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LOYOLA UNIVERSITY CHICAGO

# THE MOLECULAR PATHOPHYSIOLOGY OF SEPSIS-ASSOCIATED DISSEMINATED INTRAVASCULAR COAGULATION AND ITS PHARMACOLOGIC MODULATION

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN PHARMACOLOGY AND MOLECULAR THERAPEUTICS

BY

AMANDA TEMPLE WALBORN

MAYWOOD, ILLINOIS

AUGUST 2018

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| A-a $DO_2$ | Alveolar-Arterial Oxygen Difference |
| ADP | Adenosine Diphosphate |
| ALI | Acute Lung Injury |
| ANOVA | Analysis of Variance |
| Ang-1 | Angiopoietin 1 |
| Ang-2 | Angiopoietin 2 |
| APACHE | Acute Physiology and Chronic Health Evaluation |
| APC | Activated Protein C |
| aPTT | Activated Partial Thromboplastin Time |
| ARDS | Acute Respiratory Distress Syndrome |
| AT | Antithrombin |
| ATP | Adenosine Triphosphate |
| AUC | Area Under the Curve |
| BPM | Beats per Minute |
| CASP | Colon Ascendens Stent Peritonitis |
| CBC | Complete Blood Count |
| CDC | Centers for Disease Control |
| CLP | Cecal Ligation and Puncture |
| CMF | Comparative Medicine Facility |
| DIC | Disseminated Intravascular Coagulation |
| dl | Decileter |

| | |
|---|---|
| DVT | Deep Vein Thrombosis |
| *E. coli* | *Escherichia coli* |
| ELISA | Enzyme Linked Immunosorbent Assay |
| EGF | Epidermal Growth Factor |
| EPCR | Endothelial Cell Protein C Receptor |
| F | Factor |
| F1.2 | Prothrombin Fragment 1.2 |
| $FiO_2$ | Fraction of Inspired Oxygen |
| GAG | Glycosaminoglycan |
| GP | Glycoprotein |
| HIT | Heparin Induced Thrombocytopenia |
| HMGB-1 | High Mobility Group Box 1 |
| HMWK | High Molecular Weight Kininogen |
| IACUC | Institutional Animal Care and Use Committee |
| ICAM | Intercellular Adhesion Molecule |
| ICU | Intensive Care Unit |
| IFNγ | Interferon γ |
| IL-1α | Interleukin 1α |
| IL-1β | Interleukin 1β |
| IL-2 | Interleukin 2 |
| IL-4 | Interleukin 4 |
| IL-6 | Interleukin 6 |

| | |
|---|---|
| IL-8 | Interleukin 8 |
| IL-10 | Interleukin 10 |
| INR | International Normalized Ratio |
| ISTH | International Society on Thrombosis and Hemostasis |
| IV | Intravenous |
| JAAM | Japanese Association for Acute Medicine |
| K | Thousands |
| $K^+$ | Potassium |
| K/ml | Thousands per Milliliter |
| kDa | Kilodalton |
| kg | Kilogram |
| L | Liter |
| LMWH | Low Molecular Weight Heparin |
| LPS | Lipopolysaccharide |
| MAP | Mean Arterial Pressure |
| MCP-1 | Monocyte Chemoattractant Protein 1 |
| MI | Myocardial Infarction |
| mg | Milligram |
| ml | Milliliter |
| $mm^3$ | Cubic millimeter |
| mmHg | Millimeters Mercury |
| mmol | Millimole |

| | |
|---|---|
| MODS | Multiple Organ Dysfunction Syndrome |
| MP | Microparticles |
| MP-TF | Microparticle-Derived Tissue Factor |
| Na | Sodium |
| NET | Neutrophil Extracellular Trap |
| ng | Nanogram |
| NHP | Normal Human Plasma |
| NO | Nitric Oxide |
| PAI-1 | Plasminogen Activator Inhibitor 1 |
| $PaCO_2$ | Arterial Partial Pressure of Carbon Dioxide |
| $PaO_2$ | Arterial Partial Pressure of Oxygen |
| PAR-1 | Protease Activated Receptor 1 |
| PAR-2 | Protease Activated Receptor 2 |
| PDGF | Platelet Derived Growth Factor |
| PE | Pulmonary Embolism |
| PCT | Procalcitonin |
| PF-4 | Platelet Factor 4 |
| PMN | Polymorphonuclear |
| PT | Prothrombin Time |
| qSOFA | Quick Sequential Organ Failure Assessment |
| rTM | Recombinant Thrombomodulin |
| SAC | Sepsis Associated Coagulopathy |

| | |
|---|---|
| SCCM | Society for Critical Care Medicine |
| SD | Standard Deviation |
| SEM | Standard Error of the Mean |
| SIRS | Systemic Inflammatory Response Syndrome |
| SOFA | Sequential Organ Failure Assessment |
| t-PA | Tissue Plasminogen Activator |
| TAFI | Thrombin-Activatable Fibrinolysis Inhibitor |
| TAT | Thrombin-Antithrombin Complex |
| TF | Tissue Factor |
| TFPI | Tissue Factor Pathway Inhibitor |
| TGFβ | Transforming Growth Factor β |
| TLR | Toll-Like Receptor |
| TM | Thrombomodulin |
| TNFα | Tumor Necrosis Factor α |
| TT | Thrombin Time |
| UFH | Unfractionated Heparin |
| US | United States |
| μmol | Micromole |
| μg | Microgram |
| μl | Microliter |
| VCAM | Vascular Cell Adhesion Molecule |
| VEGF | Vascular Endothelial Growth Factor |

| | |
|---|---|
| VTE | Venous Thromboembolism |
| vWF | von Willebrand Factor |
| WBC | White Blood Cell |
| WT | Wild Type |

