterogeneous trauma population. Further mechanistic studies are required to investigate the precise relationships between the endothelium, perfusion, and subsequent organ function following trauma.

It is likely that there are some genetic factors in the ability of the endothelium to respond to trauma and resuscitation, and early biomarkers may be one way of detecting these predispositions. Other biomarkers in trauma have been proposed as prognostic indicators[34], but these are not commonly used for trauma triage or goal-directed therapy in clinical practice. The utilisation of such biomarkers—including those that may indicate endotheliopathy—remains a tantalising potential avenue for future trials. If strategies to treat the endothelium are planned in the future, it seems that early treatment (within the pre-hospital environment) would be warranted since the current study suggests that early restoration of the endothelium (within 12 hours) is associated with lower morbidity. Presence or absence of endotheliopathy may be an important stratification in future resuscitation and therapeutic intervention clinical trials.

3.4.1. Limitations

The current study is limited by its number of patients when analyzing differences in relatively rare occurrences such as mortality; although there appears to be a non-survival trend in the same direction as MODS, this was not found to be statistically significant. Not all patients had samples available from both time points. These patients were excluded during subgroup analysis, which gives potential risk of selection bias. The style of opportunistic study enrolment during pre-hospital evacuation makes the study at risk of

selection bias, since only a proportion of eligible patients were recruited into the study. This risk was reduced by the maintenance of a screening log to ensure that "missed" patients were not significantly different in demographics and injury patterns than included patients.

The statistical modeling performed to evaluate the timing of endotheliopathy should be considered as an estimation only, since it requires several assumptions that have the potential to be biased; it was assumed that patients had similar biomarkers to HCs prior to injury, and that biomarker levels above the 97.5th percentile of HCs represented the development of endotheliopathy. These assumptions were required for a pragmatic estimation, since time-of-injury sampling is unlikely to be ethically justifiable or logistically realised. Furthermore, as mentioned in Chapter 2, it is not known whether some of this elevation in biomarker concentration represents a useful inflammatory response to trauma (i.e. non-pathological).

Dividing patients into "abnormal-abnormal", "abnormal-normal" and "normal-normal" (as described in Table 3.3) is likely to be an over-simplification, and ought to be considered exploratory analysis only. Although on univariate analysis there did not appear to be a difference in ISS between groups, no meaningful multivariate analysis could be performed to determine whether the changes in biomarkers were indeed related to injury burden. Further investigations of biomarkers and their relationships to injury and treatments in the pre-hospital domain may facilitate a greater understanding of their relationships.

There were no pre-hospital blood products available in the trauma network in which this study took place, and patients were given 0.9% saline if they required fluid resuscitation during pre-hospital treatment and evacuation. Trauma patients during this study period were given a medium of 750ml of 0.9% saline according to a recent large collaborative study

that I designed and facilitated[35]. It is currently unknown what influence pre-hospital crystalloid fluids may have on the endothelium, or on the concentration of endothelial biomarkers when compared to blood products. Furthermore, it is unknown what influence the type of resuscitation fluid may have on the development of MODS, or the duration of shock in humans. Although these questions will be further explored in Chapter 7 of this thesis for pre-clinical (animal) models of haemorrhagic shock, there are very limited data for humans. These questions may be further addressed in a clinical context by investigating biomarkers and outcomes in patients who have been randomized to crystalloid fluids or blood products in the pre-hospital period, such as those patients enrolled in the RePHILL trial[36].

The reliability of out-of-hospital blood samples has been questioned in simulations of "pre-hospital" delay[37], which has the potential to bias results based on time difference between sampling and arrival hospital. Since this was a pragmatic, real-life study, such a risk may exist, but was kept to a minimum by maintaining strict and consistent preparation techniques as soon as the sample was received. The interquartile range of times that the second blood samples were taken was 4.2h – 10.5h. The study used a pragmatic target of sampling between 4 – 12h in order to facilitate blood sampling for patients without interfering with clinical activities. However, such a range in timescales puts this time point at some risk of bias due to variations in interventions may have occurred during that period between patients.

3.5. Conclusion

Endothelial cell injury and glycocalyx shedding were observed within minutes following injury, and that statistical modeling predicted that this may occur at

approximately 5–8 minutes. Failure to reverse pre-hospital endotheliopathy is associated with poorer clinical outcome in terms of multiple organ dysfunction syndrome. Biomarker dynamics did not appear to be related to injury severity as defined by anatomic injuries in the ISS. In our non-randomised study, TXA was not associated with reduced levels of biomarkers of endotheliopathy. These findings, together with other reports of early coagulopathy and inflammatory dysregulation, suggest that early interventions aimed at restoration of the endothelium may represent a clinically meaningful target for resuscitation. The most appropriate treatment for this purpose in the clinical context remains uncertain.

3.6. References

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Chapter 4

Endotheliopathy is associated with cell-free DNA following major trauma

The following chapter is adapted from the published article:

Naumann DN, Hazeldine J, Dinsdale RJ, Bishop JR, Midwinter MJ, Harrison P, Hutchings SD, Lord JM. Endotheliopathy is associated with higher concentrations of cell-free DNA following major trauma: a prospective observational study. *PLoS ONE* 12(12): e0189870.

4.1. Introduction

In the previous two chapters we have seen that endotheliopathy occurs very early after injury, and is associated with both poor microcirculatory flow and subsequent organ failure. In this chapter, further analysis was performed on the blood samples of patients in both of these study cohorts in order to determine whether there was a relationship between those endothelial biomarkers and levels of cell-free deoxyribonucleic acid (cfDNA)—a potential aetiological factor in their observed endotheliopathy and microcirculatory dysfunction.

cfDNA is defined as extracellular DNA present within the circulation. It consists of a combination of nuclear and mitochondrial DNA (mtDNA) that enters the circulation following cell necrosis[1], tissue injury, and the generation of extracellular traps by activated immune cells such as neutrophils[2] and monocytes[3]. cfDNA may be elevated due to bacterial infection, and mtDNA shares structural similarities with pathogen–associated molecular patterns (such as those derived from bacteria). These fragments may therefore activate the innate immune response following aseptic injury, and contribute in part to the systemic inflammatory response observed following trauma[4-7]. The presence of cfDNA may also give rise to hypercoagulability by coagulation factor activation, platelet aggregation, and inhibition of fibrinolysis[8, 9], whilst neutrophil extracellular traps (NETs) may cause further tissue injury and endothelial barrier dysfunction[10]. cfDNA has received interest as a biomarker following injury in relation to mortality[1, 11], organ failure[12], inflammation[2], and sepsis[13].

Syndecan-1 and thrombomodulin have been reported as biomarkers of endothelial cell and glycocalyx injury in multiple recent studies of trauma[14-16], and as described in the previous chapters. The former is a transmembrane heparan sulfate proteoglycan that forms

part of the endothelial glycocalyx, and the latter is an integral membrane protein present on the surface of endothelial cells throughout the circulation. Raised levels of these biomarkers are associated with coagulopathy[14, 15, 17, 18], inflammation[15], poor microcirculatory flow (as seen in Chapter 2)[19], organ failure (as seen in Chapter 3)[20], and mortality[15-17].

Experimental models have demonstrated that mtDNA directly increases endothelial permeability through both neutrophil-independent and dependent pathways[21], and that it may induce a pro-thrombotic phenotype within the endothelium[22]. These findings suggest that there is likely to be a relationship between cfDNA and endotheliopathy, and that each may be mutually detrimental to the other. Indeed, there is some pre-clinical evidence that endothelial cell damage by cfDNA is dose-dependent[23]. Other investigators have reported an association between endotheliopathy and elevation of cfDNA levels on admission to hospital following injury[15, 24, 25], but to our knowledge this has not been investigated within the pre-hospital period following trauma.

The current study aimed to investigate the levels of cfDNA and biomarkers of endotheliopathy following injury in a prospective cohort of trauma patients, and whether these biomarkers were associated with each other over time, from the pre-hospital period to hospital admission. We also aimed to investigate whether there was an association between levels of cfDNA and clinical outcomes, and with markers of coagulopathy.

It was hypothesised that higher levels of biomarkers of endotheliopathy would be associated with higher levels of cfDNA, that this would be true even within the first hour of injury, and that there would be an association with markers of coagulopathy. We further hypothesised that an increase or decrease over time in each biomarker would be associated with the same directional changes in the others, and that raised cfDNA levels would be associated with poorer clinical outcomes (Hypothesis 5; Table 1.1). We hypothesised that

patients who received a blood transfusion or had surgery would have increased levels of cfDNA when compared to those who did not.

4.2. Methods

4.2.1. Study design and setting

The current study combines patients from two prospective longitudinal observational studies conducted at a single Major Trauma Centre site in the UK (University Hospitals Birmingham NHS Foundation Trust, Birmingham); these included patients from the Brain Biomarkers after Trauma (BBATS)[20] (REC reference: 13/WA/0399) (Chapter 3) and MICROSHOCK[19, 26] (REC reference: 14/YH/0078) (Chapter 2) studies. The former study (Cohort A) included patients with a predicted ISS >8, regardless of mechanism of injury or blood product requirement between May 2014 and February 2017, and also includes the patients with isolated traumatic brain injury that were otherwise excluded in Chapter 3. The latter (Cohort B) included patients with traumatic haemorrhagic shock requiring blood product transfusion, with a lactate > 2 mmol/l, and a requirement for admission to Intensive Care between July 2015 and January 2017.

4.2.2. Capacity and consent

As described in the previous two chapters, all patients lacked capacity to consent for study participation at the time of study enrolment, and enrolment was undertaken according to the Mental Health Act 2005 and the Declaration of Helsinki. Both the MICROSHOCK and BBATS studies were approved to gain consent to participate from a Professional or Personal Consultee. Ultimately, if the patient regained capacity, they were

asked for consent to remain within the study, and for their previous data to be retained. If they did not regain capacity, then previous permissions from their Professional or Personal Consultee remained extant.

4.2.3. Study time points

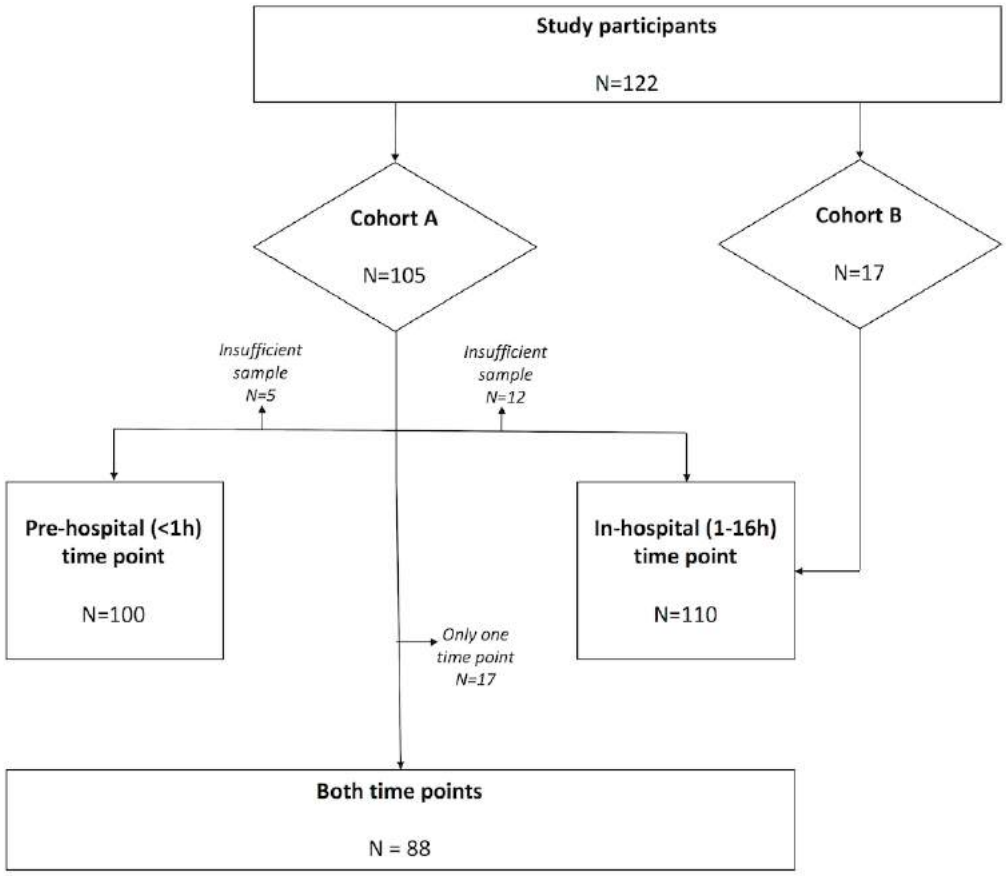
Patients in Cohort A all had blood sampled within the first 1 hour of injury by pre-hospital personnel (median time 44 (range 14–60) minutes). They then had a further sample taken between 4–12 hours following injury. Patients in Cohort B had blood sampled as soon as possible following admission to hospital, with a median time to sample of 3.5 (range 1–16) hours. For the purposes of the current study, two time points were investigated, as illustrated in Figure 4.1: (i) Pre-hospital (< 1 hour) (Cohort A only); and (ii) the first day of admission to hospital (1–16h) (Cohorts A and B combined).

4.2.4. Blood sampling and preparation

All patients underwent venepuncture to obtain blood samples for research purposes using BD Vacutainers (Becton Dickinson, Oxford, UK). For the current study, patients had already had serum samples prepared for measurement of endothelial biomarkers, as described in Chapters 2 and 3 (collected into vacutainers containing z-serum clotting activator, and then centrifuged for 10 minutes at 1,620 x g after 30 minutes at room temperature). Paired plasma samples that corresponded to the same patient time points were prepared for the measurement of cfDNA levels in the current study. For these samples, blood had been collected into vacutainers containing a 1/10 volume of 3.2% trisodium citrate. After 30 minutes at room temperature, they were centrifuged twice in order to minimise platelet contamination. First, samples were centrifuged for 20 minutes at

2,000 x g, then the top two thirds of fluid were spun again for 2 minutes at 13,000 x g. All centrifugation was performed at 4°C. Only patients with paired serum and plasma samples could be included in the current study (the former for endothelial biomarkers and the latter for cfDNA levels).

Figure 4.1. Flow diagram of study patients and samples available for analysis.



4.2.5. Measurement of endothelial biomarkers

The concentrations of thrombomodulin (CD 141) and syndecan-1 (CD 138) in serum samples were quantified using commercially available enzyme-linked immunosorbent assay kits (Abcam, Cambridge, MA) in precise accordance with manufacturer instructions, as

described in detail in Chapter 2. Each 96 well plate included a standard curve to interpolate the concentrations of biomarkers, as well as blank and control wells to validate the results.

4.2.6. Measurement of cell-free DNA

In order to measure the cfDNA concentration in plasma samples, a fluorometric assay using SYTOX® Green Dye (Life Technologies, Cheshire, UK) was used as described previously [13]. For each plate, a standard curve was generated using samples of λ-DNA (Fisher Scientific, UK) ranging from 0–1000 ng/ml. To ensure that the assay was reliable between samples and assays, the inter-assay and intra-assay coefficients of variation were calculated; these were 5.3% and 5.1% respectively. 140 µl of SYTOX® Green Dye (Final concentration 1 µM) was added to 10 µl of plasma, and incubated for 10 minutes at room temperature in the dark. All samples were run in duplicate. Fluorescence was then measured using a BioTek Synergy 2 fluorometric plate reader (NorthStar Scientific Ltd, UK). Calibration was set at 485 nm and 528 nm for excitation and emission respectively. The average values of the duplicate wells were then used to derive cfDNA concentrations by interpolating from the standard curve.

4.2.7. Data collection

Demographic and clinical data were obtained prospectively for all patients using a combination of electronic medical records and bedside medical notes. Data included age, sex, comorbidities, body mass index (BMI), smoking and alcohol status, injury severity score (ISS), mechanism of injury, lactate, systolic blood pressure, heart rate (HR), international normalised ratio (INR), partial thromboplastin time (PTT) ratio, and Glasgow Coma Scale (GCS). A Charlson Comorbidity Index was calculated for all patients based on the presence

of comorbidities. Since it is possible that blood transfusion might also increase levels of cfDNA in transfused patients due to the cfDNA within donated blood products[27], patients who had been transfused between time points were recorded. Similarly, since surgery may also increase levels of cfDNA due to direct injury, patients who had surgery between time points were noted.

4.2.8. Outcomes

Outcomes included 30-day mortality, thromboembolic events, as well as hospital-free and ICU-free days (calculated as 30 minus the number of days in hospital and ICU respectively).

4.2.9. Data analysis

Normality of data was tested using the Shapiro-Wilk test. Continuous data are presented as mean and standard deviation (SD) for normal data, and median and interquartile range (IQR) for non-normal data. Categorical data are presented as N (%). Continuous data are compared using *t*-tests for normal data and Mann-Whitney U tests for non-normal data as appropriate. Categorical data are compared using Fisher's exact tests. Correlations have been tested using Spearman's rank correlation co-efficient. The relationships between cfDNA and endothelial biomarkers were examined at both time points using multivariable linear regression models that included the covariates of gender, age, ISS, GCS, lactate, SBP, and HR. These covariates were pre-specified before data analysis, on the basis of clinical suspicion that they may be confounding variables. In order to determine whether levels of endothelial biomarkers followed the same directional change as cfDNA between time points, a model was fitted to examine the value of cfDNA at the

second time point as a function of the change in Syndecan-1 and thrombomodulin values, modified by other covariates (gender, age, ISS, GCS, lactate, SBP, and HR) and the value of cfDNA at the first time point. A *p*-value of < 0.05 was considered significant. Analyses were performed using R version 3.2.2 (http://www.r-project.org) and GraphPad Prism version 7.0 (GraphPad Software, California, USA).

4.3. Results

4.3.1. Patient characteristics

There were 122 patients included (105 from Cohort A and 17 from Cohort B), with a mean age of 41 (SD 19) and median ISS of 25 (IQR 12–34). A flow diagram of patients and available cfDNA at each time point is illustrated in Figure 4.1. Five of the patients in Cohort A did not have sufficient plasma and serum samples at the pre-hospital time point for comparison between biomarkers, making the denominator for the pre-hospital time point N=100. There were a further 12 patients in Cohort A that had insufficient samples for the second time point, making the denominator for the in-hospital time point N=110 (N=93 from Cohort A, and N=17 from Cohort B). There were therefore 88 patients with samples from both time points in Cohort A.

The patient demographic and injury-related details are shown in Table 4.1, and are compared between the two cohorts. Patients in Cohort B had worse physiological parameters for haemodynamic shock and coagulopathy when compared to Cohort A, with lower median SBP (90 *vs.* 114; $p = 0.0002$) and HR (105 *vs.* 87; $p = 0.0004$), as well as higher INR and PTT ratio (both $p = 0.020$). Otherwise their demographic and injury patterns were not significantly different between cohorts. Cohort B had a significantly lower number of

ICU-free days than those in Cohort A (8 *vs.* 22; $p = 0.003$), but there were no significant differences in other outcomes between groups.

Table 4.1. Patient characteristics according to patient cohort

Characteristic	All (N=122)	Cohort A (N=105)	Cohort B (N=17)	*p*-value
Age, mean (SD)	41 (19)	41 (20)	40 (18)	0.816
Male, N (%)	108 (87)	90 (86)	16 (94)	0.466
BMI, median (IQR)	25 (23–28)	25 (23–28)	25 (22–29)	0.891
Smoker, N (%)	55 (45)	47 (45)	8 (47)	>0.999
Alcohol intoxication, N (%)	19 (16)	19 (18)	0 (0)	0.071
Co-morbidities				
N (%)	26 (21)	23 (22)	3 (18)	>0.999
CCI, median (range)	0 (0–5)	0 (0–5)	0 (0–4)	0.814
ISS, median (IQR)	25 (12–34)	24 (10–36)	27 (17–34)	0.446
Injury mechanism, N (%)				
Blunt	101 (81)	86 (82)	13 (76)	0.738
Penetrating	23 (19)	19 (18)	4 (24)	
GCS, median (IQR)	12 (4–15)	13 (4–15)	9 (3–14)	0.232
Lactate (mmol/l), median (IQR)	3.7 (2.5–6.4)	3.5 (2.5–6.3)	6.0 (3.6–10.2)	0.071
SBP (mmHg), median (IQR)	111 (97–122)	114 (99–124)	91 (61–108)	0.0002*
HR (min^{-1}), median (IQR)	89 (79–100)	87 (76–99)	108 (98–118)	0.0004*
INR, median (IQR)	1.1 (1.0–1.2)	1.1 (1.0–1.2)	1.2 (1.1–1.2)	0.020*
PTT ratio, median (IQR)	0.9 (0.8–1.0)	0.9 (0.8–0.9)	0.9 (0.9–1.1)	0.020*
Outcomes				
Hospital-free days, median (IQR)	6 (0–20)	8 (0–20)	0 (0–5)	0.097
ICU-free days, median (IQR)	20 (8–29)	22 (10–29)	8 (0–18)	0.003*
Mortality, n (%)	19 (16)	16 (15)	3 (18)	0.728
PE, n (%)	1 (0.8)	1 (1.0)	0 (0)	>0.999

All continuous data are presented as either mean or median, with standard deviation or interquartile range in parentheses respectively, as indicated (with the exception that Charlson Comorbidity Index is shown with range in parentheses); categorical data are presented as N, with percentage in parentheses.

*Significant according to Mann-Whitney test

BMI: body mass index; CCI: Charlson Comorbidity Index; ISS: injury severity score; GCS: Glasgow Coma Scale; SBP: systolic blood pressure; HR: heart rate; INR: international normalised ratio; PTT: partial thromboplastin time; ICU: Intensive Care Unit; PE: pulmonary embolism

When patients who received blood product transfusion were compared to those with no transfusion, they had a higher median lactate (6.2 mmol/l *vs.* 3.2 mmol/l; $p < 0.0001$), lower median SBP (93 mmHg *vs.* 116 mmHg; $p < 0.0001$), and higher median HR (97 min^{-1} *vs.* 87 min^{-1}; $p = 0.018$) (Table 4.2). Although the transfused group had a higher median ISS than the non-transfused group, this did not reach statistical significance.

Table 4.2. Patient characteristics according to requirement for transfusion

Characteristic	All (N=122)	Received transfusion (N=31)	No transfusion (N=91)	*p*-value
Age, mean (SD)	41 (19)	43 (21)	41 (19)	0.503
Male, n (%)	108 (87)	27 (87)	79 (87)	>0.999
ISS, median (IQR)	25 (12–34)	27 (16–43)	23 (10–30)	0.061
Injury mechanism, n (%)				
Blunt	101 (81)	23 (74)	76 (84)	0.290
Penetrating	23 (19)	8 (26)	15 (16)	
GCS, median (IQR)	12 (4–15)	10 (3–14)	13 (4–15)	0.133
Lactate (mmol/l), median (IQR)	3.7 (2.5–6.4)	6.2 (4.0–9.5)	3.3 (2.3–5.1)	<0.0001*
SBP (mmHg), median (IQR)	111 (97–122)	93 (69–109)	116 (103–124)	<0.0001*
HR (min^{-1}), median (IQR)	89 (79–100)	97 (81–115)	87 (76–99)	0.018*

All continuous data are presented as mean or median, with standard deviation or interquartile range in parentheses respectively; categorical data are presented as N, with percentage in parentheses.
*Significant according to Mann-Whitney test
ISS: injury severity score; GCS: Glasgow Coma Scale; SBP: systolic blood pressure; HR: heart rate

4.3.2. Association between endothelial biomarkers and cfDNA

When samples were analysed at each specific time point (N=100 for pre-hospital, and N=110 for 1–16h time points), there were significant correlations between cfDNA and both syndecan-1 and thrombomodulin at both time points (Figure 4.2). When Cohorts A

and B were individually analysed, there were similarly significant correlations between cfDNA concentration and concentrations of syndecan-1 and thrombomodulin (all $p < 0.05$). When the covariates of gender, age, GCS, lactate, SBP, HR, and ISS were compared to all biomarkers using univariate analyses (Table 4.3), pre-hospital syndecan-1 was significantly associated with lactate ($R^2 = 0.084$; $p = 0.004$) and SBP ($R^2 = 0.040$; $p = 0.045$), and pre-hospital cfDNA was significantly associated with ISS ($R^2 = 0.098$; $p = 0.002$). In-hospital syndecan-1 was significantly associated with lactate ($R^2 = 0.198$; $p < 0.0001$) and SBP ($R^2 = 0.100$; $p = 0.001$); in-hospital cfDNA was significantly associated with GCS ($R^2 = 0.051$; $p = 0.016$) and ISS ($R^2 = 0.86$; $p = 0.002$); in-hospital thrombomodulin was associated with lactate ($R^2 = 0.094$; $p = 0.001$).

Table 4.3. Univariate analyses of covariates

Covariate Goodness of Fit		Prehospital			In-hospital		
		cfDNA	SD-1	TM	cfDNA	SD-1	TM
Age	R^2	0.006	0.036	<0.0001	<0.0001	0.001	0.005
	***p*-value**	0.432	0.055	0.969	0.833	0.778	0.472
GCS	R^2	0.032	0.011	0.003	0.051	0.008	0.015
	***p*-value**	0.069	0.297	0.613	0.016*	0.356	0.197
Lactate	R^2	0.006	0.084	0.017	0.032	0.198	0.094
	***p*-value**	0.455	0.004*	0.201	0.064	<0.0001*	0.001*
SBP	R^2	0.014	0.040	<0.0001	0.031	0.100	0.027
	***p*-value**	0.242	0.045*	0.905	0.063	0.001*	0.085
HR	R^2	<0.0001	0.003	0.001	0.018	0.027	<0.0001
	***p*-value**	0.913	0.579	0.806	0.16	0.082	0.898
ISS	R^2	0.098	0.011	0.001	0.086	0.002	0.029
	***p*-value**	0.002*	0.314	0.809	0.002*	0.679	0.081

cfDNA: cell free DNA; SD-1: syndecan-1; TM: thrombomodulin; GCS: Glasgow Coma Scale; SBP: systolic blood pressure; HR: heart rate; ISS: injury severity score.
*Indicates statistically significant using linear regression

Using multivariable linear regression, the relationships between cfDNA and both syndecan-1 and thrombomodulin were independent of gender, age, GCS, lactate, SBP, and HR. However, there was an additional significant relationship between ISS and cfDNA. The modelled relationship between cfDNA and syndecan-1 at increasing ISS values can be visualised in Figure 4.3.

4.3.3. Blood transfusion and surgery

When patients were divided into those who had surgery in between time points and those that did not, there were 43/122 (35%) patients who had surgery. There were no significant differences in cfDNA, syndecan-1, or thrombomodulin levels at either baseline or at the second time point between those who had surgery between time points and those who did not (Table 4.4).

When patients were divided into those who had blood transfusion in between time points and those who did not, there were 31/122 (25%) who were transfused. There were no significant differences in cfDNA, syndecan-1, or thrombomodulin levels between groups at baseline (Table 4.5). After blood product transfusion in ED, there were no differences in cfDNA or thrombomodulin between the transfused and non-transfused groups, but there was a significantly higher concentration of syndecan-1 amongst the transfused group (107 ng/ml *vs.* 43 ng/ml; $p = 0.0005$) (Table 4.5).

Figure 4.2. Association between (a) syndecan-1 and (b) thrombomodulin and cell-free DNA (cfDNA) according to time points. *P*-values are indicated according to Spearman's rank correlation coefficient. For both concentrations of (a) syndecan-1 and (b) thrombomodulin, there is a significant correlation with concentration of cfDNA at both time points.

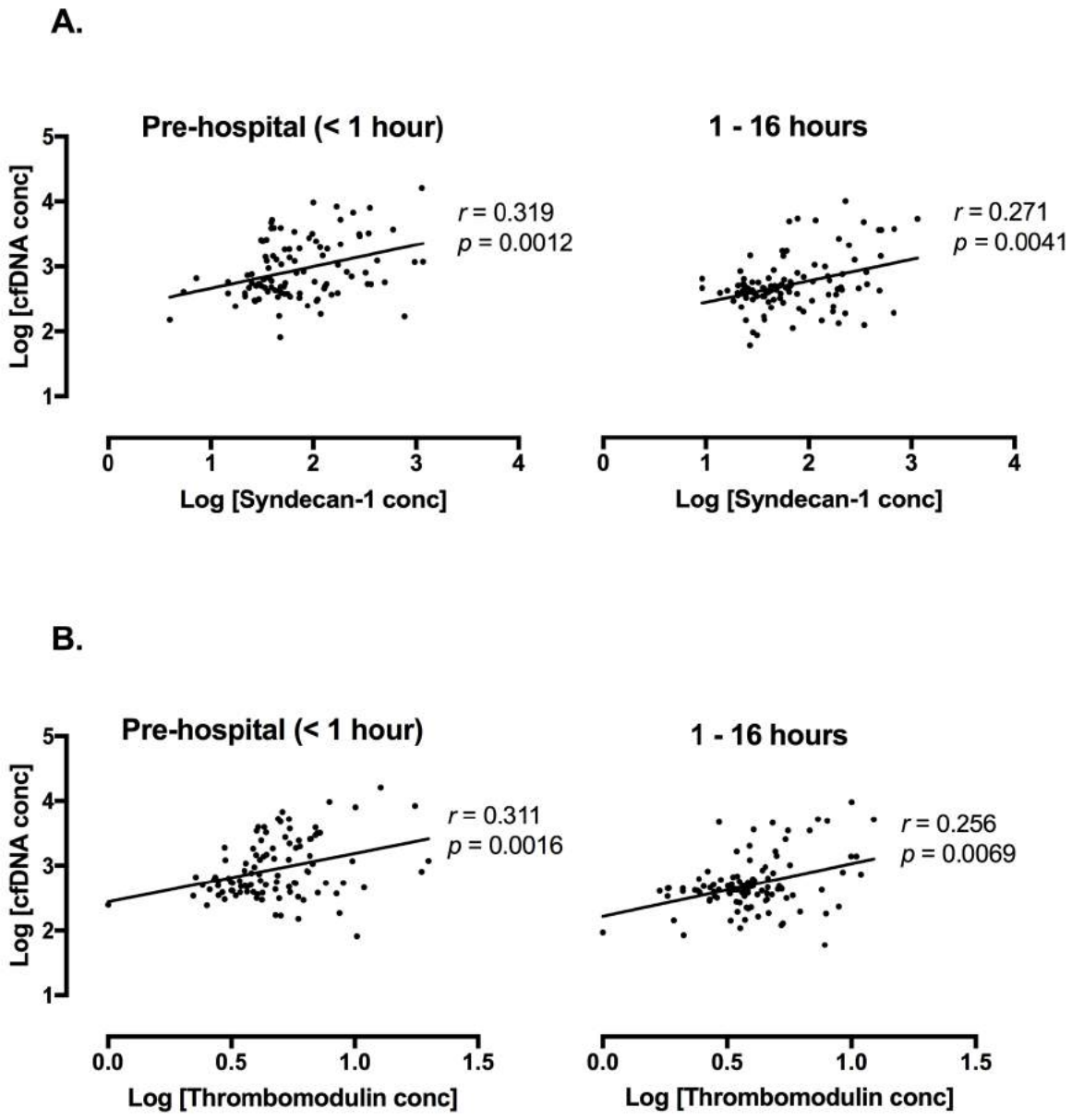


Figure 4.3. Relationship between cell-free DNA and syndecan-1 at (a) first (pre-hospital) time point and (b) second (in-hospital) time point, according to category of increasing injury severity. Solid lines represent the mean predicted values (based on the fitted model), and the shaded areas represent the range of values covered by the 95% confidence intervals associated with those predicted values.

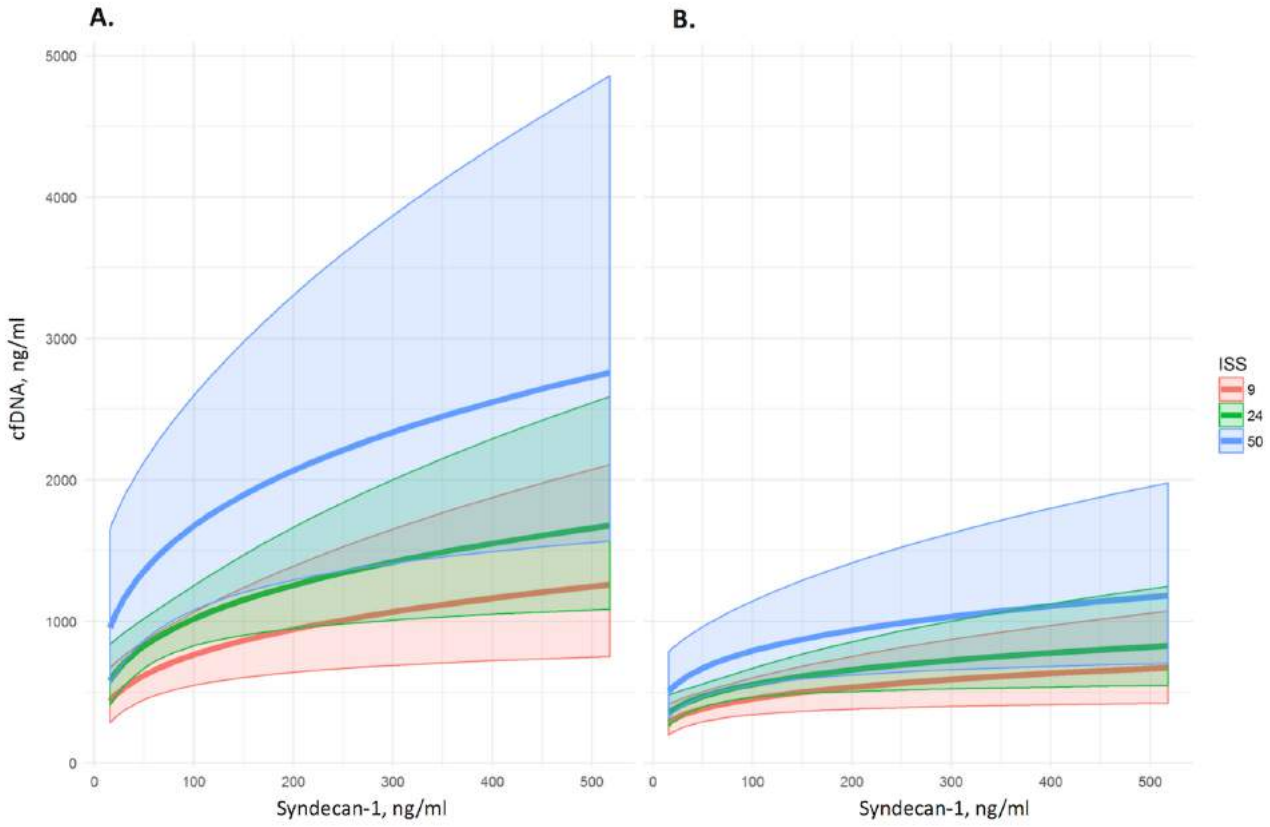


Table 4.4. Biomarker concentrations at both time points compared between patients that had surgery between time points and those who did not.

Biomarker	Had surgery (N=43)	No surgery (N=79)	*p*-value
Pre-hospital biomarkers, ng/ml			
cfDNA	558 (384–956)	817 (422–2442)	0.132
Syndecan-1	49 (35–118)	59 (33–136)	0.700
Thrombomodulin	4.3 (3.4–6.2)	4.7 (3.7–6.3)	0.508
1-16h biomarkers, ng/ml			
cfDNA	411 (233–1141)	457 (368–609)	0.231
Syndecan-1	65 (37–197)	46 (26–111)	0.110
Thrombomodulin	4.0 (3.5–5.4)	3.7 (3.0–4.6)	0.087

All summary data are presented as median, with interquartile range in parentheses.
cfDNA: cell-free DNA

Table 4.5. Biomarker concentrations at both time points compared between patients that received a blood transfusion and those who did not.

Biomarker	Received transfusion (N=31)	No transfusion (N=91)	*p*-value
Pre-hospital biomarkers, ng/ml			
cfDNA	828 (379–3174)	645 (410–1839)	0.501
Syndecan-1	109 (35–281)	50 (34–118)	0.095
Thrombomodulin	5.7 (3.5–7.1)	4.4 (3.6–5.9)	0.248
1-16h biomarkers, ng/ml			
cfDNA	466 (299–1790)	433 (346–570)	0.191
Syndecan-1	107 (43–256)	43 (25–89)	0.0005*
Thrombomodulin	4.1 (3.5–5.2)	3.7 (2.9–4.7)	0.073

All summary data are presented as median, with interquartile range in parentheses.
*Significant according to Mann-Whitney test
cfDNA: cell-free DNA

4.3.4. Biomarkers and coagulopathy

Table 4.6 shows the correlations between the three biomarkers (cfDNA, syndecan-1, and thrombomodulin) at both time points, and both admission INR and PTT ratios. Syndecan-1 levels were significantly correlated with INR and PTT ratios at both the pre-hospital ($p = 0.036$ and $p = 0.002$ respectively) and in-hospital ($p = 0.010$ and $p < 0.0001$ respectively) time points. cfDNA levels were only significantly correlated with INR at the pre-hospital time point (with a borderline significant *p*-value of 0.048), and with PTT ratio at the in-hospital time point ($p = 0.028$). Thrombomodulin was only significantly correlated with PTT ratio at the in-hospital time point ($p = 0.001$).

Table 4.6. Correlations between biomarker concentrations at both pre-hospital (<1h) and in-hospital (1-16h) time points and admission INR and PTT ratios

	Correlation with biomarkers, *r* (95% CI)			
Biomarker	**INR**	***p*-value**	**PTT ratio**	***p*-value**
Pre-hospital biomarkers				
cfDNA	0.195 (0.00–0.380)	0.048*	0.000 (-0.218–0.220)	0.993
Syndecan-1	0.208 (0.01–0.392)	0.036*	0.338 (0.125–0.521)	0.002*
Thrombomodulin	0.119 (-0.084–0.311)	0.119	0.195 (-0.029–0.399)	0.078
1-16h biomarkers				
cfDNA	0.054 (-0.157–0.259)	0.607	0.222 (0.018–0.407)	0.028*
Syndecan-1	0.243 (0.053–0.415)	0.010*	0.428 (0.244–0.582)	<0.0001*
Thrombomodulin	0.085 (-0.109–0.272)	0.377	0.328 (0.132–0.500)	0.001*

All summary data are presented as *r*, with 95% confidence intervals in parentheses.

*Significant according to Spearman's rank correlation coefficient

INR: international normalised ratio; PTT: partial thromboplastin time; cfDNA: cell-free DNA

4.3.5. Biomarker levels over time

For patients in Cohort A with cfDNA results for both time points (n=88), the differences in cfDNA concentrations between time points were compared to differences in concentrations of biomarkers of endotheliopathy. When a model was fitted to examine the value of cfDNA at the second time point as a function of the change in syndecan-1 and thrombomodulin levels, increases in the change in syndecan-1 and thrombomodulin between time points were associated with statistically significant increases in cfDNA levels, even accounting for the cfDNA value at the first time point. With all other covariates held at fixed values, a 50 ng/ml change in syndecan-1 between time points corresponded to a 15% increase in cfDNA levels (95% CI 7–23%; $p = 0.0002$) (Figure 4.4.a), and a 1 ng/ml change in thrombomodulin between time points corresponded to a 20% increase in cfDNA levels (95% CI 12–29%; $p < 0.0001$) (Figure 4.4.b).

4.3.6. Biomarker levels and outcomes

When cfDNA levels were compared between those who died within 30 days and those who survived, those who died had significantly higher levels at both the pre-hospital (1170 (IQR 538–5279) ng/ml *vs.* 617 (IQR 391–1731) ng/ml; borderline *p*-value of 0.049) and in-hospital (636 (IQR 410–1569) ng/ml *vs.* 432 (IQR 322–577) ng/ml; $p = 0.030$) time points (Figure 4.5.a). There were no significant differences between groups in terms of survival for the pre-hospital or in-hospital levels of syndecan-1 (Figure 4.5.b) or thrombomodulin (Figure 4.5.c).

Figure 4.4. Relationship between cfDNA levels at the second time point and change in (a) syndecan-1 and (b) thrombomodulin concentrations between time points. Solid lines represent the mean predicted values (based on the fitted model), and the shaded areas represent the range of values covered by the 95% confidence intervals associated with those predicted values.

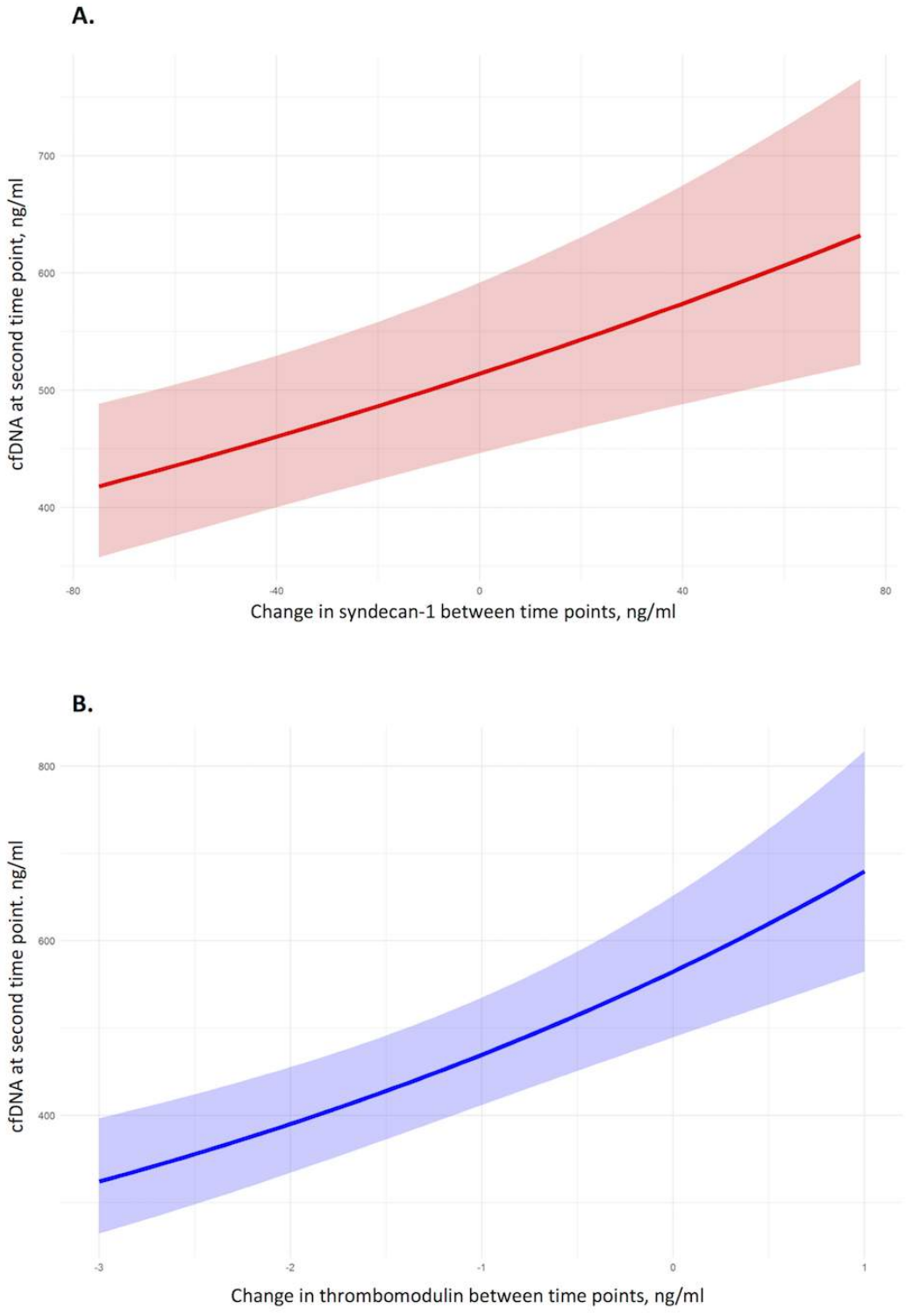
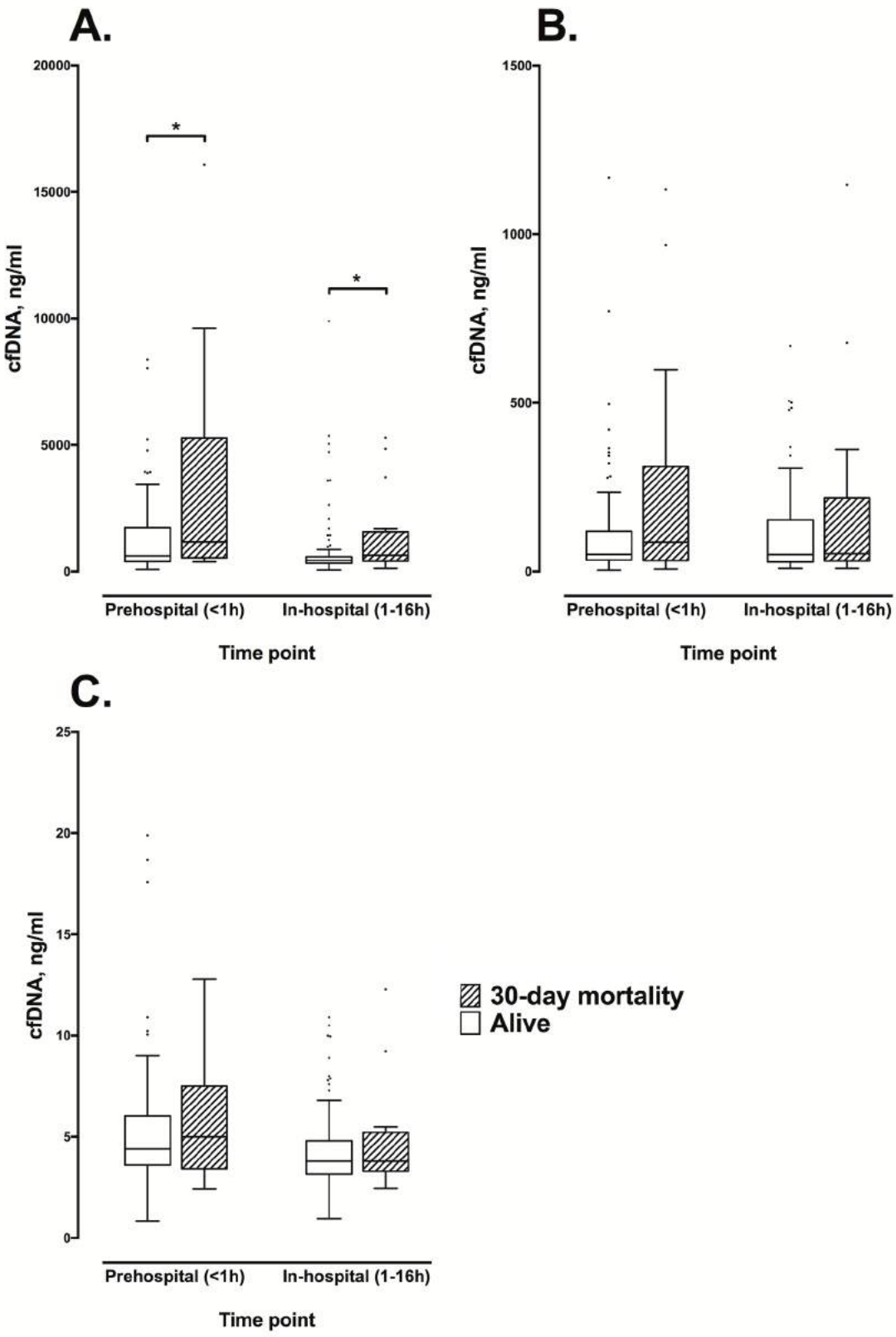


Figure 4.5. Comparison at both time points according to 30-day mortality for levels of (a) cell-free DNA; (b) syndecan-1; and (c) thrombomodulin. **p* < 0.05 according to Mann-Whitney *U* test



When hospital-free days and ICU-free days were compared to both pre-hospital and in-hospital biomarkers, there were significant negative correlations between cfDNA and syndecan-1 levels and these outcomes, but thrombomodulin levels did not have any significant correlations (Table 4.7). Only one patient had a thromboembolic event (Table 4.1), therefore no comparisons could be made with biomarker levels.

Table 4.7. Correlations between biomarker concentrations at both pre-hospital (<1h) and in-hospital (1-16h) time points and length of stay outcomes

	Correlation with outcomes, *r* (95% CI)			
Biomarker	**Hospital-free days**	***p*-value**	**ICU-free days**	***p*-value**
Pre-hospital biomarkers				
cfDNA	-0.390 (-0.546 – -0.207)	<0.0001*	-0.385 (-0.542 – -0.201)	<0.0001*
Syndecan-1	-0.217 (-0.399 – -0.017)	0.029*	-0.187 (-0.373 – 0.014)	0.060
Thrombomodulin	-0.140 (-0.331 – 0.062)	0.161	-0.130 (-0.321 – 0.072)	0.194
1-16h biomarkers				
cfDNA	-0.331 (-0.490 – -0.150)	0.0003*	-0.334 (-0.493 – -0.154)	0.0003*
Syndecan-1	-0.224 (-0.399 – -0.034)	0.018*	-0.207 (-0.384 – -0.016)	0.029*
Thrombomodulin	-0.165 (-0.346 – 0.028)	0.083	-0.178 (-0.357 – 0.015)	0.062

All summary data are presented as *r*, with 95% confidence intervals in parentheses.
*Significant according to Spearman's rank correlation coefficient

ICU: intensive care unit; cfDNA: cell-free DNA

4.4. Discussion

The main finding from the current study is that there is an association between circulating levels of cfDNA and biomarkers of endotheliopathy following trauma, independent of physiological parameters. Neither transfusion nor surgery were associated with higher levels of cfDNA. There were associations between levels of cfDNA and clinical outcomes. These associations were observed in both pre-hospital (<1h) and in-hospital (1-16h) environments, and changes in endothelial biomarkers had the same directional change as those of cfDNA between these time points. These findings suggest that the presence of cfDNA within the circulation and endotheliopathy of trauma may be mechanistically linked, confirming findings of previous pre-clinical [10, 23, 28, 29] and clinical [15, 24, 25] studies. To our knowledge, our study is the first to report this association within the pre-hospital period of evacuation following injury, supporting the hypothesis that this is an early phenomenon, occurring within minutes, rather than hours, of injury. The half-life of cfDNA has been reported as under 2 hours [30], which implies that persistently elevated levels of cfDNA over subsequent days may be due to sustained production or reduced clearance by DNase. Since the half-lives are similarly short for syndecan-1 [31, 32] and thrombomodulin [33], our findings that the rise or fall in cfDNA is associated with similar patterns in endothelial biomarker levels suggest that they may share a common pathway.

An observational clinical study of this kind cannot demonstrate a causal link between endotheliopathy and increase in release of cfDNA, or whether an increase in cfDNA from injured tissues causes endotheliopathy, making pre-clinical data the best source for hypotheses. There is evidence that NETs cause disruption to the cell-cell contacts between endothelial cells due to the elastase-mediated proteolysis of VE-cadherin and nuclear translocation of β-catenin[29]. When NETs are free in the circulation, they may promote tissue

injury and oedema [34], and can bind to endothelial cells and cause direct injury [28]. After prolonged exposure to NETs, endothelial cells are more likely to die than non-exposed cells or those only exposed for a short period of time [35]. We propose that the most likely sequence of events is that cfDNA from cellular injury, surgical intervention, and the production of NETs by circulating activated immune cells injures the vascular endothelium, which in turn releases new cfDNA.

There has been recent evidence that in addition to endogenous sources of cfDNA, injured patients may be exposed to further fragments of mtDNA from transfused packed red blood cells, plasma, and platelets, and that delivery of these to patients may increase the risk of transfusion-related lung injury and acute respiratory distress syndrome [36, 37]. In the current study, blood transfusion was not associated with higher levels of cfDNA, even when the transfusion group had worse parameters for perfusion (lactate) and haemodynamic compromise (SBP and HR). The significantly higher syndecan-1 levels in the transfused group may represent a greater amount of glycocalyx shedding amongst the patients with haemorrhagic shock, a finding in keeping with other studies of the endothelium following trauma[38]. Although we were not able to confirm the previous findings that transfusion may increase the amount of cfDNA, these were a heterogeneous group of patients, and non-randomised. It is unknown whether differences may have occurred after our study time points (i.e. >16 hours).

Biomarkers such as cfDNA, syndecan-1, and thrombomodulin have not yet entered clinical utility, and their roles within diagnosis and management of trauma are still uncertain. Since the current study has shown that raised levels of these biomarkers are associated with poorer clinical outcomes such as mortality and length of stay in hospital or ICU, they may therefore have some prognostic value. Their potential role in diagnosis and

treatment is not yet known. Best practice guidelines recommend the use of goal-directed therapy aimed at correcting coagulopathy using viscoelastic assays such as rotational thromboelastometry or thromboelastography [39]. Since cfDNA, thrombomodulin, and syndecan-1 have all been shown to be associated with coagulopathy (both hypercoagulability[8, 9] and hypocoagulability with fibrinolysis[14, 15])—a finding that was confirmed in the current study—they represent possible sources of further information to the trauma clinician during resuscitation in addition to viscoelastic assays. A previous investigation reported better patient outcomes when there was a decrease in cfDNA between time points [40]. In our study, a decrease in cfDNA was associated with a similar decrease in both syndecan-1 and thrombomodulin. The reduction in these biomarkers suggests a relative restoration of the endothelium and reduction in cell injury and NET production, which may partly explain any better clinical outcomes previously observed.

The current study combines analysis of blood samples from two different cohorts of trauma patients; Cohort A included patients who had any form of injury as long as it was likely to have an ISS >8, whereas Cohort B included patients with severe injuries and the additional burden of haemorrhagic shock. Despite the differences in these cohorts, there was an equally compelling relationship between cfDNA and endotheliopathy within each cohort as well as within the whole group. Furthermore, the association between syndecan-1, thrombomodulin, and cfDNA remained consistent independently of physiological and transfusion status. It is tempting to conclude that these biomarkers represent a common pathway. However, it is possible that other confounding variables are responsible for the elevation and decrease in both cfDNA and endothelial biomarkers. For example, levels of mtDNA have been linked to surgical trauma, injury severity and volume of intravenous fluids delivered [41, 42]. The current study shows a relationship between ISS and cfDNA. The precise

relationship between cfDNA and the endothelium, although examined in detail in pre-clinical studies, requires further analysis in humans. In particular, attention should be given to causality between these biomarkers, so that a full narrative of trauma from initial injury to endotheliopathy, coagulopathy, and organ dysfunction might be better understood.

4.4.1. Limitations

The current study reports a relatively high burden of injury (median ISS of 25) amongst patients, and therefore the findings may not necessarily be translatable to less severely injured patients. However, the range in ISS and injury mechanisms within the study cohorts may increase the reliability and translatability of the main findings. The number of patients is relatively low, and from a single Major Trauma Centre in a developed trauma network. Our findings may benefit from corroboration in further sites and other trauma systems. In particular, our trauma network does not currently deliver pre-hospital blood products, and it is unknown what effects these may have on cfDNA and endothelial biomarkers before arrival in hospital.

Although we report markers of endotheliopathy, the clinical impact (such as the potential for soft tissue oedema and alterations to normal fluid balance) was not examined in the current study. Thromboelastography was not available during this study. Instead, INR was used as a marker of acute traumatic coagulopathy as previously described[43]. Further clinical investigations of the impact of endotheliopathy on vascular permeability and thromboelastography are warranted.

4.5. Conclusion

There is an association between concentrations of cfDNA and markers of endotheliopathy (syndecan-1 and thrombomodulin) within the circulation following injury that is independent of physiological parameters. This relationship is present within the first hour of injury, persists over time, with an increase or decrease in concentration of one biomarker being reflected by a similar change in the others. There is an association between cfDNA and injury severity, and higher levels of cfDNA are associated with longer lengths of stay and mortality. Although causality cannot be established, these findings are in keeping with previous evidence that there is a close mechanistic relationship between circulating DNA, vascular endothelial injury, and poorer clinical outcomes.

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PART II

CLINICAL APPLICATION OF EARLY MICROCIRCULATORY MONITORING FOLLOWING TRAUMATIC HAEMORRAGIC SHOCK

Chapter 5

Microcirculatory assessment is safe and feasible for patients with traumatic haemorrhagic shock in the emergency department

The following chapter is adapted from the published article:

Naumann DN, Mellis C, Smith IM, Mamuza J, Skene I, Harris T, Midwinter MJ, Hutchings SD. Safety and feasibility of sublingual microcirculation assessment in the emergency department for civilian and military patients with traumatic haemorrhagic shock: a prospective cohort study. *BMJ Open*. 2016;6(12):e014162.

5.1. Introduction

In Part 1 of this thesis, we discussed the relationship between endotheliopathy of trauma and microcirculatory flow disruption, and that these pathological processes are likely to occur very early after injury, and are associated with organ failure. Early detection of microcirculatory failure may be desirable to the clinician during the assessment and resuscitation of patients, and may offer a target for goal-directed therapy. However, such utility has not yet been realised in clinical practice. In order to determine whether the clinician might have access to microcirculatory flow dynamic parameters during trauma resuscitation, it is necessary to first demonstrate that this is both feasible and safe, even for very unwell patients. Such an exercise has not yet been undertaken within the Emergency Department, and the following study addresses this.

There has been considerable interest in the disruption of the microcirculatory endothelium and endothelial glycocalyx following traumatic haemorrhagic shock (THS)[1], as discussed in Part 1 of this thesis. Dysfunctional sublingual microcirculation following THS has been reported to be a good predictor of subsequent organ failure when measured in patients admitted to the Intensive Care Unit (ICU)[2]. The ability to maintain microcirculatory perfusion during early THS has been shown to be associated with more rapid reversal of the shock state during resuscitation in a large animal experimental model[3]. There may be some circumstances where microcirculatory flow does not follow global haemodynamics and parameters such as cardiac output and blood pressure no longer act as reliable surrogate markers for perfusion[4]. In such circumstances microcirculatory monitoring may offer more reliable guidance for resuscitation by adding information about true end-organ perfusion. The implications of bedside point-of-care microcirculatory parameters have not yet been

realised but may have far-reaching utility in both civilian and military contexts (and will be discussed in the next chapter).

Although it seems intuitive that microcirculatory readings from earlier time points closer to point of injury—especially before the definitive cessation of bleeding—may offer diagnostic and prognostic value following major trauma, this has not yet been investigated. Some investigators have performed sublingual microcirculatory assessment in the Emergency Department (ED) for patients with sepsis[5] and acute decompensated heart failure[6], but this has not yet been undertaken for trauma patients. It is possible that researchers have not attempted sublingual video-microscopy for trauma patients in the ED because of the constraints imposed by clinical urgency and environmental uncertainty, lack of capacity to consent, multiple interventions, and rapid transfer of the patient. Such a scenario is also likely to be noisy and crowded, with limited space and time at the bedside – conditions that may be even more hostile in the deployed military context. Conversely, the ICU offers a more 'placid' environment with a stationary patient, increased space and time, and more stable physiology, even when patients are critically unwell. However, by the time of ICU arrival, patients may have received multiple resuscitative interventions, with unknown impact on the predictive value of sublingual video microscopy. It is therefore important to establish the feasibility of microcirculatory monitoring within the ED as a basis for studies to determine its clinical utility.

I will present for the first time the feasibility of obtaining sublingual video-microscopy video clips during the emergency presentation of patients with THS in the ED. It was hypothesised that non-invasive microcirculatory imaging in this emergency context is safe, feasible, does not interfere with clinical management, and provides data of sufficient quality for meaningful analysis (Hypothesis 6; Table 1.1).

5.2. Methods

5.2.1. Study design and setting

A prospective observational pilot study was undertaken to assess whether sublingual video-microscopy to image the microcirculation was feasible and safe for both civilian and military patients with THS, and whether the captured video clips were of high enough quality for analysis. Both civilian Research Ethics Committee (REC Ref 14/YH/0078) and Ministry of Defence Research Ethics Committee (MODREC Ref PPE 281/12) approvals were granted before the start of the study.

5.2.2. Patient selection

Patients were enrolled into the MICROSHOCK study (ClinicalTrials.gov Identifier: NCT02111109)[7]. Patients were eligible for inclusion if there was evidence of haemorrhagic shock, and all of the following features: (i) injury mechanism consistent with blood loss; (ii) the patient is intubated and ventilated; (iii) plasma lactate concentration >2 mmol/L; and (iv) the patient has received any blood products during initial resuscitation. Patient were recruited as soon as possible after arrival at three UK Major Trauma Centres (Queen Elizabeth Hospital, Birmingham; Kings College Hospital and Royal London Hospital, London). This was either in the ED or Intensive Care Unit (ICU). The current study includes the first 13 civilian patients recruited in ED and a further 2 deployed soldiers enrolled in the ED at the Role 3 medical facility in Camp Bastion during the Afghanistan conflict.

5.2.3. Sublingual video-microscopy

Sublingual microcirculation was visualised in the civilian patients using incident dark field (IDF) video-microscopy (Cytocam, Braedius Medical B.V., Huizen, The Netherlands).

Military patients were scanned using a sidestream dark field (SDF) device (Microvision Medical, Amsterdam, The Netherlands). IDF is a newer technology with higher resolution and larger field of view, but produces comparable results[8]. The devices are positioned towards the sublingual mucosa and maneuvered until a clear image of the microcirculation is acquired. Video clips (preferably lasting at least 5 seconds each) are then recorded and stored for offline analysis using dedicated computer software (Automated Vascular Analysis V.3.02, Microvision Medical, The Netherlands). At least 3 (but preferably 5) individual video clips are required for data analysis according to consensus agreement[9], but this does not limit the number of videos that can be captured. In this study as many videos as possible were recorded to ensure a sufficient number of analysis quality. For SDF video images continuous video was taken rather than short clips; this was later spliced into high quality segments (each lasting 5 seconds) for computer analysis.

5.2.4. Training

Sublingual video-microscopy was undertaken by dedicated research clinicians and research nurses who had been trained in the technique by an expert user and the study's Chief Investigator (S.D.H.) to a standard suitable for clinical research. Training was undertaken paying particular attention to standard quality assessment variables[10], including the optimisation of stability, focus and illumination, as well as reducing pressure artefact and ensuring that the field of view contained microcirculatory vessels. The rationale and details of these quality domains have been described in detail elsewhere[11]. Since all patients in the MICROSHOCK study are intubated, users are trained to access the sublingual area with the endotracheal tube *in situ*.

5.2.5. Capacity and consent

As described in Part I of this thesis, due to the nature of the injuries sustained and physiological status of patients, capacity to consent was absent. The REC-approved consent process for enrolment in the study was guided by the Mental Health Act, UK (2005) and is explained in more detail in the study protocol[7] and in previous chapters. In short, the physician in charge of the care of the patient (Professional Consultee) agreed on the participation of the patient. A close friend or relative could also be approached if appropriate to act as a Personal Consultee. Ultimately if the participant regained capacity they were asked for their permission to retain data already collected.

5.2.6. Data collection

Patient demographics (age, sex) and injury-related details (mechanism of injury, injury severity score (ISS)) were recorded. Physiological parameters from the pre-hospital evacuation and ED included lowest systolic blood pressure (SBP), lowest Glasgow Coma Score (GCS), and highest lactate (as a surrogate for perfusion). The number and type of blood products were recorded as a measure of haemorrhagic burden. Details regarding sublingual video-microscopy included timings of video capture, profession of user, mechanism of notification of user, number of video clips stored, total length of video capture, and type of consent were also noted.

5.2.7. Outcomes

The outcomes of interest were: (i) safety (absence of adverse events or interference with clinical management); (ii) feasibility (successful acquisition and storage of video clips); and (iii) the attainment of videos of high enough quality for meaningful data analysis.

Quality assessment was undertaken according to a standardised technique that grades 6 domains for each video (including illumination, duration, focus, content, stability, and pressure artefact)[10] by a single assessor (D.N.N) who was blinded to clinical status of the patient. Each domain was graded as optimal (0 points), suboptimal but still useable (1 point), or unacceptable and unusable (10 points). If any video clip has a score of 10 in any domain then the video was deemed unusable.

5.2.8. Minimising potential sources of bias

All patients that triggered a trauma team activation were screened for inclusion in the study, and a log was kept in order to ensure that risk of selection bias was minimised. The training of all video-microscopists was supervised and regularly assessed by the Chief Investigator to minimise the risk of inter-user heterogeneity. Quality assessment of videos was kept blinded to clinical status of the patient, study site, and video-microscopist, so that quality grading was as unbiased and consistent as possible.

5.3. Results

5.3.1. Patient characteristics

There were 15 patients (13 civilians and 2 military) included in the study. The majority of patients (12/15, 80%) were male; the median age was 41 (IQR 30 – 55) years. All patients were unconscious and intubated at time of study enrolment, and recruited into the study with agreement by a Nominated Consultee. There were no cases of subsequent withdrawal of consent from the patient once they regained capacity.

5.3.2. Injury burden and physiology

For civilian patients, the most common injury mechanism was road traffic accident (n=7), followed by crush injury (n=2), fall (n=2), penetrating trauma (n=1), and struck by a train (n=1). One military patient had been injured in an improvised explosive device (IED) blast; the other had been crushed by an armoured vehicle. The median ISS for all patients was 26 (23 – 34). The median lactate in ED was 4.6 (interquartile range (IQR) 2.8 – 7.9) mmol/L. Median SBP was 79 (IQR 68 – 105) mmHg, and median lowest GCS before intubation was 9 (IQR 5 – 12). Patients in this group received a median of 4 (IQR 1.5 – 6) units of RBCs, 2 (IQR 0 – 5) units of FFP, and 0 (IQR 0 – 0.5) units of platelets within the first 24 hours. The military patient injured by the IED received 32 units of RBCs, 31 units of FFP and 5 units of platelets.

5.3.3. Video-microscopy

The IDF device was used for 13 civilian patients, and the SDF device was used for the 2 military patients. Figure 5.1 illustrates a flow diagram of microcirculatory video acquisition. Video-microscopy was performed by a doctor for 12 patients and nurse for 3 patients. On all occasions these healthcare professionals were alerted to the arrival of the patient by phone call from the relevant ED. Video-microscopy was performed a median of 80 (IQR 58–138) minutes after arrival of the patient at the hospital. Where a CT was performed as part of trauma management (all patients), this preceded sublingual video-microscopy in all instances.

5.3.4. Safety and feasibility

Video-microscopy was successfully performed and videos stored for analysis for all

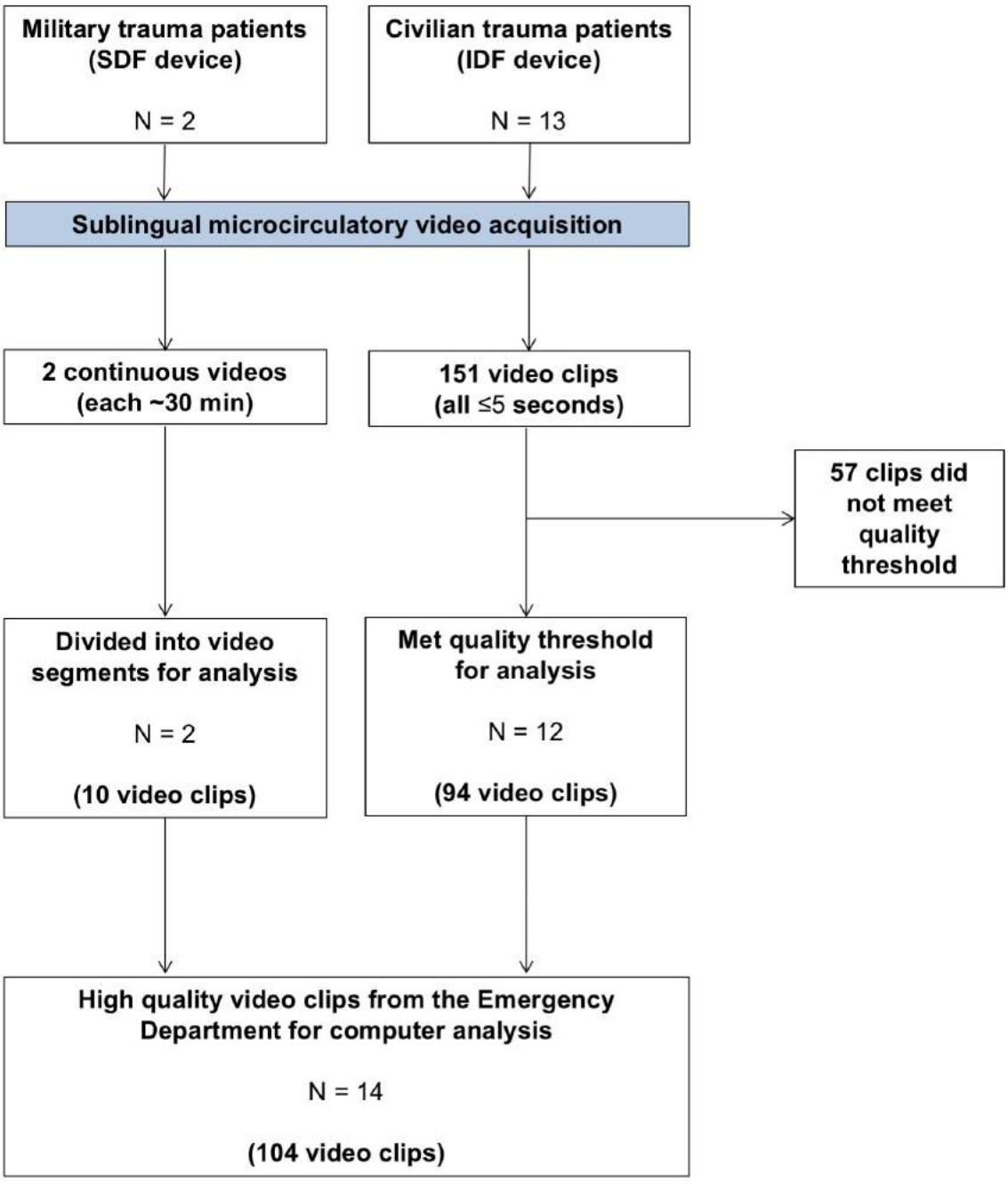
patients enrolled in ED. 161 video clips were stored for analysis, including 151 from civilian patients and 10 from military patients (the long continuous videos acquired for the military patients were spliced into 5 clips each). The median time at the bedside for video capture was 6 (IQR 5 – 8) minutes. There were no adverse events, and no incidents reported where clinical management was affected or patient care interrupted.

5.3.5. Quality assessment of videos

Of all videos retained for analysis, 104/161 (64.6%) were of suitable quality for computer analysis. These videos were acquired from 14 of the patients, with one civilian patient having no useable data. A median of 6 (IQR 5 – 10) video clips per patient were eligible for analysis, exceeding the 3 – 5 clips recommended by consensus guidance[9]. The median quality assessment score for useable videos was 2 (IQR 1 – 2). Of the 57 video clips that were unusable, 18 failed quality assessment on more than one domain. The remaining 39 video clips that failed due to a single quality domain included content (n=14), pressure (n=13), stability (n=6), illumination (n=3), focus (n=2) and duration (n=1)

Figure 5.1. Flow diagram of microcirculatory video clip acquisition for computer analysis

N indicates the number of study participants at each stage



5.4. Discussion

The main finding from this study is that early sublingual microcirculatory monitoring in the Emergency Department is feasible and safe for patients with THS, and yields videos that can be used for analysis. Investigation of patients with THS can be performed using this technique without apprehension of interference in clinical management or detriment to the patient. Such non-invasive scanning modalities are commonplace during trauma resuscitation when they are considered to add valuable information, including focused assessment with sonography for trauma (FAST), and ultrasound to guide fluid therapy[12]. Associated training and ongoing validation would be essential components if this technique were to be used in clinical practice.

Patients in this study had a considerable injury burden, with additional haemodynamic compromise according to their physiological and biochemical parameters. Sublingual microcirculatory monitoring was still feasible in this context within the very first hours of their arrival in hospital. Although the clinical utility of such readings is yet to be realised, it is possible that the availability of additional data relating to tissue perfusion may be of value in the resuscitation of such patients. Point-of-care microcirculatory monitoring is not currently used in clinical practice, but innovations to move this technique from research to the clinical domain have been proposed by our group[13] (as discussed in the next chapter) and others[14]. If point-of-care microcirculatory monitoring is deemed to be a useful resuscitation end point then it would be important to obtain readings before, during and after interventions so that changes might be recorded. The current study did not use such methodology, but further investigations into the utility of this technique are warranted.

5.4.1. Obstacles and limitations

There are known obstacles in the acquisition of early microcirculatory data, which were confirmed in this feasibility study. Patients with THS are critically unwell, and their treatment is urgent and needs to progress uninterrupted. Transfers to radiology, ICU or operating theatre cannot be paused for data acquisition without strong justification. Sublingual video-microscopy has potential to overcome some of these limitations because it is mobile and can follow the patient. We report that it takes a matter of minutes to undertake, and that there was a point in the patient pathway in all cases before patient transfer during which opportunistic video-microscopy was suitable. In all occasions where cross-sectional imaging was undertaken, video-microscopy was performed afterwards. The study investigators did not wish to interfere with the preparation or transfer of patients who needed urgent imaging. If the technique is found to have clinical utility, then there may be some justification in obtaining even earlier readings, and incorporating the technique into the resuscitative pathway.

Although feasibility has been demonstrated, one civilian patient had no videos clips of high enough quality for assessment. Time constraints and interference with video acquisition may increase the risk of such occurrences, and would require continued education, training, and maintenance of appropriate skills for data capture in less than ideal (and sometimes adverse) circumstances. User-dependency is a common feature of scanning modalities. Clinical judgment continues to be the optimal management strategy for these emergency scenarios with or without the additional data that microcirculatory monitoring might yield. There were only two military patients included in this study, and it is acknowledged that firm conclusions cannot be made with these limited data. Further validation is required in such an environment.

The majority of sublingual microcirculatory monitoring is conducted in the research domain, and early bedside point-of-care monitoring of the microcirculation for patients with THS has not been reported. Although limited by a small number of patients, the current study adds to the growing body of evidence that may justify and facilitate the transition of microcirculatory monitoring from research into clinical practice. Restoration of tissue perfusion by directing fluid and inotropic resuscitation towards microcirculatory targets appears to be a viable technique, but is yet to be tested. Some investigators have proposed that plasma may improve microcirculatory function due to its restorative properties[15]. Detection of microcirculatory dysfunction may have a role in guiding the choice or volume of fluids. Since acquisition of early microcirculatory data is feasible, it is timely to design and implement appropriate studies to examine whether microcirculatory goal-directed therapy is of benefit to patients.

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Chapter 6

The microcirculation can be assessed at the bedside using a novel point-of-care microcirculation tool

The following chapter is adapted from the published article:

Naumann DN, Mellis C, Husheer SL, Hopkins P, Bishop J, Midwinter MJ, Hutchings SD. Real-time point of care microcirculatory assessment of shock: design, rationale and application of the point of care microcirculation (POEM) tool. *Crit Care*. 2016;20(1):310.

6.1. Introduction

In the previous chapter, we discussed the safety and feasibility of bedside microcirculatory monitoring for unwell patients following trauma. However, this technology is still limited to use in research (rather than clinical practice) because the videos derived from the technique need to be analysed at length by specialist computer software, and away from the patient. In order to bring this technique into clinical practice at the bedside for unwell patients, it is necessary to create a way of assessing the videos in real time, without the need for complex analysis away from the patient. The following chapter will describe a novel assessment tool to assess the microcirculation for exactly this purpose.

Since the microcirculation is the anatomical location of oxygen and substrate exchange, its behaviour during shock is of interest to those involved in patient resuscitation. The term "haemodynamic coherence" has been used to describe a situation in which resuscitation aimed at restoring systemic haemodynamic parameters (such as cardiac output) also makes a corresponding improvement to the microcirculation[1]. Some pathological circumstances such as sepsis may cause an imbalance between global and microcirculatory parameters so that the microcirculation no longer corresponds to the macrocirculation (i.e. loss of haemodynamic coherence); in this circumstance goal-directed resuscitation targeted towards global parameters may lead to harm[1]. There is some evidence that when microcirculatory flow is impaired during circulatory shock it may not be restored even when blood pressure is improved[2]. Microcirculatory parameters may also predict clinical outcomes better than global measurements in sepsis[3] and traumatic haemorrhagic shock[4]. Improvement of microcirculatory parameters during resuscitation may also predict better outcomes following major surgery[5] and sepsis[6].

The use of hand held non-invasive sublingual video-microscopes (such as sidestream dark field (SDF) or incident dark field (IDF) microscopy) has allowed researchers to study microcirculatory flow *in vivo*, both in experimental models and in patients, as discussed in previous chapters. However, despite over a decade of detailed investigations and demonstrations of the rationale of monitoring the microcirculation, this technology and associated techniques have still not advanced from the research to clinical domains. A major limitation in the use of current video-microscope technology is that analysis depends on the capture of video clips that require offline analysis. This takes a considerable amount of time (after the clinical window of diagnostic utility). Real-time point-of-care automated (computerised) analysis has not yet been validated against traditional offline analysis. Even if computerised automated analysis were to yield accurate, validated parameters, their clinical applicability is unlikely to be meaningful without user interpretation and some form of clinical grading system that might determine particular therapeutic pathways based on target readings. This is because such a system may yield parameters of unknown clinical relevance. What is required in real-life clinical practice is a simple grading system that acts as a trigger or guide for the delivery of particular interventions. In the future, based on such algorithms, artificial intelligence may even allow this process to be automated.

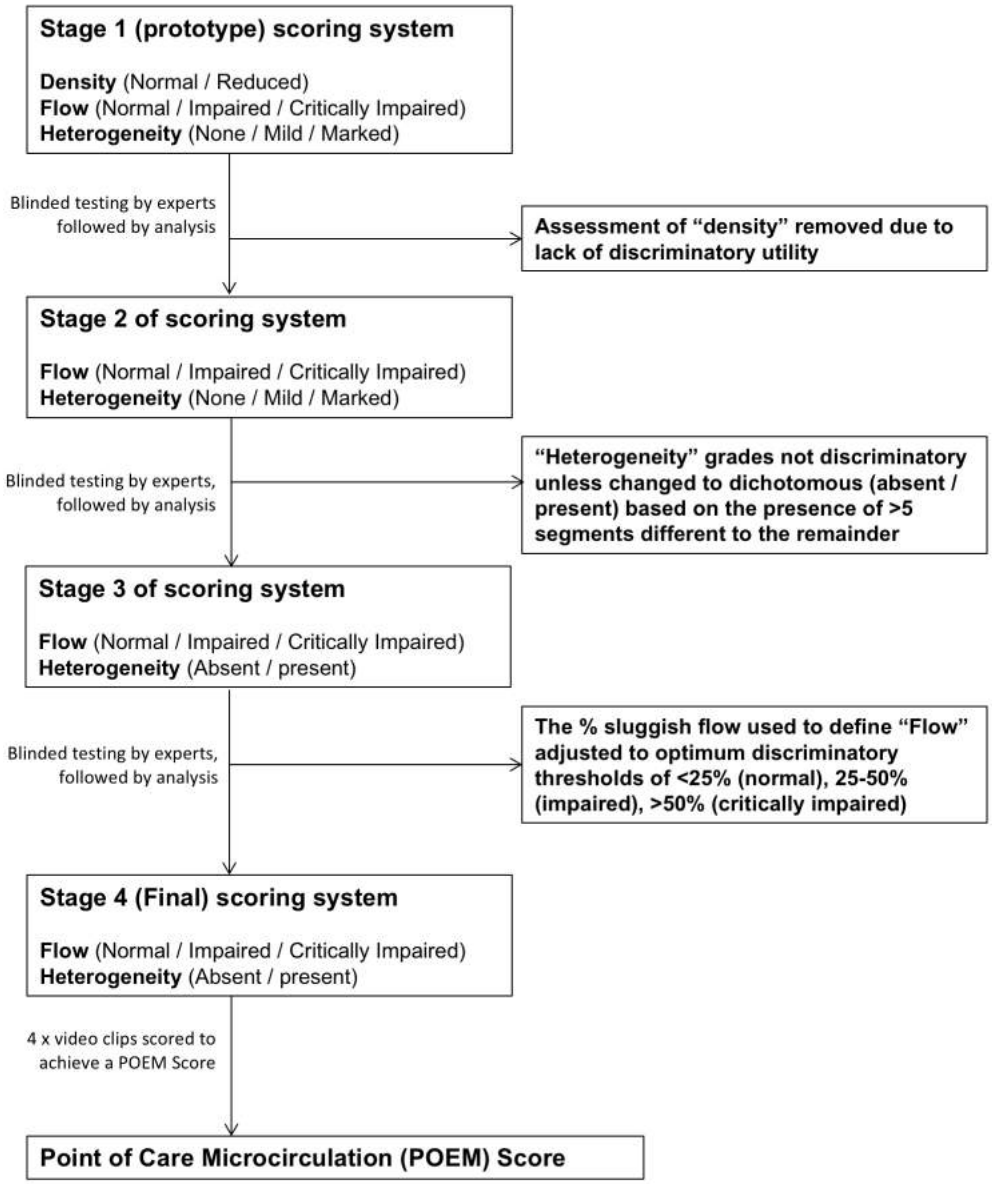
Here we describe for the first time a 5-point ordinal grading scale of microcirculatory function based on a composite of flow and heterogeneity in vessel segments viewed by sublingual video-microscopy. The score is relatively simple and can be assigned at the point-of-care. It may be one way of facilitating goal-directed therapy using microcirculatory parameters. It was hypothesised that trained professionals could use this point-of-care tool as accurately as offline computer analysis (Hypothesis 7; Table 1.1).

6.2. Methods

6.2.1. Design of the Point-of-Care Microcirculation grading system

A schematic diagram (Figure 6.1) summarises the stages in the design of the Point-of-Care Microcirculation (POEM) Score. This process was undertaken by two expert users (D.N.N. and S.D.H.), who have extensive experience in video-microscopy technique analysis.

Figure 6.1. Schematic flow diagram of the stages in development of the final POEM scoring system



The scoring system is based on the premise that flow and heterogeneity of vessel segments are the key components of interest. The final POEM score does not account for vessel density, because early trials of the scoring system that incorporated assessment of density did not demonstrate its discriminatory utility.

6.2.2. Assigning a POEM score

The final POEM scoring system is a 5-point ordinal scale that integrates assessments of both flow and heterogeneity (Table 6.1). It enables a user to assess real time microcirculatory videos obtained during sublingual IDF microscopy. It is derived from the assessment of 4 video clips from the same patient at the same time point (since a recommendation of 3 – 5 video clips corresponds to the consensus opinion in traditional analysis[7]). For each of the 4 individual video clips, the user determines the flow and heterogeneity as such:

6.2.2.1. Flow

(i) Normal: <25% of vessel segments in view are sluggish/stopped

(ii) Impaired: 25 – 50% of vessel segments in view are sluggish/stopped

(iii) Critically impaired: >50% of vessel segments in view are sluggish/stopped

6.2.2.2. Heterogeneity

Only if the user determines that a clip has “Normal” overall flow are they prompted to also assign whether heterogeneity is present or absent. This is because during the pilot phase of developing the scoring system (Figure 6.1) we found that heterogeneity was universally present in clips with “impaired” and “critically

impaired" overall flow (i.e. having an assessment of heterogeneity for such scenarios did not make any difference overall). Heterogeneity is determined for "normal" clips as "present" if >5 vessel segments demonstrate different flow to the remainder. The threshold for 5 vessels segments was used because this was found to be the most discriminating (Figure 6.1).

Each individual video clip is therefore assigned either: "critically impaired", "impaired", "normal flow with heterogeneity", or "normal flow without heterogeneity". The combination of 4 clips gives the POEM score (Table 6.1; Figures 6.2 and 6.3)

Table 6.1. Point-of-care microcirculation (POEM) grade and corresponding definitions

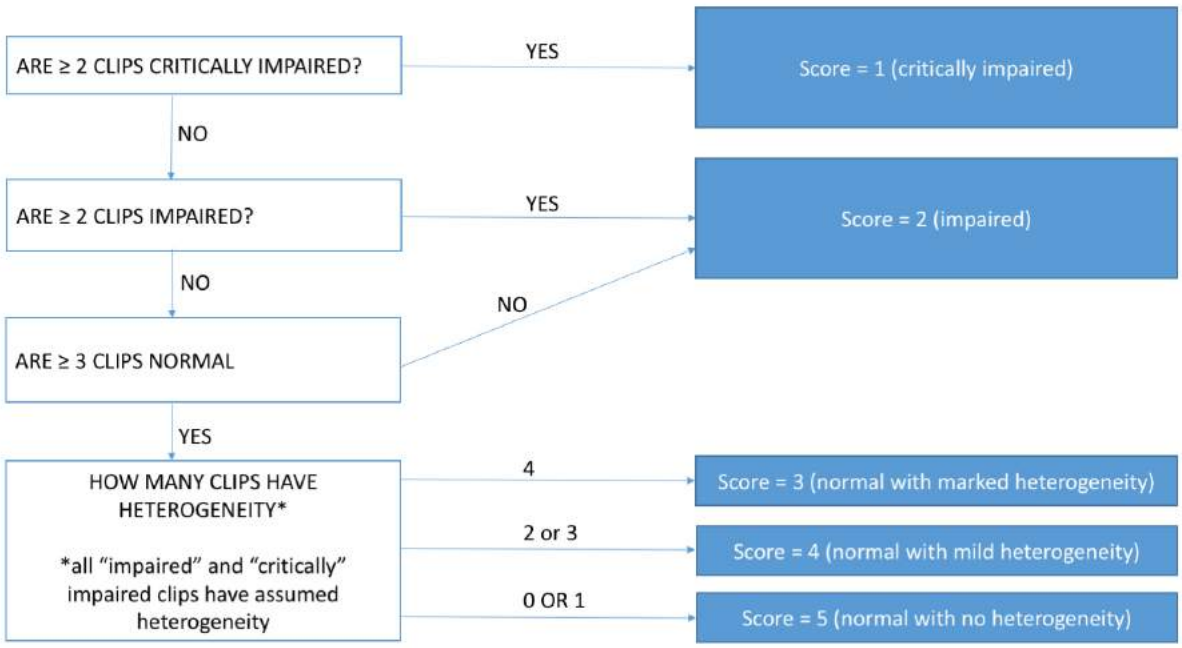
POEM Score	Microcirculatory function
5	Normal flow*, with no heterogeneity†
4	Normal flow*, with mild heterogeneity††
3	Normal flow*, with marked heterogeneity†††
2	Impaired flow** (heterogeneity is also present)
1	Critically impaired flow*** (heterogeneity is also present)

* Less than 25% of vessel segments in view are sluggish/stopped
** 25–50% of vessel segments in view are sluggish/stopped
*** More than 50% of vessel segments in view are sluggish/stopped
† 0 or 1 clips have heterogeneity present
†† 2 or 3 clips have heterogeneity present
††† All 4 clips have heterogeneity present

6.2.3. POEM Score calculation

It is the combination of all 4 video clips that gives overall POEM Score as per the algorithm in Figure 6.2. In short, if two or more clips have "critically impaired" flow, then the overall POEM score is 1 (Critically Impaired). If 2 or more clips have "impaired" flow then the overall POEM score is 2 (Impaired). If 3 or more clips have "normal" flow then they can be one of three different scores: POEM score 3 (Normal with marked heterogeneity) if all 4 clips show heterogeneity; POEM score 4 if two or three clips show heterogeneity; and POEM Score 5 if ≤1 clip has heterogeneity.

Figure 6.2. Algorithm for overall POEM Score using the parameters from 4 video clips

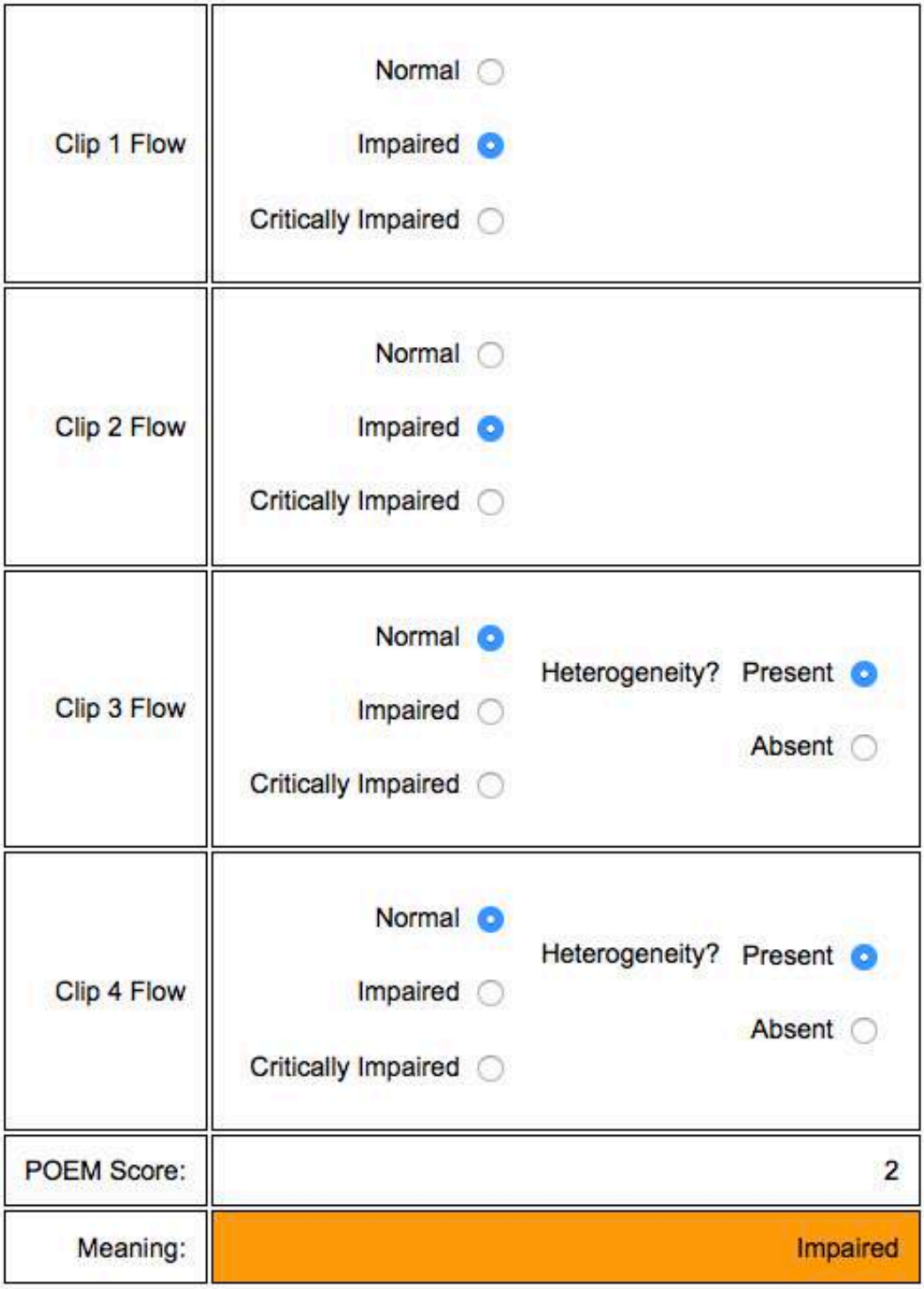


6.2.4. Online tool

An online tool (http://www.POEMscore.com) can be used to perform the calculation for the POEM Score based on the scores for all 4 video clips with minimal effort. An example

of an online POEM score calculation is shown in Figure 6.3, and is based on the algorithm illustrated in Figure 6.2.

Figure 6.3. An example of a POEM grade being assigned using the online tool. In this case the overall POEM Score is 2, indicating "Impaired" flow



6.2.5. Validation of the scoring system by healthcare professionals

A group of 32 healthcare professionals volunteered as study participants to assess the utility of the POEM score. These participants were from two UK collaborating sites (University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK, and King's College Hospital NHS Foundation Trust, London, UK). All participants were current Intensive Care Unit clinicians or nurses and had not previously used sublingual microcirculatory monitoring or interpreted video-microscopy clips.

6.2.5.1. Training

All study participants undertook a standardised 60-minute interactive training session using 18 slides and a pre-selected set of videos of varying microcirculatory dysfunction as examples. All video clips were obtained from the MICROSHOCK study[8], from patients with traumatic haemorrhagic shock (mixed blunt and penetrating trauma) and taken on day 0 or 1 of their hospital stay. The aim of the training session was to teach the participants how to assign "normal", impaired", or "critically impaired" flow to individual video clips, as well as whether heterogeneity was "present" or "absent" for "normal" clips. Teaching sessions were delivered by subject matter experts (D.N.N. and S.D.H.). The participants were given an opportunity to ask questions and re-look at some example videos before being asked to score the test sequence of video clips.

6.2.5.2. Testing

Straight after the training session, the participants were asked to view and score 5 video sequences (corresponding to 5 different patients), each of which

consisted of 4 video clips (20 clips in total). These videos were taken from the MICROSHOCK study[8], and had been recorded using an IDF video microscope recently validated for use in shock states[9] and all had been assessed as high quality according to guidelines[10]. The participants were blinded to each other's scores and the clinical status of the patients. They were allowed up to 2 minutes for each individual video clip, which was played in 'loop' until the allocated time was reached. Once all 4 clips had been watched and scored for a given sequence, the participants were not allowed to revise their scores.

6.2.6. Human *versus* offline computer analysis of video clips

A random selection of 68 individual video clips, taken at 15 time points from 8 patients that had been acquired by sublingual IDF video-microscopy during the MICROSHOCK study[8] were analysed offline using Automated Vascular Analysis V.3.02 (Microvision Medical, The Netherlands). Semi-automated analysis was utilised as described in greater detail elsewhere[8] (fully automated analysis was not performed). All video clips were rated for quality[10], and then semi-quantitative data were recorded for each video clip according to consensus guidelines[7]. These included total vessel density (TVD), perfused vessel density (PVD), proportion of perfused vessels (PPV), and microcirculatory flow index (MFI) for individual clips; and microcirculatory heterogeneity index (MHI) for each time point. Each individual video clip was assigned a random number at time of analysis, and played in random order for an expert user (S.D.H.) to grade according to the POEM scoring tool. Therefore the expert user was blinded to both the computer analysis parameters and the clinical status of the patients.

Using the assessments from these individual video clips, POEM scores were applied

to all 15 time points according to the algorithm in Figure 6.2 and web-based tool (Figure 6.3). No time limit was imposed for the expert to assign POEM scores or computer analysis. The time taken to assign POEM scores and perform computer analysis was recorded.

6.2.7. Ethics approval and consent to participate

All videos were obtained as part of the MICROSHOCK study. Research Ethics Committee (Yorkshire and Humberside – Leeds West) approval was granted for all sites before any data were collected (Ref: 14/YH/0078). Consent to participate in the study was given by all patients except where they lacked capacity, in which case either a Personal Consultee (close friend or relative) or Nominated Consultee (Physician looking after the patient but not involved in the study) gave consent on behalf of the patient, in accordance with the approved protocol.

6.2.8. Data analysis

Inter-user variability for the ordinal 5-point scale was assessed using the intra-class correlation coefficient (ICC) in terms of both consistency (e.g. do participants tend to score item A higher than item B but lower than item C) and agreement (e.g. do participants all tend to give a score of X to item A and a score of Y to item B). Values are presented with 95% confidence intervals (CI). Analysis assumed a two-way model (where both the video clips and individual participants are regarded as random samples from a potential larger pool of video clips and participants). Naïve participant scores were also compared to expert scores by subtracting the true expert score from the observed rater scores and then perform a linear regression on these 'error scores', including rater and video sequence as independent predictors.

Values from computer analysis (PVD, TVD, PPV, MFI, and MHI) were compared to ordinal POEM scores using linear regression. Further comparison was made between computer analysis parameters and both individual video scores and POEM scores as categorical variables (using Kruskal-Wallis analysis followed by step-wise paired analysis using Dunn's multiple comparisons test). A p-value of < 0.05 was considered significant.

6.3. Results

6.3.1. Study participants

There were 32 study participants, including 12 consultants, 18 training-grade doctors, and 2 intensive care nurses. All of these participants worked in their hospital's ICU. None had used sublingual video-microscopy before, and all were naïve to IDF video analysis. All participants completed the training and assessment sessions. The expert user (S.D.H.) who conducted the offline computer analysis and POEM scoring has previously analysed over 1000 video-microscopy clips.

6.3.2. Inter-user variability

When the naïve user POEM scores were analysed to determine the inter-user variability between clips and between each other, the ICC values for consistency and agreement were 0.83 (95% CI 0.626, 0.976) and 0.815 (95% CI 0.602, 0.974) respectively. From the analysis of variance between users and expert, there was greater agreement for "critically impaired" and "normal" but higher variability for the scores in between; both item and rater were significant predictors of the level of error in the scores when compared to expert ($p < 0.001$ and $p = 0.046$ respectively).

6.3.3. Human *versus* computer analysis of video clips

When traditional offline computer analysis parameters were compared to expert assigned POEM scores there was good correlation with PPV (R^2 = 0.71; p < 0.001), PVD (R^2 = 0.39; p < 0.05), MFI (R^2 = 0.75; p < 0.001), and MHI (R^2 = 0.68; p < 0.001), but not TVD (R^2 = 0.03; p = 0.535) (Figure 6.4).

In addition, when offline computer analysis parameters were compared to expert assigned grades for the 68 individual video clips, these scores corresponded well to perfusion (PPV and PVD) and flow (MFI) parameters, but there was no statistically significant relationship to the pure density parameter (TVD) (Figure 6.5). When computer analysis of heterogeneity (MHI) and flow (MFI) were compared to POEM scores for the 15 time points, there was also a significant association (Figure 6.6).

When the timings were compared between the assignment of POEM scores and completion of computer analysis they were 2 minutes and 44 minutes respectively (p < 0.001).

Figure 6.4. Relationship between traditional offline computer analysis and individual POEM scores.

Dashed lines indicate 95% confidence interval

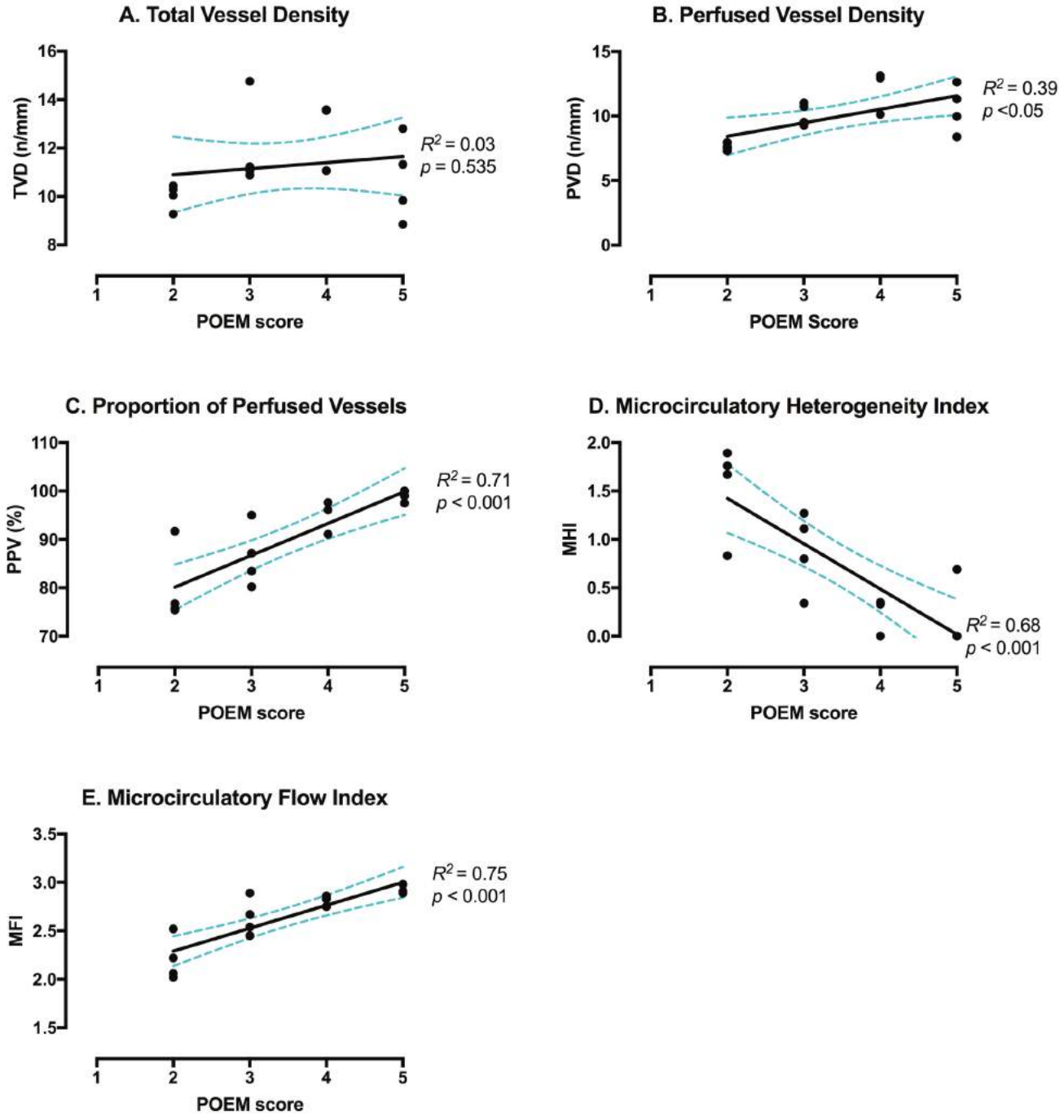


Figure 6.5. Relationship between traditional offline computer analysis and individual scores for video clips using the POEM score as a categorical variable

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

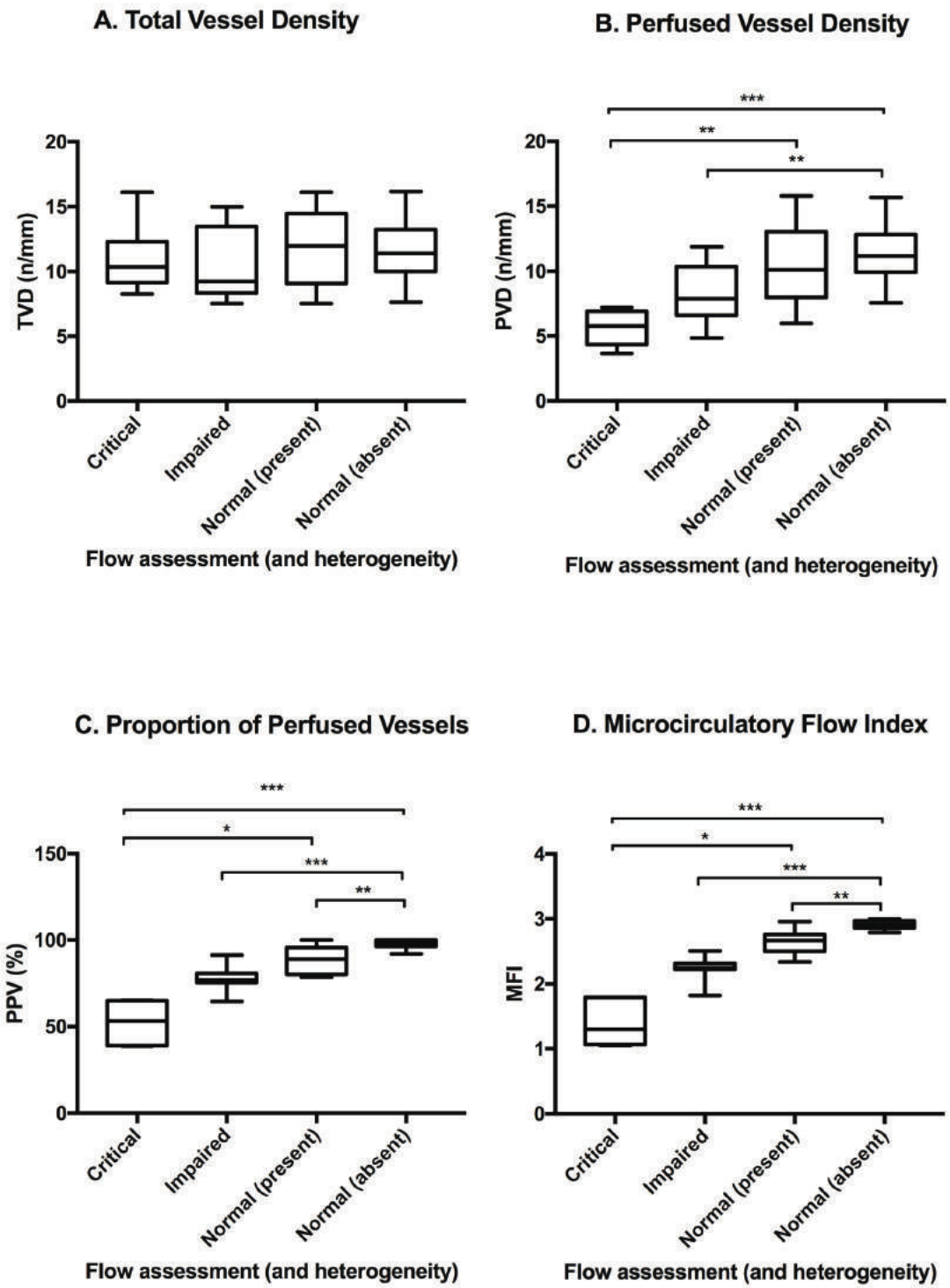
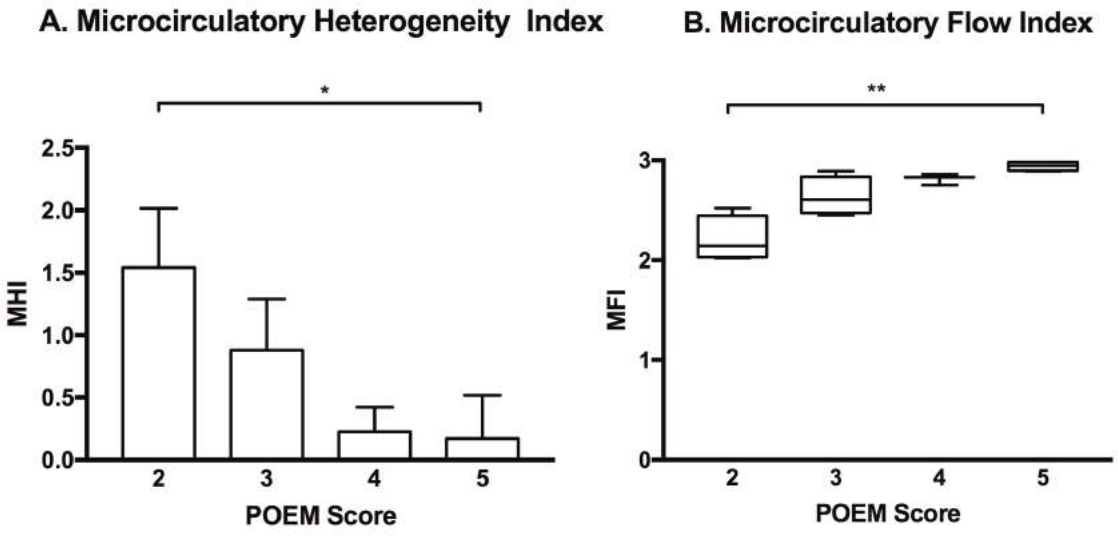


Figure 6.6. Relationship between offline computer analysis of microcirculatory heterogeneity and flow indexes and POEM scores, with the POEM score as a categorical variable.

*$p < 0.05$; **$p < 0.01$



6.4. Discussion

The current study reports the first use of a novel 5-point grading system (the POEM score) for the hybrid assessment of microcirculatory flow and heterogeneity. Naïve users can be trained to assess the microcirculation using this system, and have produced scores with relative consistency and agreement even after just one teaching session. Furthermore, the scoring is in keeping with offline computer generated analysis of flow, perfusion and heterogeneity and takes several minutes rather than an hour to perform. It is a user-friendly, straightforward tool that may be used as a point-of-care test in the observation of patients who are being treated for shock. Clinical validation and further examination of the real-time applicability are required before the 5-point grading system can be introduced

into clinical practice, in particular in relation to other parameters such as CO_2 gap and lactate. The POEM scoring system has the potential to alert the clinician to lack of haemodynamic coherence, and also to the degree of microcirculatory disruption in terms of flow and heterogeneity.

The semi-quantitative "Boerma Method" of assigning MFI values during sublingual video-microscopy[11] has the potential to be used in real-time. Although it is usually performed offline as part of the full panel of agreed parameters[7], it has also been used at the bedside as a real-time measurement of microcirculatory flow with good reliability when compared to offline computer analysis[12, 13]. The current study shows that POEM scores correspond well to MFI scores from the same video sequences for both individual videos and patient time points. The potential advantage of the POEM scores over MFI is that it also appears to correspond well to traditional values of heterogeneity (MHI), flow (PPV) and PVD (composite flow and density) rather than flow alone. The MFI approach involves superimposing a quadrant grid over the video images and then grading the MFI score for each quadrant before performing a calculation to determine the difference in minimum and maximum MFI scores compared to the average. By contrast the POEM score requires the user to identify whether more than 5 vessel segments in the entire field have flow abnormalities, removing the potentially artificial quadrant approach. We consider that such a technique is easier to perform for naïve users, and has the advantage of producing a single composite ordinal score that still gives accurate information about the state of overall flow and flow heterogeneity.

Although heterogeneity is usually presented as MHI by calculating the differences in MFI values, the POEM score uses a simplified method that does not require calculation, in order to make it easier to use. The clinician is only required to determine whether there are

5 vessel segments different to the rest in the field of view, regardless of quadrant. Since POEM scores reflect MHI well, this method may not necessarily be objectively precise but appears to be suitable in determining the level of heterogeneity for the purposes of a 5-point ordinal scale as well as full traditional MHI calculations.

Recently some investigators have demonstrated that visual inspection by clinicians may have good agreement with more detailed offline computer analysis, making the prospect of point-of-care microcirculatory analysis more of a realistic prospect[14]. A further study has demonstrated feasibility of bedside microcirculatory monitoring by critical care nursing staff[15]. If a clinician is to utilise sublingual microcirculatory monitoring to direct treatment in real-time, the video microscopy must be interpreted in a validated, graded, replicable manner with minimum inter-user variability. If the clinician can determine that the patient has lost haemodynamic coherence using a grading system of microcirculatory dysfunction, then alterations to therapy might be made that otherwise would have been guided by macrocirculatory parameters alone. A grading system of severity of microcirculatory dysfunction may aid in diagnosis, prognosis and management.

Although it has been an aspiration for some time, the real-time monitoring of the microcirculation at the point-of-care has been more of a futuristic prospect[16, 17] rather than a present-day clinical tool in the armoury of the clinician. It is timely to bring the available expertise and technology into clinical practice. Technology has advanced so that handheld, ergonomic instruments (such as the IDF videomicroscope (Cytocam, Braedius Medical B.V., Huizen, The Netherlands)) can be used to visualise the sublingual microcirculation with ease and speed. There has been a decade of research since the 2006 microcirculation consensus meeting[7], and yet microcirculatory monitoring has been confined to the realm of research during that time. The microcirculation community eagerly awaits automated computer-

generated analysis so that the microcirculation can be assessed without the requirement for offline analysis. However, it is likely even with such technology that a more simplified clinical grading system will be of maximal utility for the clinician at the point-of-care. Essentially the clinician's two primary aims when monitoring the microcirculation are (i) to determine whether there is loss of haemodynamic coherence; and (ii) to determine the degree of microcirculatory disruption. These factors have the potential to guide therapy.

Traditional offline measurements of microcirculatory function will be of continued value in the research context for studies that utilise microcirculatory parameters as resuscitation outcome measures. They will also be important when testing particular therapeutic measures following shock, and in providing diagnostic information (such as type of shock and classification of subtypes), and in tailoring therapy to specific requirements. The benefit of the POEM score in the clinical context is that it is faster to obtain, can more easily be used in real-time, and has the potential to guide and direct therapy at the bedside. Rather than replacing traditional numerical values, the POEM score is designed so that it can be used concurrently. One of the potential uses of the POEM score is as an early warning marker for microcirculatory dysfunction and loss of coherence. More detailed assessment of the microcirculation will still be required to determine the precise nature of microcirculatory dysfunction.

6.4.1. Future avenues of research

The POEM score does not replace traditional parameters in their role as research endpoints. Rather, it brings the overall assessment of the microcirculation forward to the point-of-care, in order to act as guidance of initial therapy. It is not currently known whether this will improve outcomes for patients, but future prospective randomised studies

may wish to test the utilisation of the POEM score versus standard practice in the management of patients in shock (such as septic or traumatic haemorrhagic shock). Furthermore, it is possible that the clinician might direct resuscitative fluids in a manner that addresses microcirculatory dysfunction when such a phenomenon is detected at the point-of-care (rather than being falsely reassured by satisfactory global haemodynamic parameters). Recent studies have reported that the delivery of plasma appears to have restorative function on the endothelial glycocalyx and ameliorate the endotheliopathy of trauma [18, 19]. Could the detection of microcirculatory failure and subsequent delivery of plasma in a circumstance where such therapy would be omitted be beneficial to patients? Such a question remains unanswered in a clinical context and may be a promising avenue for future research. However, such research depends entirely on a rapid and reproducible assessment of the microcirculation at the point-of-care – something that the POEM assessment tool may provide.

The introduction of user-dependency on a point-of-care test is not without precedence; cardiac echo and ultrasonography are some obvious examples of techniques that require high quality assessment by expert users for reliable readings. Trained users are accustomed to the practice of a fast, efficient assessment of patients in the Emergency Department (ED) in the case of focused assessment with sonography in trauma (FAST). Quality assurance and regular training, experience and revalidation should be of upmost importance amongst microcirculation monitoring users if their point-of-care assessment were to enter the domain of clinical utility. The current study did not test the level of skill fade or utility of the scoring system after a time interval, but these are aspects of training and professional development that would require attention if the POEM score were to be used clinically. Similarly, devices and technology would need to come under the scrutiny of

clinical point-of-care governance to ensure safety and consistency in clinical practice.

If real-time microcirculatory assessment is to be performed at the bedside, then quality assessment of video clips must also be undertaken before an assessment of flow and heterogeneity can be made. The current study only utilised videos that were high quality; whether poorer quality videos in a real-world scenario might lead to more variability in grading is unknown. The assessment of quality needs to be consistent, and reproducible, and with little inter-user variability. In particular, attention should be paid to guarding against pressure artefact, perhaps by ensuring that there are visible flowing venules within the field of view.

A decision was made early in the formation of the POEM score to not incorporate an assessment of vessel density, instead favouring assessments of flow and heterogeneity. Assessment of density was not a good discriminator between video clips when testing the early forms of the scoring system. Early pilot testing of the scoring system also showed that density assessment was difficult to apply in a manner that would accurately reflect TVD values. Nevertheless, POEM scores do correspond well to PVD, which is in itself a mixed density and perfusion variable. Since the POEM score corresponds well to PPV, PVD, MFI, and MHI, it is the first tool to yield a composite assessment of flow and heterogeneity and also yield potentially meaningful data regarding perfusion and perfused vessel density.

6.4.2. Limitations

None of the POEM study subjects were physically next to a patient's bedside when assigning POEM scores. It is unknown whether this physical proximity and patient contact would influence the scoring or performance of the test. Instead, the study subjects were blinded to patient status in order to establish the scoring system without any bias that

clinical exposure might bring. Further validation at the patient's bedside may be warranted in future studies that utilise the POEM score.

This study utilised video-microscopy clips of patients with traumatic haemorrhagic shock. Further validation in other clinical scenarios such as sepsis and following major surgery are required in order to determine whether this scoring tool has more generalisable utility.

Although there is a good association between POEM assessments and PVD, the POEM scoring system does not take into account pure density parameters of the microcirculation (such as TVD). This may limit its usefulness in determining the aetiology of the microcirculatory derangement. Measures to determine density parameters must rely on alternative techniques, which are currently not available at the point-of-care.

6.5. Conclusion

A new 5-point ordinal scoring system of microcirculatory flow and heterogeneity has been tested amongst healthcare professionals at two large UK teaching hospitals, and has relatively high consistency and agreement even after just 1 hour of training. POEM scores take a matter of minutes to assign, and correspond well to computer-analysis variables of flow, perfusion and heterogeneity. This novel point-of-care microcirculatory assessment tool is quick, reliable, and gives potentially meaningful clinical parameters that might guide resuscitation. Prospective randomised trials utilising goal-directed therapy targeted at the POEM score are required to test its real-life clinical utility.

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Chapter 7

What is the optimal fluid to resuscitate the microcirculation following haemorrhagic shock?

The following chapter is adapted from these two published articles:

Naumann DN, Dretzke J, Hutchings S, Midwinter MJ. Protocol for a systematic review of the impact of resuscitation fluids on the microcirculation after haemorrhagic shock in animal models. *Systematic reviews*. 2015;4:135.

Naumann DN, Beaven A, Dretzke J, Hutchings S, Midwinter MJ. Searching for the optimal fluid to restore microcirculatory flow dynamics after haemorrhagic shock: a systematic review of preclinical studies. *Shock.* 2016;46(6):609-22.

7.1. Introduction

In the previous two chapters we have discussed that early microcirculatory monitoring can be safely performed in the Emergency Department, even for very unwell patients, and that the microcirculation can be assessed in real-time rather than waiting to perform offline analysis away from the patient. If bedside monitoring of the microcirculation is performed in the clinical domain, then it has the potential to guide fluid resuscitation of patients with traumatic haemorrhagic shock. It is unknown what type of fluid is optimal for the restoration of microcirculatory flow in this setting, since there is a sparsity of data from humans. However, there are many pre-clinical (animal) studies that examine the effects of different fluids on the microcirculation after haemorrhagic shock. The following chapter is a review of all the available pre-clinical literature in order to determine which fluid—or which components of fluids—are most effective at restoring the microcirculation following haemorrhagic shock in animal models.

Alterations in the microcirculation have been reported as more reliable than global parameters in predicting clinical outcome following septic shock[1] and traumatic haemorrhage[2]. Improved microcirculatory flow during resuscitation is associated with reduced organ failure even when there is no difference in global haemodynamic factors[3]. Even when global parameters are improved following shock, this may not be associated with improvements in the microcirculation and tissue perfusion[4]. In such circumstances there appears to be a clinically meaningful discrepancy between the macro and microcirculatory behaviour. During normal physiological conditions both microcirculatory flow and tissue perfusion are determined by the circulatory pressure and volume. This phenomenon is known as 'haemodynamic coherence'[5]. This coherence may be lost during circulatory shock, and therefore global surrogate markers may no longer be relied on as

markers of microcirculatory function in that context – a rationale for monitoring of the microcirculation following shock.

During resuscitation of patients with haemorrhagic shock, fluid delivery is intended to increase oxygen delivery to tissues to meet demand, repay oxygen debt, eliminate lactate, and normalise pH. These processes all occur at the level of the microcirculation, representing a key anatomical location during shock and resuscitation. Although pre-clinical studies have been conducted to measure global haemodynamic parameters (such as blood pressure and heart rate) following haemorrhagic shock and resuscitation[6, 7], these surrogate markers of microcirculatory flow may not be relevant in the case of loss of haemodynamic coherence. Microcirculatory flow and dynamics therefore represent relevant study endpoints for the assessment of resuscitation fluid delivery after haemorrhagic shock, and would be parameters of importance if point-of-care microcirculatory monitoring were to be considered.

Current clinical resuscitative practice favours the utilisation of blood products (rather than crystalloid fluids) following haemorrhagic shock, but there are no randomised clinical studies that compare the microcirculation during different fluid resuscitation regimes. In order to guide clinical investigation and form credible hypotheses for testing in clinical research, it is timely to review the pre-clinical literature and determine the current state of evidence. No previous systematic reviews on this topic were identified during preliminary searches on MEDLINE and the Cochrane library. It was hypothesised that provision of haemoglobin and plasma, in particular by whole blood, may be superior to other fluid characteristics in the restoration of the microcirculation following haemorrhagic shock (Hypothesis 8; Table 1.1).

7.1.1. Aim

The current systematic review aims to examine all available pre-clinical studies of haemorrhagic shock that use microcirculatory parameters as research endpoints and compare the efficacy of at least one type of fluid for resuscitation.

7.2. Methods

This systematic review is intended to address the impact of resuscitation fluids on the behaviour of the microcirculation in animal models of haemorrhagic shock.
The protocol for this systematic review has been published previously[8], and made freely available through Open Access and registration at the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES)[9]. Systematic review methodology is reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[10].

7.2.1 Research questions

This is the first review to examine the impact of different fluid resuscitation techniques on the physical structure and function of the microcirculation in animal models. The following questions will be addressed:

- What is the impact of intravascular fluid resuscitation on the microcirculation following haemorrhagic shock, compared to haemorrhagic shock alone?
- Which type of fluid has the most impact on the microcirculation following haemorrhagic shock?

7.2.2. Study subjects

This systematic review includes studies that utilise animal models of haemorrhagic shock (any size, age, strain and species). Any volume of haemorrhage (survivable or non-survivable) was allowed as long as the intention was to create a period of circulatory shock after which fluid resuscitation was delivered. Study protocols with additional elements such as trauma were still eligible for inclusion. Studies that utilised isovolaemic exchange transfusion, ischaemia-reperfusion, or septic shock models were excluded unless they also contained a subgroup of haemorrhagic shock.

7.2.3. Interventions

Studies that used at least one type of fluid intervention for resuscitation following haemorrhage were eligible for inclusion. There were multiple interventions of interest, broadly categorised as: (i) blood products (e.g. whole blood, packed red cells (PRBCs), plasma); (ii) haemoglobin-based oxygen carriers (HBOC) (e.g. modified bovine haemoglobin); (iii) crystalloids (e.g. Ringer's lactate, 0.9% or hypertonic saline); and (iv) colloids (e.g. albumin, dextran, starch).

7.2.4. Comparisons

The studies included in this systematic review were varied in both methodology and research question. Multiple permutations of fluid comparisons were made (for example blood product versus crystalloid, and colloid versus crystalloid). Some studies use haemorrhagic shock alone as a control, and some use surgical instrumentation (sham) as a control. These comparisons are summarised in narrative form.

7.2.5. Outcomes

The outcomes of interest included any parameter that was intended to represent the physical microcirculatory behaviour. These included: flow rate (nL/s); red blood cell velocity (mm/s), vessel diameter (μm); Functional capillary density (%); glycocalyx thickness (μm); Shear rate (s^{-1}); proportion of perfused vessels (%); vessel density (n/mm); perfused vessel density (n/mm); microcirculatory flow index; blood flow intensity; and heterogeneity index. Studies that only examined physiological aspects of the microcirculation such as lactate, oxygen partial pressures, and delivery of oxygen but did not report physical (flow dynamics) parameters[11-19] were excluded.

7.2.6. Study design

Studies were included if they were controlled prospective animal studies with detailed reporting of the type and amount of fluid(s) used and at least one microcirculatory physical parameter. Although randomised studies with blinded outcome assessment were considered preferable, prospective studies without such design were still eligible for inclusion. Single case reports and letters were rejected. Conference proceedings and abstracts were screened for new data and adequate methodological detail. They were only included if they contained new data (not replicated by full papers from the same authors and time period). Uncontrolled studies were recorded but not included in the analysis.

7.2.7. Search strategy

The following sources were searched for primary studies: EMBASE and Medline (via OVID SP) and SCOPUS. Before starting the review, scoping searches were undertaken to identify any existing systematic reviews of the impact of resuscitative fluids on the

microcirculatory changes during haemorrhagic shock and to gauge the number and type of relevant primary studies. No systematic reviews were identified, but there are a number of pre-clinical studies. The systematic search was undertaken using a combination of text and MeSH terms relating to the intervention (e.g. “bleed”, “transfusion”, “fluid resuscitation”, “colloid”, “red blood cells”), to the condition (e.g. “haemorrhage”, “trauma”, “injury”, “shock”), and to the microcirculation (e.g. “microcirculation’, “endothelium”, “capillary”). The search strategies were broad in order to capture any study that has explored resuscitation fluids and microcirculatory changes in the context of haemorrhagic shock, regardless of study design, study subjects, method of assessment, or outcomes reported. There were no language or date restrictions, or restrictions by publication type. Citations were collated using EndNote reference management software (V.X7, Thomson Reuters). An example of a search strategy used is shown in Table 7.1.

7.2.8. Study Selection

All titles and abstracts were screened by two independent reviewers, and full texts were obtained for studies that appeared to be of interest. Eligible studies were identified from reading the full texts. References from full texts were further screened for potentially relevant papers using pre-defined selection criteria. Discrepancies between reviewers were resolved by discussion or by referring to a third reviewer.

Table 7.1. Example of a search strategy using MEDLINE

Step	Search term	Results
1	haemorrhag$.mp.	42370
2	hemorrhage.mp. or exp Hemorrhage/	317823
3	bleed$.mp.	152764
4	trauma.mp. or exp "Wounds and Injuries"/	815484
5	shock.mp. or exp Shock, Hemorrhagic/ or exp Shock, Traumatic/ or exp Shock/	188261
6	microcirculation.mp. or exp Microcirculation/	41855
7	microcirculat$.mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]	44370
8	capillar$.mp. or exp Capillaries/	144927
9	exp Endothelium/ or endothelium.mp.	149398
10	1 or 2 or 3 or 4 or 5	1342437
11	7 or 8 or 9	308688
12	10 and 11	21552
13	(fluid$ adj3 resuscitat$).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]	4445
14	(fluid$ adj3 administrat$).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]	3158
15	normal saline.mp.	15704
16	exp Fluid Therapy/ or crystalloid$.mp.	20028
17	(colloid$ adj3 resuscitat$).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]	212
18	(red blood cell$ or RBC or plasma or platelet$ or whole blood or blood product$ or blood component$).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]	1033868
19	exp Blood/	962483
20	18 or 19	1806182
21	(resuscitat$ or transfus$).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]	182117
22	20 and 21	54184
23	13 or 14 or 15 or 16 or 17 or 22	91548
24	12 and 23	1126

7.2.9. Data extraction

Data extraction was performed by one reviewer (D.N.N.) and confirmed by another (A.B.). Data were extracted with regards to study characteristics and design (author, year, type of study, hypothesis), animal model (species, age, experimental groups, size/weight, housing), number of animals (haemorrhagic shock and resuscitation only), and haemorrhage protocol (technique, percentage and volume of bleeding, timings, and target pressures). Details regarding interventions (type and timings), and microcirculatory monitoring (technique, anatomical location) were also extracted. Primary method for data extraction was to take the numerical values directly from the results sections and tables of individual studies. Where only percentages were reported in the place of raw numerical data, the numerical values were calculated based on the reported percentage and denominator. Where a numerical value is missing but the data were displayed graphically, a digital screen ruler was used to extract the numerical data.

7.2.10. Quality assessment

Two reviewers (D.N.N. and A.B.) assessed the included studies based on the Systematic Review Centre for Laboratory animal Experimentation (SYRCLE) risk of bias tool[20]. This tool assesses selection, performance, detection, attrition, and reporting biases.

7.2.11. Translatability

The validity of study design with regards to translatability to clinical practice was examined by two authors (D.N.N. and A.B.) for each study based on the three domains described by Henderson et al: (i) threats to internal validity; (ii) threats to construct validity; and (iii) threats to external validity[21]. For the "threats to internal validity" domain, there are

points for power calculation, blinding, randomisation, appropriate controls, demonstrating the flow of animals through the experiments, and study of a dose-response relationship. For the "construct validity" domain, there are points for baseline characteristics of animals, matching the animal model (including age) to the human disease, and characterisation of the mechanistic pathway of the treatment response. For the "external validity" domain, there are points for replication of experiments in different animals, laboratories, and models of the same disease. The domain relating to outcome measure validity was omitted since this systematic review only includes studies with pre-defined outcome measures (microcirculatory physical parameters) that are considered valid endpoints of shock resuscitation.

7.3. Results

7.3.1. Search results

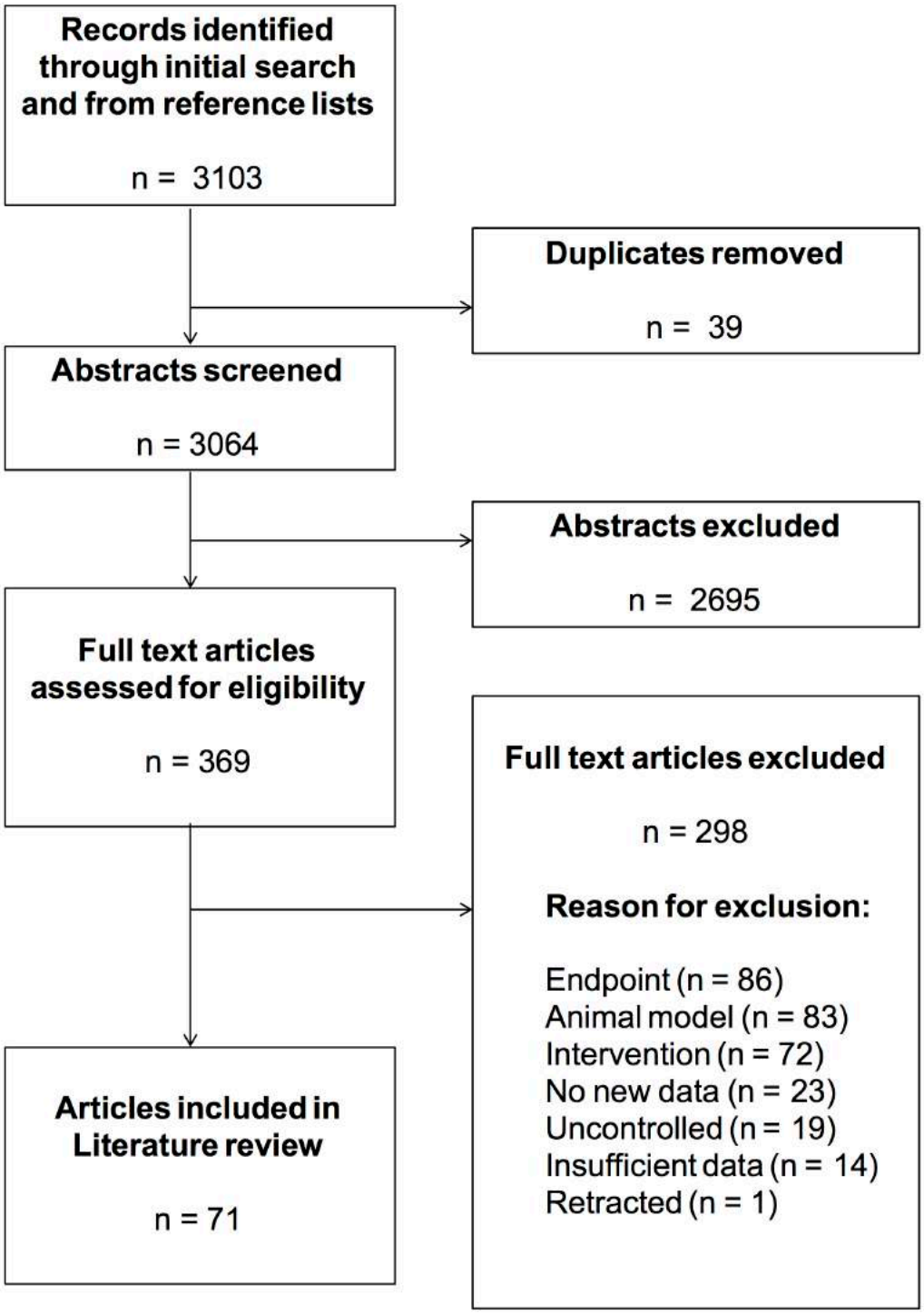
Figure 7.1 shows the PRISMA diagram for the study selection. The initial search strategy identified 3103 studies, from which 369 full texts were examined. There were 71 studies that measured the impact of resuscitation fluids on the microcirculation in an animal model of haemorrhagic shock[22-92].

7.3.2. Study characteristics

The 71 included studies were published between 1990 and 2015, and include 67 original articles and 4 conference proceedings/abstracts with 45 individual first authors. Countries of origin are listed as: Austria, Brazil, Canada, China, France, Germany, Hungary, Italy, Japan, Spain, Taiwan, and the USA. All studies were prospective, experimental controlled studies. Although random allocation of intervention and control arms was

implied by all studies, the words "random" or "randomly" were only explicitly reported in 52/71 (73%) of studies.

Figure 7.1. PRISMA diagram to illustrate search results



7.3.3. Animal characteristics

All studies included a single species experimental model. Animal characteristics are summarised in Table 7.2, and included 62 rodent, 5 canine, and 4 porcine studies. A total of 1959 animals underwent haemorrhagic shock and resuscitation in the included studies. There were 55 studies that reported the sex of the animals, of which 48 included only male animals, 4 studies had mixed male and female animals, and 3 included only female animals.

Table 7.2. Animal characteristics of included studies

Species	Animals	Studies	Weight range	Awake, %	References
Rat	1149	31	120–460 g	0	22-24, 35, 40-42, 46, 48, 50, 51, 54-57, 64, 68, 72-77, 81-83, 88-92
Hamster	518	26	40–75 g	100	25-34, 47, 49, 52, 60, 62, 63, 65, 70, 71, 78-80, 84-87
Rabbit	94	3	0.8–3.5 kg	0	53, 59, 61
Pig	74	4	7–45 kg	0	43-45, 58
Mouse	68	2	25–30 g	0	66, 67
Dog	56	5	22–35 kg	0	36-39, 69

7.3.4. Haemorrhagic shock protocols

A haemorrhagic shock protocol was described in all studies, and was heterogeneous, as illustrated in Table 7.3. Some studies bled the animals to target values or percentage of mean arterial pressures, whereas others bled to a percentage of total blood volume or weight. The length of time of the “shock” phase was also variable.

Table 7.3. Summary of haemorrhage protocol targets and timings

Haemorrhage protocol	N	Target % of blood volume lost	Target MAP, mmHg	Target % of MAP	Target mL/kg bled	Period of shock, minutes	References
Volume controlled haemorrhage							
% of total blood volume lost	27	50 (50–50)	N/A	N/A	N/A	60 (60–60)	26-34, 53, 58, 60, 63-65, 73-76, 78-80, 84-87, 91
Bled a specific volume of blood per kg	6	N/A	N/A	N/A	30 (30–30)	40 (30–57.5)	43, 44, 48, 50, 88, 89
Pressure controlled haemorrhage							
Bled to a target MAP	26	N/A	40 (37.5–40)	N/A	N/A	60 (45–60)	22-24, 35, 40-42, 46, 47, 49, 51, 54-57, 61, 66-69, 77, 81-83, 90, 92
Bled to both % of total blood volume and target MAP	11	50 (40–50)	40 (37.5–48.8)	N/A	N/A	45 (36–60)	25, 36-39, 45, 52, 59, 62, 70, 71
Bled to a % of MAP	1	N/A	N/A	50	N/A	30*	72

All values are expressed as median, with interquartile range in brackets unless otherwise specified
*This is a single value rather than a median

7.3.5. Microcirculatory monitoring techniques

Several different techniques and anatomical locations were used to determine microcirculatory parameters, as summarised in Table 7.4. Five studies[41, 56, 69, 88, 89] examined the microcirculation in multiple regions, and the remainder studied only one anatomical location. One study conducted haemorheological analysis externally (from blood samples) without specific anatomical location[92]. Some studies used a combination of techniques at the same anatomical location[40, 58]. Intravital microscopy was the most common technique used for microcirculatory visualisation, with dorsal skin fold and bowel/mesentery being the most common anatomical locations. Only five studies used sidestream dark field microscopy

(SDF)[43, 44, 58, 61, 69], which is considered the most appropriate technique for clinical assessment[93].

Table 7.4. Anatomical location and techniques for microcirculatory parameter acquisition

		References					
Anatomical location	**Studies**	**Intravital microscopy**	**Laser speckle contrast imaging**	**Sidestream dark field microscopy**	**Laser Doppler flowmetry probe**	**Electron microscopy**	**Orthogonal polarisation spectral imaging**
Dorsal skin fold	26	25-34, 47, 49, 52, 60, 62, 63, 65, 70, 71, 78-80, 84-87					
Bowel / mesentery	15	35, 41, 48, 50, 51, 64, 72, 91	88, 89	61, 69	46	54	77
Liver	9	22, 23, 40, 55, 57, 68, 81	88, 89		40		
Cremaster	5	66, 67, 73, 75, 76					
Skeletal muscle	6	24, 41, 42, 59	88, 89				
Conjunctiva	4	36-39					
Sublingual	5			43, 44, 58	45, 58		
Kidney	5	41	88, 89		46, 56		
Pancreas	2	82, 83					
Brain	2				46, 56		
Internal spermatic fascia	1	90					
Buccal mucosa	1			69			
Ear chamber	1	53					
Heart	1				46		

7.3.6. Fluid comparisons

There were multiple comparisons made between fluids in the included studies, summarised in Table 7.5. There were 29 permutations of sham (no haemorrhage), haemorrhage only, blood product, HBOC, crystalloid and colloid fluid administration.

Table 7.5. Resuscitative fluid comparisons in included studies

Studies	Sham (no haemorrhage)	Haemorrhage only	Blood product(s)	Oxygen carrier(s)	Crystalloid(s)	Colloid(s)
Torres 2013	✔	✔	✔		✔	✔
Kozar 2011	✔	✔	✔		✔	
Zhao 2009, Wu 2015(b)	✔	✔			✔	✔
Wu 2015(a)	✔	✔			✔	
Paxian 2003	✔		✔	✔	✔	✔
Von Dobschuetz 1999	✔		✔	✔		✔
Kubulus 2009	✔		✔	✔		
Paes-da-Silva 2003	✔		✔		✔	✔
Ni 2013, Torres 2014	✔		✔		✔	
Torres 2015(a), Zakaria 2006	✔		✔		✔	
Bauer 1993, Pascual 2001, Vajda 2004, Vollmar 1994	✔				✔	✔
Bauer 1995, Cabrales 2007(b), Maier 2004	✔					✔
Gulati 1998	✔			✔	✔	
Pascual 2002, Yada-Langui 2004	✔				✔	
Bi 2004		✔	✔	✔		✔
Cabrales 2004, Cabrales 2005(b), Wettstein 2006		✔				✔
Cheung 2001, Ortiz 2014, Wettstein 2004(b)			✔	✔		
Cheung 2006, Cheung 2007, Kerger 1997			✔	✔	✔	✔
Cheung 2004, Hungerer 2006, Nolte 1997, Sakai 2002, Wettstein 2003			✔	✔		✔
Casali 2002, Kao 2010, Torres 2015(b)			✔		✔	
Cabrales 2007(c), Sakai 1999, Scalia 1990			✔			✔
Cabrales 2007(a), Villela 2009			✔			
Peruski 2014				✔		
Kumar 1997				✔	✔	
Botzlar 1996				✔	✔	✔
Cabrales 2009, Hermann 2007, Palmer 2011, Vazquez 2011, Wettstein 2004(a)				✔		✔
Kao 2011, Villela 2011					✔	
Corso 1999, Cryer 2005, Gierer 2004, Gonzalez 2012, Gonzales 2016, Guerci 2014, Horstick 2002, Komori 2005, Mazzoni 1990, Vollmar 1996					✔	✔
Cabrales 2005(a), Cabrales 2008(a), Cabrales 2008(b), Maier 2009, Messmer 2012						✔

7.3.7. Risk of bias

The majority of studies fulfilled SYRCLE criteria to control for risk of bias by specifically reporting a random allocation sequence generation for the different interventions, and also reporting similar baseline characteristics (illustrated in Table 7.6). No studies met all SYRCLE criteria. All studies were at risk of selection bias due to lack of reporting of allocation concealment, and performance bias due to lack of reporting of blinding of caregivers/investigators. All studies were also at risk of detection bias due to lack of reporting of random outcome assessment.

7.3.8. Translatability

Assessment of the translatability of the animal model to clinical relevance found some consistently under-reported details, which threaten the validity of the studies in humans (illustrated in Table 7.7). Most of the studies did not describe a power calculation in their methodology, did not have blinded outcome assessment, and did not include a dose-response relationship. Only one study reported a rationale for the age of the animals[43], and none reported their experimental model had been tested with different transgenic strains, different species, or in collaboration with different research groups (for the same experiment).

Table 7.6. Assessment of bias based on the SYRCLE's risk of bias tool for animal studies

Author	Year	Sequence generation	Baseline characteristics	Random housing	Blinding (outcome)	Incomplete outcome data	Selective outcome reporting	Total
Bauer	1993		*		*			**2**
Bauer	1995	*	*		*			**3**
Bi	2004	*	*			*		**3**
Botzlar	1996		*					**1**
Cabrales	2004	*	*					**2**
Cabrales	2005 (a)	*	*					**2**
Cabrales	2005 (b)	*	*					**2**
Cabrales	2007 (a)	*	*			*		**3**
Cabrales	2007 (b)	*	*			*		**3**
Cabrales	2007 (c)	*	*			*		**3**
Cabrales	2008 (a)	*	*			*		**3**
Cabrales	2008 (b)	*	*			*		**3**
Cabrales	2009	*	*			*		**3**
Casali	2002							**0**
Cheung	2001	*	*			*		**3**
Cheung	2004	*	*					**2**
Cheung	2006	*	*					**2**
Cheung	2007	*	*			*		**3**
Corso	1999		*					**1**
Cryer	2005		*					**1**
Gierer	2004		*					**1**
Gonzalez	2012	*						**1**
Gonzalez	2016	*	*		*			**3**
Guerci	2014	*	*			*		**3**
Gulati	1998		*					**1**
Hermann	2007	*	*					**2**
Horstick	2002	*	*					**2**
Hungerer	2006	*	*					**2**
Kao	2010	*	*			*		**3**
Kao	2011	*						**1**
Kerger	1997		*			*		**2**
Komori	2005		*					**1**
Kozar	2011		*					**1**
Kubulus	2009	*	*		*			**2**
Kumar	1997		*					**1**
Maier	2004	*			*			**2**
Maier	2009	*	*		*			**3**
Mazzoni	1990	*	*			*		**3**
Messmer	2012	*	*			*		**3**
Ni	2013	*						**1**
Nolte	1997	*	*					**2**
Ortiz	2014	*	*			*		**3**
Paes-da-Silva	2003	*	*					**2**
Palmer	2011	*	*			*		**3**
Pascual	2001	*	*		*			**3**
Pascual	2002	*	*	*	*			**4**
Paxian	2003		*					**1**
Peruski	2014	*	*		*			**3**
Sakai	1999		*	*		*		**3**
Sakai	2002		*	*				**2**
Scalia	1990		*					**1**
Torres	2013	*	*					**2**

Author	Year	Sequence generation	Baseline characteristics	Random housing	Blinding (outcome)	Incomplete outcome data	Selective outcome reporting	Total
Torres	2014	*	*			*		**3**
Torres	2015 (a)		*					**1**
Torres	2015 (b)		*					**1**
Vajda	2004	*	*					**2**
Vazquez	2011	*	*			*		**3**
Villela	2009	*	*			*		**3**
Villela	2011	*	*			*		**3**
Vollmar	1994	*	*		*			**3**
Vollmar	1996	*	*					**2**
von Dobschuetz	1999	*	*					**2**
Wettstein	2003	*	*			*		**3**
Wettstein	2004 (a)		*					**1**
Wettstein	2004 (b)	*	*					**2**
Wettstein	2006		*			*		**2**
Wu	2015 (a)	*	*					**2**
Wu	2015 (b)	*	*					**2**
Yada-Langui	2004	*						**1**
Zakaria	2006	*	*					**2**
Zhao	2009	*	*					**2**

Columns for allocation concealment, caregiver blinding, and random outcome assessment are not included, because no studies fulfilled these criteria

Table 7.7. Assessment of translatability according to most frequent recommendation for pre-clinical research

Author	Year	Choice of sample size (power calculation)	Random allocation of animals	Blinded outcome assessment	Flow of animals through experiment	Selection of appropriate control groups	Dose-response relationship	Baseline characteristics	Characteristic pathway	Age matching	Total
Bauer	1993			*				*	*		**3**
Bauer	1995		*	*		*		*	*		**5**
Bi	2004		*		*		*	*	*		**5**
Botzlar	1996					*		*	*		**3**
Cabrales	2004		*			*		*	*		**4**
Cabrales	2005 (a)		*					*	*		**3**
Cabrales	2005 (b)		*			*		*	*		**4**
Cabrales	2007 (a)		*		*			*	*		**4**
Cabrales	2007 (b)		*		*	*		*	*		**5**
Cabrales	2007 (c)		*		*			*	*		**4**
Cabrales	2008 (a)		*		*	*		*	*		**5**
Cabrales	2008 (b)		*		*			*	*		**4**
Cabrales	2009		*		*			*	*		**4**
Casali	2002								*		**1**
Cheung	2001		*		*	*		*	*		**5**
Cheung	2004		*			*		*	*		**4**
Cheung	2006		*		*			*	*		**4**
Cheung	2007		*		*			*	*		**4**
Corso	1999				*	*		*	*		**4**
Cryer	2005							*	*		**2**
Gierer	2004					*		*	*		**3**
Gonzalez	2012		*								**1**
Gonzalez	2016		*	*				*	*	*	**4**
Guerci	2014		*		*			*	*		**4**
Gulati	1998						*	*	*		**3**
Hermann	2007		*				*	*	*		**4**
Horstick	2002		*		*			*	*		**4**
Hungerer	2006		*					*	*		**3**
Kao	2010		*		*			*	*		**4**
Kao	2011		*								**1**
Kerger	1997				*			*	*		**3**
Komori	2005					*		*	*		**3**
Kozar	2011					*		*	*		**3**
Kubulus	2009		*			*		*	*		**4**
Kumar	1997					*		*	*		**3**
Maier	2004		*	*			*		*		**4**
Maier	2009		*	*				*	*		**4**
Mazzoni	1990	*	*		*	*		*	*		**6**
Messmer	2012	*	*		*			*	*		**5**
Ni	2013		*			*			*		**3**
Nolte	1997		*			*		*	*		**4**
Ortiz	2014		*		*		*	*	*		**5**
Paes-da-Silva	2003		*			*		*	*		**4**
Palmer	2011		*		*			*	*		**4**
Pascual	2001		*	*		*		*	*		**5**
Pascual	2002		*	*		*		*	*		**5**
Paxian	2003					*	*	*	*		**4**
Peruski	2014		*	*				*	*		**4**

Author	Year	Choice of sample size (power calculation)	Random allocation of animals	Blinded outcome assessment	Flow of animals through experiment	Selection of appropriate control groups	Dose-response relationship	Baseline characteristics	Characteristic pathway	Age matching	Total
Sakai	1999				*			*	*		**3**
Sakai	2002						*	*	*		**3**
Scalia	1990					*		*	*		**3**
Torres	2013		*			*		*	*		**4**
Torres	2014		*		*	*		*	*		**5**
Torres	2015 (a)					*					**1**
Torres	2015 (b)							*			**1**
Vajda	2004		*			*		*	*		**4**
Vazquez	2011		*		*			*	*		**4**
Villela	2009		*		*		*	*	*		**5**
Villela	2011		*		*			*	*		**4**
Vollmar	1994		*	*		*		*	*		**5**
Vollmar	1996		*						*		**2**
von Dobschuetz	1999		*			*		*	*		**4**
Wettstein	2003		*		*			*	*		**4**
Wettstein	2004 (a)							*	*		**2**
Wettstein	2004 (b)		*					*	*		**2**
Wettstein	2006				*	*		*	*		**4**
Wu	2015 (a)		*			*		*	*		**4**
Wu	2015 (b)	*	*			*		*	*		**5**
Yada-Langui	2004		*			*			*		**3**
Zakaria	2006		*			*		*	*		**4**
Zhao	2009		*			*		*	*		**4**

7.3.9. Data synthesis

An assessment of feasibility of meta-analysis was made, but was considered to be unwarranted due to high heterogeneity of haemorrhage protocol (5 permutations), interventions (29 permutations), and endpoints (6 permutations) between studies. In particular, there were variations in study hypotheses and research questions that rendered meta-analytic synthesis impossible. Descriptive narrative is therefore utilised to synthesise findings across similar studies. Studies were summarised according to study hypothesis and research question. The included studies could be broadly divided into 5 hypotheses as summarised in Tables 7.8 – 7.12. These primarily investigated fluids containing

haemoglobin, physical properties (viscosity and oncotic/osmotic potential), and the restorative and anti-inflammatory properties of the resuscitation fluids. Some studies reported data related to more than one of these hypotheses. Of these studies, 55/71 (77.5%) reported one fluid being superior to another, with the remainder reporting equivalence between fluids or only testing one fluid.

7.3.10. Fluids containing haemoglobin

There were 27 studies that considered resuscitation fluids containing haemoglobin for the restoration of microcirculatory flow dynamics as summarised in Table 7.8. There were 21 studies that examined HBOC fluids, including 10 studies that tested HBOC versus both whole blood and non-haemoglobin (Hb) carrying fluids; 8 studies that tested HBOC versus non Hb carrying fluids alone; and 3 studies that tested HBOC versus whole blood only. HBOC preparations included bovine haemoglobin[24, 25, 34, 78], modified human haemoglobin[87], mixed human/bovine haemoglobin[65], diaspirin cross-linked haemoglobin (DCLHb) [46, 56, 62, 83], o-raffinose cross-linked oligomerized haemoglobin[52], and nitric oxide-scavenging recombinant haemoglobin[47]. In the 13 studies in which HBOC fluids were directly compared to whole blood, three HBOCs (bovine haemoglobin glutamer-250[63], modified human haemoglobin[87], and DCLHb[62]) were superior to whole blood in the restoration of microcirculatory flow dynamics. All except one[71] of the remaining studies reported that HBOC fluids were equivalent to whole blood in terms of flow dynamics. HBOC fluids were superior to non Hb carrying fluids in 13 studies, and equivalent to non Hb carrying fluids in 5 studies.

Some of the studies that tested HBOC fluids also addressed the potential unwanted side effects of HBOCs such as vasoconstriction, nitric oxide (NO) scavenging and

leucocyte/endothelial interactions. They reported that HBOCs did not cause vasoconstriction[25], did not increase leucocyte/endothelial interactions[25, 49, 62, 83], and do not have toxic or lethal effects[38] when compared to other non-HBOC fluids. Some preparations of Hb are superior to others[65], and lower Hb concentrations appeared to be superior to higher concentrations with regards to vasoconstriction[34, 63]. Furthermore specific modification of HBOCs to reduce NO scavenging has been reported as effective[47]. Conversely, one study did report hepatotoxic effects of HBOC[55].

There were 6 studies that tested fluids containing Hb that were not HBOCs. These included 4 studies testing whole blood versus crystalloid[35, 51, 61] or colloid[70], and two studies examining preparations of PRBCs[68, 79]. These studies reported that whole blood was superior to crystalloid and colloid. When PRBCs were tested, one oxygen carrying emulsion (perflubron emulsion) was reported as being superior to red cells with respect to microcirculatory flow dynamics[68]. Another study reported that lower oxygen affinity PRBCs were superior to higher oxygen affinity[79].

Table 7.8. Summary of study findings regarding haemoglobin-carrying fluids

Study	Animal	Method	Test fluid(s)	Control fluid(s) or sham	Endpoint(s)	Main finding
A. Haemoglobin based oxygen carriers						
Bi 2004	Rat	IVM	PEG-Hb (3 different volumes)	Whole blood (2 different volumes) and dextran and HC	Velocity, flow, diameter	HBOC superior to non Hb carrying fluid, and equivalent to whole blood
Botzlar 1996	Hamster	IVM	U-PBHb 11g/dL and 13g/dL	Dextran and LR	Diameter, FCD	HBOC superior to non Hb carrying fluid
Cabrales 2009	Hamster	IVM	PBH 13g/dL and PBH 4g/dL	Albumin	Velocity, flow, diameter, FCD	HBOC superior to non Hb-carrying fluid
Cheung 2001	Canine	IVM	Bovine Hb glutamer-200	Whole blood	Velocity, diameter	HBOC equivalent to whole blood
Cheung 2004	Canine	IVM	Oxyglob	Whole blood and 6% hetastarch	Velocity, diameter	HBOC equivalent to non Hb-carrying fluid and whole blood
Cheung 2006	Canine	IVM	Oxyglobin	Whole blood and NS and 6% hetastarch	Velocity, diameter	HBOC equivalent to non Hb-carrying fluid and whole blood
Cheung 2007	Canine	IVM	Oxyglobin	Whole blood and NS and 6% hetastarch	Velocity, diameter	HBOC equivalent to non Hb-carrying fluid and whole blood
Gulati 1998	Rat	LDF	DCLHb (3 different concentrations)	LR	Velocity, perfusion	HBOC superior to non Hb carrying fluid
Hermann 2007	Hamster	IVM	Recombinant Hb wild type and recombinant Hb nitric-oxide scavenging	6% dextran	Velocity, diameter, FCD	Nitric-oxide scavenging HBOC superior to HBOC or non Hb carrying fluid
Hungerer 2006	Hamster	IVM	DCLHb	Whole blood and dextran	Velocity, FCD	HBOC equivalent to whole blood and non Hb carrying fluid
Kerger 1997	Hamster	IVM	Cell-free o-raffinose cross-linked oligomerized Hb	Whole blood and LR and dextran	Velocity, flow, diameter, FCD	HBOC superior to non Hb-carrying fluid and equivalent to whole blood
Kubulus 2009	Rat	IVM	Hb glutamer-200	Whole blood	Velocity, flow, diameter, PVD	HBOC equivalent to whole blood
Kumar 1997	Rat	LDF	DCLHb	LR	Velocity, perfusion	HBOC superior to non Hb carrying fluid
Nolte 1997	Hamster	IVM	DCLHb	Whole blood and dextran	Velocity, diameter, FCD	HBOC superior to whole blood and non Hb carrying fluid
Ortiz 2014	Hamster	IVM	Bovine Hb glutamer-250 at 4, 8, and 12 g/dL	Whole blood	Velocity, flow, FCD	HBOC superior to whole blood; lower Hb preparations superior to higher
Palmer 2011	Hamster	IVM	Polymerised human Hb and polymerised bovine Hb	HSA	Velocity, flow, FCD	HBOC superior to non Hb carrying fluid; bovine preparation of Hb superior to human
Sakai 2002	Hamster	IVM	Vesicle-encapsulated Hb 3g/dL and 7g/dL	Whole blood and HSA	Velocity, flow, diameter, FCD	HBOC superior to non Hb carrying fluid but inferior to whole blood

Study	Animal	Method	Test fluid(s)	Control fluid(s) or sham	Endpoint(s)	Main finding
Vazquez 2011	Hamster	IVM	Oxyglobin	HES	Diameter, FCD	HBOC superior to non Hb carrying fluid
von Dobschuetz 1999	Rat	IVM	DCLHb	Whole blood and HES	FCD	HBOC superior to non Hb carrying fluids and equivalent to whole blood
Wettstein 2003	Hamster	IVM	PEG-Hb	Whole blood and HES	Velocity, flow, diameter, FCD	HBOC superior to whole blood and non Hb carrying fluid
Wettstein 2004(a)	Hamster	IVM	PEG-Alb	PEG-Hb (from earlier experiment)	Velocity, flow rate, diameter, FCD	HBOC equivalent to non Hb carrying fluid
B. Red cells and whole blood						
Casali 2002	Rat	IVM	Whole blood	LR	Velocity	Whole blood superior to crystalloid
Kao 2010	Rat	IVM	Whole blood and whole blood/EPO	NS and NS/EPO	Flow, perfusion	Whole blood superior to crystalloid
Ni 2013	Rabbit	SDF	Whole blood and LR and whole blood/LR	Sham	TVD, PVD, PPV, MFI	Blood and crystalloid combined superior to either fluid on its own
Paxian 2003	Rat	IVM	PRBC and perflubron emulsion/HES and PRBC/perflubron emulsion	LR and HES and whole blood	Diameter, velocity, flow	O_2 emulsion superior to whole blood, red cells, or non-oxygen carrying fluid
Sakai 1999	Hamster	IVM	Whole blood	HSA	Velocity, flow, diameter, FCD	Whole blood is superior to colloid
Villela 2009	Hamster	IVM	High O_2-affinity PRBC (50mmHg)	Low O_2-affinity PRBC (10mmHg)	Velocity, flow, diameter, FCD	Lower O_2 affinity of red cells superior to higher

Techniques: IVM: intravital microscopy; LDF: laser doppler flowmetry; SDF: sidestream dark field microscopy
Endpoints: PVD: perfused vessel density; TVD: total vessel density; PPV: proportion of perfused vessels; MFI: microvascular flow index; HI: heterogeneity index; FCD: functional capillary density;
Fluids: HBOC: haemoglobin based oxygen carrier; PRBC: packed red blood cells; LR: Ringer's lactate; NS: normal saline; HTS: hypertonic saline; HSA: human serum albumin; HC: haemorrhage control; HES: hydroxyl-ethyl starch; PBH: polymerized bovine haemoglobin; PEG-Alb: polyethylene glycol-conjugated albumin; PEG-Hb: polyethylene glycol-conjugated haemoglobin; UPBHb: ultrapurified polymerised bovine haemoglobin solution; DCLHb: diaspirin cross-linked haemoglobin; Hb: haemoglobin; EPO: erythropoietin

7.3.11. Osmotic and oncotic potential

There were 19 studies that tested the hypothesis that the osmotic/oncotic properties of a resuscitative fluid are most important in the restoration of the microcirculation following haemorrhage, of which 14 studies reported findings in keeping with this hypothesis. These are summarised in Table 7.9. Hypertonic-hyperosmotic solutions of saline-dextran[59], saline-HES[64, 77, 81], and HES[42, 82] were reported as superior to isotonic solutions. One study reported that hypertonic saline/dextran fluid improved microcirculatory parameters better than whole blood[72]. One study reported that hypertonic saline resuscitation was superior to isotonic fluid but only if whole blood was also returned to the animal[91]. Increase colloid pressure and volume expansion was reported as superior for microcirculatory restoration when comparing colloid to crystalloid solutions[53, 88], and when using modified colloids[29]. One study reported that the duration of time of oncotic force was important in restoring the microcirculation[28], and another showed that hypertonic solutions may restore microcirculatory flow for longer than isotonic solutions[41]. One study reported that hypertonic fluid is superior due to its reduced effects on red blood cell deformability when compared to isotonic fluids[92].

Five studies did not report superiority of higher osmotic/oncotic potential fluids; these included reports of equivalence[23] or inferiority[50]. Although some studies reported that the microcirculatory fluid dynamics were unaffected by higher oncotic/osmotic properties when compared to isotonic fluids, it was noted that the permeability of micro-vessels[66, 67] and haemoglobin oxygen saturation[58] may be improved with such solutions nevertheless.

Some studies reported that the hypertonic-hyperosmotic nature of the resuscitative fluids influenced the behaviour of leucocytes to a greater effect than isotonic solutions in their actions towards improving the microcirculation[66, 67, 81], as described later.

Table 7.9. Summary of study findings regarding osmotic/oncotic pressure

Study	Animal	Method	Test fluid(s)	Control fluid(s) or sham	Endpoint(s)	Main finding
Bauer 1993	Rat	IVM	7.2% HTS/Dextran and 7.2%HTS/10% HES	LR and Sham	Velocity, flow, diameter	Hypertonic and isotonic fluids are equivalent
Cabrales 2005(b)	Hamster	IVM	5% PEG-Alb	10% HES and HC	Velocity, flow, diameter, FCD	Length of time of oncotic pressure is important
Cabrales 2008(b)	Hamster	IVM	4% PEG-Alb	5% HSA and 10% HSA	Velocity, flow, shear stress, diameter, FCD	Increased plasma expansion superior with conjugated molecule
Cryer 2005	Rat	IVM	7.2% HTS/6% dextran and NS/dextran	NS	Velocity, flow, diameter	Effects of hypertonic/hyperosmotic fluid last longer than isotonic
Gierer 2004	Rat	IVM	10% HES and 7.2% HTS/6% HES	NS	FCD	Hypertonic/hyperosmotic fluid is superior
Kao 2011	Rat	IVM	7.5% HTS and 7.5% HTS/EPO	NS and NS/EPO and LR and LR/EPO	PVD	Hypertonic crystalloid inferior to isotonic
Komori 2005	Rabbit	IVM	HES	LR	Velocity, flow, diameter	Hypertonic/hyperosmotic fluid is superior
Maier 2009	Porcine	LDF and SDF	Gelatine and 7.2% HTS/6% HES	6% HES	Flow, capillary density, MFI	Hypertonic and isotonic fluids equivalent
Mazzoni 1990	Rabbit	IVM	HTS/dextran	LR	Diameter	Hypertonic/hyperosmotic fluid is superior
Paes-da-Silva 2003	Rat	IVM and LDF	7.5% HTS and 5% BSA and NS/HES and HTS/HES	Whole blood and NS	Diameter, flow	Hypertonic/hyperosmotic fluid is superior
Pascual 2001	Mouse	IVM	Pentastarch and LR	Sham	Diameter, velocity, shear rate	Hypertonic and isotonic fluids equivalent
Pascual 2002	Mouse	IVM	7.5% HTS and LR	Sham	Velocity, shear rate, shear stress	Hypertonic and isotonic fluids equivalent
Scalia 1990	Rat	IVM	HTS/dextran and dextran	Whole blood	Diameter	Hypertonic fluid superior to whole blood
Vajda 2004	Rat	OPS	HTS/HES	NS	Velocity, flow, FCD	Hypertonic/hyperosmotic fluid is superior
Vollmar 1994	Rat	IVM	10% HES and 7.2% HTS/10% HES	LR and Sham	Velocity, perfusion	Hypertonic/hyperosmotic fluid is superior
Vollmar 1996	Rat	IVM and LDF	10% HES and 7.2% HTS/10% HES	LR	Velocity, diameter, flow, FCD	Hypertonic/hyperosmotic fluid is superior
Wu 2015(b)	Rat	LSCI	NS and HTS and gelatine and HES	Sham and HC	Flow	Hypertonic/hyperosmotic fluid is superior
Zakaria 2006	Rat	IVM	Whole blood/NS and whole blood/HTS and HTS/NS	Sham	Diameter, flow	Hypertonic/hyperosmotic fluid is superior when given with whole blood
Zhao 2009	Rat	IVM	NS and HTS and HTS/dextran	Sham and HC	Shear rate	Hypertonic/hyperosmotic fluid improves RBC deformability

Techniques: IVM: intravital microscopy; LDF: laser doppler flowmetry; LSCI: laser speckle contrast imaging; SDF: sidestream dark field microscopy; OPS: orthogonal polarization spectral imaging
Endpoints: MFI: microvascular flow index; FCD: functional capillary density; PVD: perfused vessel density
Fluids: LR: Ringer's lactate; NS: normal saline; HTS: hypertonic saline; HSA: human serum albumin; HC: haemorrhage-only control; HES: hydroxyl-ethyl starch: BSA: bovine serum albumin; EPO: erythropoietin

7.3.12. Viscosity

There were 12 studies which tested the hypothesis that increased fluid viscosity was superior to normal or reduced viscosity in the restoration of microcirculatory flow following haemorrhage, as summarised in Table 7.10. Ten of these studies had findings in keeping with this hypothesis. Higher viscosity preparations of hydroxylethyl starch (HES) are reported as superior to lower viscosity HES[26, 33, 85]. Higher viscosity preparations of Ringers Lactate (by addition of 0.3% alginate) were superior to conventional Ringers Lactate in restoring the microcirculation[80]. Solutions with increased molecular weight (with higher viscosity) were show to be superior to lower molecular weight (and therefore lower viscosity) solutions; for example, using higher density polymerised human serum albumin (HSA)[60] or higher molecular weight HES[32].

The viscosity—rather than the oxygen carrying capacity—has been reported as the factor of importance even when using oxygen-carrying solutions[27, 31]. Furthermore increase in viscosity was reported as more important than the increase in oncotic pressure[30]. High viscosity preparations of pegylated bovine albumin were superior than the same preparations combined with red blood cells, demonstrating that transfusion haemoglobin triggers might be lowered if higher viscosity fluids are used[86].

Two studies did not find that higher viscosity was superior to lower; one of these compared higher and lower viscosity HBOCs[69] and another higher versus lower viscosity non-oxygen carrying fluids[45].

Table 7.10. Summary of study findings regarding viscosity

Study	Animal	Method	Test fluid(s)	Control fluid(s) or sham	Endpoint(s)	Main finding
Cabrales 2004	Hamster	IVM	0.7% and 0.8% LVM alginate	5% HES and HC	Velocity, flow, shear stress, diameter, FCD	Higher viscosity superior to lower
Cabrales 2005(a)	Hamster	IVM	10% HES/0.3% alginate and 10% HES/0.6% alginate	10% HES	Velocity, flow, diameter, FCD	Higher viscosity superior to lower
Cabrales 2007(a)	Hamster	IVM	OxyRBC and MetRBC	Fresh plasma	Velocity, flow, shear stress, diameter, FCD	Higher viscosity superior to lower, independent of O2 carrying capacity
Cabrales 2007(b)	Hamster	IVM	High-MW HES	Low-MW HES and Sham	Velocity, flow, FCD	Higher viscosity superior to lower
Cabrales 2007(c)	Hamster	IVM	OxyRBC and MetRBC	10% HES	Velocity, flow, shear stress, diameter, FCD	Higher viscosity superior to lower, independent of O2 carrying capacity
Cabrales 2008(a)	Hamster	IVM	6% hetastarch /0.4% alginate	6% hetastarch and HC	Velocity, flow, shear stress, FCD	Higher viscosity superior to lower
Guerci 2014	Porcine	LDF	HES/7% HTS	LR	Perfusion	No difference between viscosities
Messmer 2012	Hamster	IVM	High MW Polymerised HSA (3 different concentrations)	HSA	Velocity, flow, shear stress, diameter, FCD	Higher viscosity superior to lower
Peruski 2014	Canine	SDF	HBOC-alginate (hyperviscous)	Standard HBOC	PVD, TVD, MFI, PPV	Higher viscosity equivalent to lower
Villela 2011	Hamster	IVM	LR-alginate	LR	Velocity, flow, diameter, FCD	Higher viscosity superior to lower
Wettstein 2004(b)	Hamster	IVM	PEG-BSA/PRBC at 4 and 8 g/dL	PEG-BSA	Velocity, flow, diameter, FCD	Higher viscosity superior to lower and more important than oxygen carrying capacity
Wettstein 2006	Hamster	IVM	5% HES and 10% HES and 20% HES	HC	Velocity, flow, FCD	Higher viscosity superior to lower

Techniques: IVM: intravital microscopy; LDF: laser doppler flowmetry; SDF: sidestream dark field microscopy
Endpoints: PVD: perfused vessel density; TVD: total vessel density; PPV: proportion of perfused vessels; MFI: microvascular flow index; HI: heterogeneity index; FCD: functional capillary density
Fluids: HBOC: haemoglobin based oxygen carrier; PRBC: packed red blood cells; LR: Ringer's lactate; HSA: human serum albumin; HTS: hypertonic saline; MW: molecular weight; HC: haemorrhage-only control; HES: hydroxyl-ethyl starch; OxyRBC: oxygen-carrying red blood cells; MetRBC: methemoglobin red blood cells; PEG-BSA: pegylated bovine albumin; LVM: low viscosity high-mannuronic acid

7.3.13. Attenuation of inflammation

There were 9 studies that considered the anti-inflammatory properties of resuscitation fluids, as summarised in Table 7.11. Using albumin as a resuscitative fluid has been reported to both improve the microcirculatory parameters as well as reducing the inflammatory response[48]. A number of studies have proposed that small volume resuscitation with hypertonic-hyperosmotic solutions may affect the flow behaviour of leucocytes and reduce their stagnation[40], margination[22], rolling[67] and adhesion to the

endothelium[81], as well as attenuating the number of endothelium-leukocyte interactions[23, 66, 90]. Reduction of leucocyte adhesion has also been reported when using gelatin serum protein solutions as a resuscitative fluid[57].

Table 7.11. Summary of study findings regarding attenuation of inflammation

Study	Animal	Method	Test fluid(s)	Control fluid(s) or sham	Endpoint(s)	Main finding
Bauer 1993	Rat	IVM	7.2% HTS-Dextran and 7.2% HTS/10% HES	LR and Sham	Velocity, flow, diameter	Modified fluid can reduce leucocyte adhesion and restore microcirculatory flow
Bauer 1995	Rat	IVM	HES-desferoxamine conjugate	HES and Sham	Velocity, flow	Modified fluid to scavenge free radicals can reduce leucocyte adhesion and restore microcirculatory flow
Corso 1999	Rat	IVM and LDF	6% dextran and 7.2% HTS/10% dextran	LR	Velocity, flow, shear stress	Hypertonic fluid no difference in flow but attenuates leukocyte adhesion
Horstick 2002	Rat	IVM	20% albumin	NS	Velocity, shear rate	Albumin has anti-inflammatory properties
Maier 2004	Rat	IVM	Gelatin and 5%HSA and SPS	Sham	Diameter, velocity, flow	Serum protein solution can reduce inflammation
Pascual 2001	Mouse	IVM	Pentastarch and LR	Sham	Diameter, velocity, shear rate	Hypertonic fluid attenuates leukocyte adhesion
Pascual 2002	Mouse	IVM	7.5% HTS and LR	Sham	Velocity, shear rate, shear stress	Hypertonic fluid attenuates leukocyte adhesion
Vollmar 1994	Rat	IVM	10% HES and 7.2% HTS/10% HES	LR and Sham	velocity, perfusion	Hypertonic fluid attenuates leukocyte adhesion
Yada-Langui 2004	Rat	IVM	HTS	LR	Velocity, shear rate	Hypertonic fluid attenuates leukocyte adhesion

Techniques: IVM: intravital microscopy; LDF: laser doppler flowmetry;
Fluids: LR: Ringer's lactate; NS: normal saline; HTS: hypertonic saline; HES: hydroxyl-ethyl starch; SPS: serum protein solution; HSA: human serum albumin

7.3.14. Restorative properties

There were 5 recent studies from the USA that tested the hypothesis that endothelial glycocalyx is shed following haemorrhagic shock, and may be restored by components of plasma (but not crystalloid), restoring the microcirculatory dysfunction. All

studies reported that their results were in keeping with that hypothesis as summarised in Table 7.12 [54, 73-76].

Table 7.12. Summary of study findings regarding restorative properties of the fluid

Study	Animal	Method	Test fluid(s)	Control fluid(s) or sham	Endpoint(s)	Main finding
Kozar 2011	Rat	Electron microscopy	Fresh plasma	LR and Sham and HC	Glycocalyx thickness	Plasma can restore the endothelial glycocalyx
Torres 2013	Rat	IVM	FFP	LR and HES and HC and sham	Velocity, diameter	Plasma can restore the endothelial glycocalyx
Torres 2014	Rat	IVM	1:1 PRBC/LR and 1:1 washed PRBC/LR and whole blood	LR and Sham	Glycocalyx thickness, flow	Constituents of plasma can restore the endothelial glycocalyx
Torres 2015(a)	Rat	IVM	FFP	NS and 3% HTS and Sham	Glycocalyx thickness	Plasma can restore the endothelial glycocalyx
Torres 2015(b)	Rat	IVM	FFP	NS	Glycocalyx thickness, flow	Plasma can restore the endothelial glycocalyx and flow

Techniques: IVM: intravital microscopy
Fluids: PRBC: packed red blood cells; LR: Ringer's lactate; NS: normal saline; HTS: hypertonic saline; HC: haemorrhage-only control; HES: hydroxyl-ethyl starch; FFP: fresh frozen plasma

7.3.15. Notable exclusions

Some studies were ineligible for inclusion due to lack of comparator. This was either due to the same fluid being given in different volumes[94] or no control[95]. Some study protocols varied the amount of haemorrhage rather than resuscitation fluid[96, 97]. Isovolaemic exchange transfusion[98-100], haemodilution[101], and ischaemia-reperfusion[102-105] protocols were excluded. Small volume acute blood loss without haemorrhagic shock[106, 107] were also excluded.

There were multiple studies that used drug delivery as interventions (rather than purely comparing different fluids), including additives[108-111], noradrenaline[112], polydatin[113], nitric oxide[114] and naloxone[115].

Although of interest in the basic science of endothelial behaviour, in vitro studies[116] and those that measured endothelial relaxation[117, 118] and activity[119] were excluded. Similarly, conformational changes in red blood cells (such as deformability and fragility[99, 106]), and the modulation of the inflammatory components of haemorrhagic shock[120] and leucocyte behaviour[48, 81] were ineligible for inclusion.

Studies that only reported perfusion endpoints (such as delivery of oxygen) rather than any microcirculatory flow dynamics were also excluded[17, 18].

7.4. Discussion

According to the pre-clinical available evidence, the most favourable properties of resuscitative fluids for the restoration of the microcirculatory flow dynamics are: (i) the presence of a haemoglobin preparation (HBOC being mostly equivalent to whole blood); (ii) higher viscosity; (iii) higher oncotic/osmotic potential, and (iv) having the physical and constituent properties that enable attenuation of endothelial-leucocyte interactions, reduced inflammation and endothelial permeability. The evidence for these properties comes from 71 published pre-clinical studies that have each tested the basic scientific questions regarding physical properties of resuscitation fluids, as well as the influence of their constituents. Since none have tested all of these properties in a single experiment, it is only by summation and consideration of all available evidence that translatable research questions might be considered for the clinical context.

After catastrophic haemorrhage whole blood is not usually readily available, and fractionated parts of blood such as PRBCs, FFP, and platelets are precious resources. Furthermore, the most appropriate ratios of these fractions is a matter of controversy[121].

Availability is also not the only limitation, since whole blood or components may not be the ideal fluids to deliver following haemorrhagic shock; some of these pre-clinical studies have demonstrated superiority of other fluid strategies to delivery of whole blood. Regardless of the type of fluid delivered in the emergency scenario, the priority is to restore tissue perfusion by enabling the transport of oxygen at the microcirculatory surface. This goal requires consideration of which characteristics of the resuscitative fluid are most important for that task. Not only should the fluid restore the microcirculatory flow dynamics, but may also contribute to the mitigation and repair of endothelial injury that has occurred following haemorrhage. Restoration of the endothelium and endothelial glycocalyx and prevention of leucocyte-endothelial interactions may be key for longer-term outcomes, but such a question has not been answered in animal models. All of the individual fluid characteristics reported here provide a sound basis for further clinical research.

The design of an 'ideal' fluid for resuscitation after haemorrhagic shock appears to depend on several factors of importance. The careful balance of osmotic potential and viscosity in resuscitative fluids appears to allow the fluid to inhibit endothelial cell swelling, minimise shear stress, and keep the individual microcirculatory segments open long enough to allow the exchange of oxygen between the circulation and the end tissues. Some studies in this review have reported that Hb preparations and red cells improve the microcirculatory function by their osmotic and viscous effect on microcirculatory flow dynamics rather than their oxygen carrying capacity. Reduction in cell swelling and maladaptive endothelial-leucocyte interactions might lead to reduction in shunting of flow and subsequent systemic inflammatory response. The fluid itself has potential to deposit glycoproteins and essential components of the endothelial glycocalyx that may enable the microcirculation to improve its function. Although HBOCs are thought to interfere with the endothelium derived relaxing

factor and NO system and increase vasoconstriction, unwanted oxidative reactions, and endothelial-leucocyte interactions, the studies included in this review appear to report that appropriate modification of HBOCs can reduce such unwanted effects.

As well as the usual limitations in translatability that arise when attempting to apply results of animal studies to the clinical context, the studies included in this review were all at risk of threats to their validity according to the most widely common recommendations for animal studies[21]. The majority of studies were undertaken with rodent models rather than large animals, which may provide further issues for translatability; testing the same research question from a rodent study in a large animal study may result in a different answer[122]. Furthermore, all studies had a potential risk of bias according to the SYRCLE tool; indeed, the majority of studies were only assessed as positive in 3 or fewer domains (out of a possible 10). In particular, the general lack of blinding of investigators and outcome assessments mandate a cautious approach to their interpretation. It is not known whether these studies failed to fulfil all domains of the assessment tools due to methodological deficiencies or whether they simply did not report the relevant details. If further animal studies are to be conducted in this field, we would recommend that the experimental protocols *and reporting technique* are designed according to the SYRCLE tool domains of importance. This tool is based on the Cochrane Collaboration's Risk tool for assessing bias in randomised controlled trials, and should therefore be the gold standard for animal studies that hope to establish clinically sound hypotheses[123]. It is also important to note that a limited number of studies used sublingual video-microscopy, which is the most appropriate technique for human translatability, as discussed in previous chapters.

7.4.1. Limitations

Meta-analytical tests could not be undertaken for the studies included in this review since there were too many permutations of animal model, intervention, and outcome measure to provide consistent grouping of studies. Such a feasibility assessment was pre-defined in the original review protocol. Statistical heterogeneity could not be assessed and funnel plots could not be used to assess publication bias. This is a notable limitation since significant results are more likely to be published[124], and it is likely that unpublished and unavailable studies have not been included in the current systematic review. Nevertheless, this systematic review summarises the available published literature with regards to haemorrhagic shock resuscitation in pre-clinical models, and provides a basis on which to test hypotheses in the clinical context.

There are some clinically relevant omissions in the pre-clinical literature with regards to haemorrhagic shock resuscitation and the microcirculation. For example, there were no studies that tested platelets as a resuscitation fluid. When plasma was delivered and shown to be superior, the exact constituent components of benefit have not been identified. There were also no clinically relevant long-term outcomes analysed. The experimental protocols were not intended to assess clinically relevant outcomes such as 24-hour or survival to discharge, organ failure, or complications of treatment. Such questions could only be reliably tested in the clinical context.

7.5. Conclusion

Based on the available pre-clinical evidence, the ideal resuscitation fluid for restoration of microcirculatory flow following haemorrhagic shock is likely to contain a preparation of haemoglobin, favour higher oncotic/osmotic potential and viscosity, protect

and reconstitute the endothelium, and attenuate inflammation. The latter two of these are in keeping with the findings in Part 1 of this thesis, where endotheliopathy was reported as a significant pathological element in the early phase following trauma. The hypotheses here are derived from an extensive series of pre-clinical studies that have tested the basic biological questions regarding the physical properties of a wide range of fluids. Because of the potential risk of translatability, further evaluation in clinical studies are warranted in order to determine the 'ideal' resuscitative fluid to restore the microcirculation in humans.

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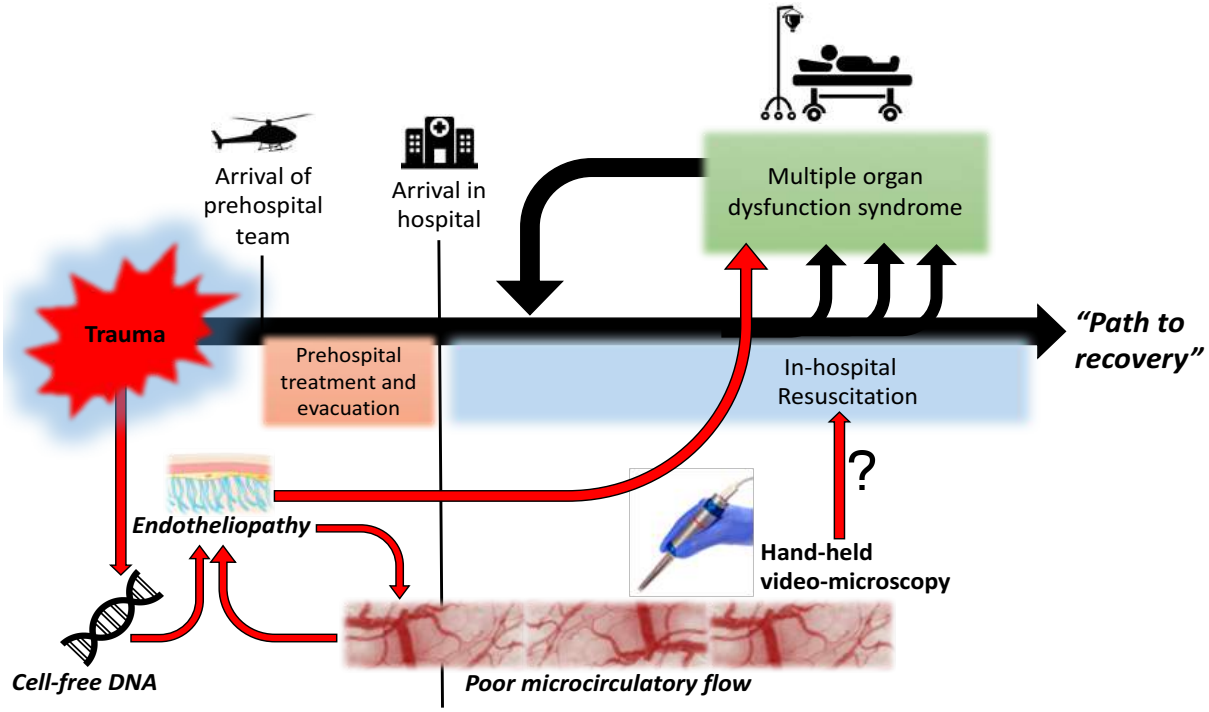
Chapter 8

Conclusions

8.1. Summary of findings

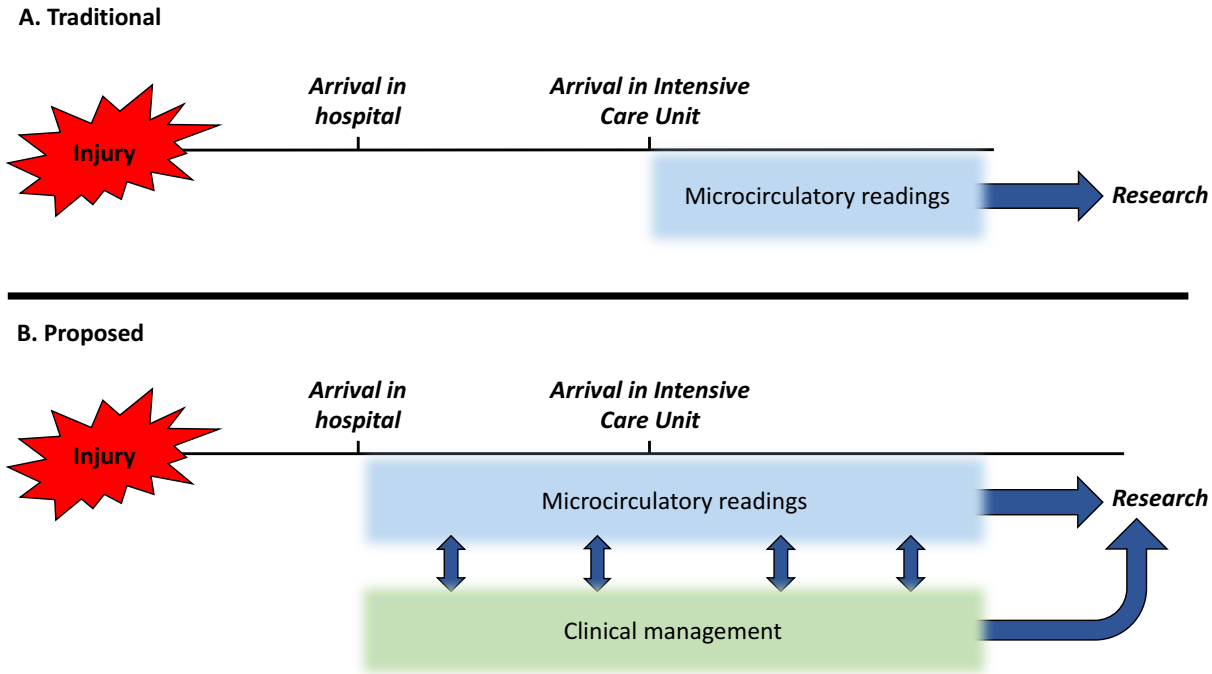
In Part 1 of this thesis I have presented some novel findings based on 5 hypotheses (Table 1.1), regarding the endothelium and microcirculation in the context of trauma and haemorrhagic shock. The first of these findings is that there is an association between the endotheliopathy of trauma and poor microcirculatory flow following haemorrhagic trauma, which is most prominent in the first hours after injury[1]. Secondly, endotheliopathy occurs just minutes after trauma, and is associated with multiple organ dysfunction syndrome[2]. Thirdly, these pathological processes are associated with the presence of cell-free DNA within the circulation, which may play a role in the mechanisms of microcirculatory impairment following trauma and haemorrhagic shock[3]. Taken together, these findings may partly contribute to the growing narrative of the early pathological response to trauma, particularly in the first minutes and hours after injury (Figure 8.1).

Figure 8.1. Schematic diagram of the mechanistic findings in Part 1



In Part 2 of this thesis, I have presented some data regarding the clinical context in which the microcirculatory derangements described in Part 1 might be important, by addressing a further 3 broad hypotheses (Table 1.1). I have presented that microcirculatory flow can be assessed safely soon after arrival in the Emergency Department[4], and that the flow and heterogeneity might be quantified at the point-of-care[5]. It might therefore be proposed that microcirculatory monitoring be performed earlier, and be integrated into the clinical management pathway if justified by further investigation (Figure 8.2).

Figure 8.2. Schematic diagram of (a) the traditional approach to microcirculatory monitoring, and (b) what might be proposed based on the findings within this thesis.



Figures 8.1 and 8.2 are deliberately over-simplified, since there are many other processes occurring following trauma, and the findings presented in this thesis are only a small part of a much larger network of complex, interconnected pathological events.

In anticipation that microcirculatory monitoring may one day be used within the realm of clinical practice, I have further presented a synthesis all of the available pre-clinical literature regarding fluids and restoration of microcirculatory flow[6,7]. From these pre-clinical studies, it appears that the optimal fluid for this aim would contain a preparation of haemoglobin, favour higher oncotic potential, higher viscosity, and mitigate the inflammation and endotheliopathy of trauma. Further clinical investigations are required in order to determine whether the hypotheses generated from these pre-clinical studies can be supported in clinical practice, and may be guided by the findings presented.

8.2. Avenues for future research

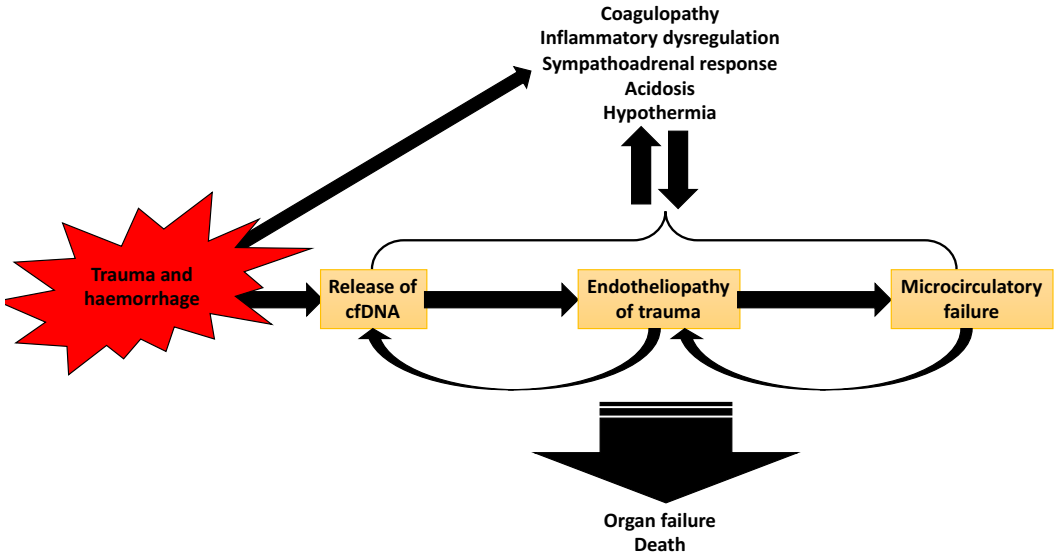
In the process of investigating the hypotheses within this thesis, several more have been generated that require further clinical studies in order to address. Some research questions warrant further discussion, and will be briefly summarised here.

8.2.1 Does endotheliopathy of trauma cause microcirculatory dysfunction, or vice versa?

In Chapters 2 – 4, we were able to see associations between cfDNA, endotheliopathy, and microcirculatory flow disruption[1-3]. Unfortunately, within the limitations of observational clinical studies, the precise causality could not be demonstrated. A linear narrative from point of injury that includes a timeline of these phenomena cannot be proven with the current data, since there was no definitive evidence of cause-and-effect. Indeed, it is likely that a continuous timeline from one phenomenon to another is an over-simplification, since cfDNA, endotheliopathy, and microcirculatory failure are just a few elements in a far greater and interconnected network of pathological processes, that includes coagulopathy[8], inflammatory dysfunction, genomic activation, and subsequent

organ failure[9,10]. From the data presented, a hypothesis might be proposed that injury leads to the release of cfDNA as one type of damage-associated molecular pattern (DAMP), which exacerbates endotheliopathy, leading to microcirculatory flow disruption. Together, these processes may each make the others worse, and may increase the likelihood of coagulopathy[8], inflammatory dysfunction, and increase the risk of subsequent organ failure and death (Figure 8.3). The finding that the microcirculatory flow dynamics mostly improve over time for all patients, even when biomarkers of endotheliopathy may remain elevated (Chapter 2)[1] remains unexplained. It may suggest that current ICU resuscitation techniques are aimed at the restoration of flow and perfusion, but not endotheliopathy—which may be responsible for poorer outcomes, even when flow is restored. Whether ICU treatment directed at both restoration of the endothelium *and* perfusion and flow would improve patient outcomes is unknown.

Figure 8.3. Schematic illustration of a hypothesis for the sequence of events after trauma.



It is also important to note that any proposed sequence of events would not exist in isolation, but must be taken in the context of the many other elements within the physiological response to trauma. Finding the answers to questions regarding timescales may be important when planning treatments that might address each pathological factor. The discovery that endotheliopathy occurs within minutes of injury may have implications for pre-hospital resuscitative care, with a potential new therapeutic target: the preservation and reconstitution of the endothelial glycocalyx. This may be particularly important in the context that coagulopathy of trauma may also be a pre-hospital phenomenon[11].

8.2.2 Why are early pathological processes associated with organ failure days later?

In Chapter 3, the failure to restore the endothelium within the first hours of injury was associated with organ failure some days later[2]. In Chapter 4, raised cfDNA was associated with poorer clinical outcomes days later[3]. The full narrative that connects these early phenomena with later clinical outcomes is not fully understood. It may be the case that these early insults to the physiology may have lasting effects on organs, and that failure to "restore" homeostasis may increase the likelihood of this occurrence. This hypothesis would be supported by data shown in Chapter 3, where patients who developed MODS were less likely to have restoration of the endothelium than those who did not[2]. A return to normal levels of endothelial biomarkers on arrival in hospital was associated with lower levels of organ failure. The exact physiological, biochemical, and anatomical effects of early endotheliopathy and microcirculatory dysfunction require further investigation. A better understanding of homeostatic mechanisms would allow insights into the pathophysiology and the potential therapeutic opportunities.

8.2.3 Would point-of-care tests for cfDNA or endotheliopathy be useful in clinical practice?

We have seen in Chapters 2 – 4 that both cfDNA and endothelial biomarkers might be used to predict clinical outcomes. Such data may have the potential to guide decisions such as early triage, or requirement for surgery or intensive care. For example, could the presence of endotheliopathy or raised cfDNA be combined with other physiological parameters in order to improve the sensitivity and specificity of pre-hospital triage systems, or the requirement for massive transfusion? Point-of-care lactate devices have already been used within the pre-hospital environment[12], and in-hospital lactate values from arterial blood gas are commonplace during trauma care, but there are no point-of-care tests for endotheliopathy in clinical practice. A rapid technique for cfDNA quantification has been described[13], but this has not yet entered clinical practice. A recent systematic review reported a low level of accuracy for current pre-hospital triage systems[14]. Could the addition of further biochemical data within the pre-hospital period improve these systems? As well as for triage and prognosis, it is possible that they may also have some potential in guiding treatment. For example, would early detection of endotheliopathy in the pre-hospital environment favour a plasma-first resuscitation strategy, in order to restore the endothelial glycocalyx? Such a question would require prospective investigation.

8.2.4. Could cfDNA levels be used as a biomarker of injury severity?

There have been some criticisms of the utility and accuracy of the Injury Severity Score (ISS), and several variations have been proposed[15,16]. Furthermore, anatomical injury severity scoring systems are vulnerable to inter-user variability[17]. These scoring systems attempt to quantify the injury burden by adding together scores from individual anatomical

areas. Rather than calculating arbitrary scores on an ordinal scale, it is possible that the concentration of cfDNA within the circulation could be a biomarker of injury burden. Although this has not been thoroughly investigated, it is an appealing hypothesis, because it may offer an objective, continuous variable that quantifies the full burden of visible and hidden tissue injury. The exact quantities of tissue or cellular sources of cfDNA would need to be determined. Further large-scale investigations are required to compare cfDNA levels to other forms of injury severity scoring systems, as well as with clinical outcomes.

8.2.5. Can bedside microcirculatory monitoring be used for goal-directed therapy for shocked patients?

Handheld video-microscopy or the detection of microcirculatory flow disruption has been used for research purposes for decades, but has not yet entered mainstream clinical practice. Two of the factors preventing clinical use are (a) the lengthy and offline computer analysis; and (b) doubts about the feasibility of using this technology at the point-of-care, for critically unwell patients. These two concerns have partly been addressed within Chapters 5 and 6[4,5]. I have demonstrated that the technique can be used safely within the ED, even for patients with haemorrhagic shock, and that it is still possible to capture high quality videos for these patients. Whether access to these extra data at the bedside might affect clinical practice or guide resuscitation is unknown, since no interventional trial has been conducted. Although clinicians might be able to grade the microcirculation as accurately as offline analysis (Chapter 6), this may not be as accurate as traditional offline analysis, especially when it comes to density parameters. This technique has also not yet been tested in real-time at the patient bedside, and its clinical utility is unknown. The next step would be to design a study that utilised the findings in both Chapters 5 and 6, so that

clinicians might assess the microcirculation at the bedside for critically unwell patients. At time of writing, I am an investigator in an ethically approved study that has been designed to do exactly this (Real time clinical validation of the Point-of-Care Microcirculation tool at the bedside; REC reference 17/SC/0274). Furthermore, a prospective randomised controlled trial with bedside handheld video-microscopy compared to normal care might test the utility of these extra data for goal-directed therapy.

8.2.6. Which therapeutic strategies are superior for the restoration of the microcirculation?

Although some hypotheses were generated for this question in Chapter 7 using a systematic review of all pre-clinical literature[6], the answer for humans is unknown. There is uncertainty regarding the optimal ratios of blood products that might be ideal following trauma and haemorrhage[18], and whether there is a place for crystalloid or colloid fluids in trauma care. More recently, whole blood transfusion has been reported to be associated with greater survival than fractionated components[19]. Furthermore, there is some pre-clinical evidence of a wide range of non-fluid therapies that might restore the microcirculation, such as sulodexide[20], tranexamic acid[21], and polyethylene oxide[22]. If randomised controlled trials are undertaken in the future to compare resuscitation strategies (such as component therapy *versus* whole blood for damage control resuscitation), data regarding microcirculatory flow would be of value, especially if those data were to be associated with clinical outcomes.

8.3. References

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