## LIST OF NON-STANDARD ABBREVIATIONS

| | |
|---|---|
| ENHANCE | Extended Evaluation of Recombinant Human Activated Protein C |
| OPTIMIST | Optimized Phase 3 Tifacogin in Multicenter International Sepsis Trial |
| PROWESS | Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis |
| PROWESS-SHOCK | Prospective Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis and Septic Shock |

# CHAPTER ONE

# REVIEW OF LITERATURE

## Hemostasis

The maintenance of blood flow through the circulation is critical to adequately supply oxygen and nutrients to organs and tissues and thereby maintain life. The ability to recover from disruptions to the integrity of the vascular system is critical to this function. Hemostasis is the process by which blood within the vasculature remains a free-flowing liquid while also permitting the rapid formation of solid clots to plug defects in ruptured or injured blood vessels. This physiological response to vascular injury prevents excessive blood loss from a damaged blood vessel through vascular constriction, platelet plug formation, and blood coagulation, forming a plug to block the vascular leakage site. This process is accomplished through the combined actions of the vascular wall (including the endothelial and subendothelial layers), platelets, and the coagulation and fibrinolytic systems. Disruption of this process, resulting in either excessive bleeding or excessive clotting, can have severe and potentially fatal consequences.

The initial response to vascular injury is vasoconstriction, mediated through neurogenic reflexes. This restricts blood flow through the damaged vessel, minimizing blood loss. Primary hemostasis is mediated by platelets and results in the formation of an initial platelet plug. This is followed by secondary hemostasis, in which the coagulation

cascade is activated and a fibrin clot is formed. The fibrinolytic system ultimately degrades this clot as tissue healing occurs.

**Coagulation.**

The coagulation cascade, shown in Figure 1, is a network of circulating zymogen proteins as well as cofactors, activators, and inhibitors. Coagulation factor proteases are produced in the liver and circulate in inactive, proenzyme forms. When a coagulation factor is activated by enzymatic cleavage, it gains the ability to proteolytically activate the next factor in the coagulation cascade. This system is responsible for the production of an insoluble, gel-like fibrin clot. Coagulation can be initiated through either the intrinsic (contact activation) or extrinsic (tissue-factor mediated) pathway. These pathways converge at the conversion of Factor X to its active form, FXa.

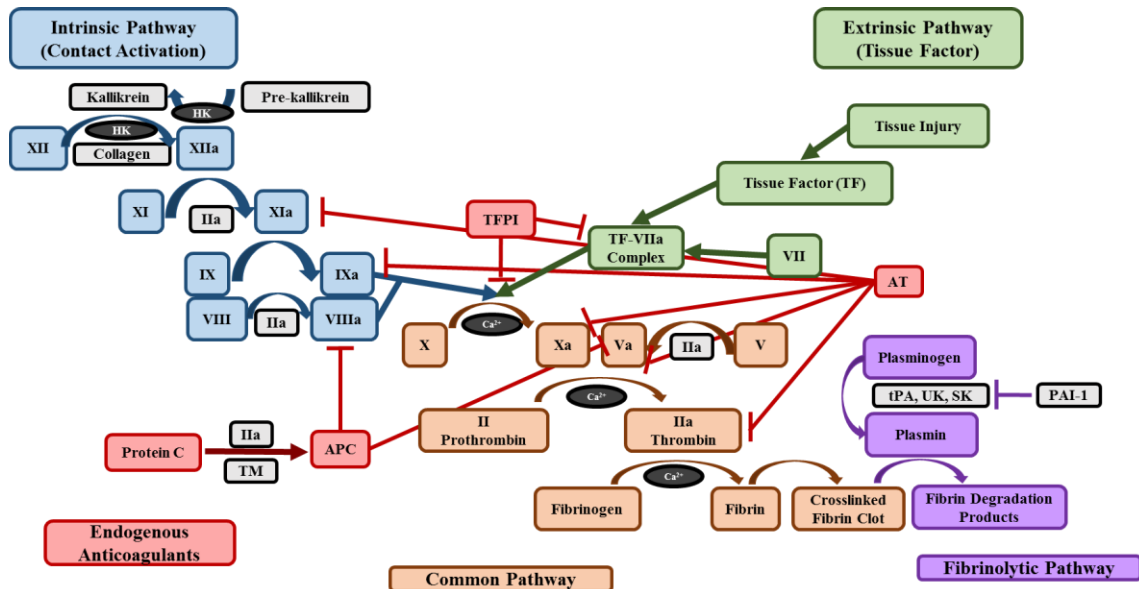


**Figure 1. The Coagulation Cascade.** The coagulation cascade is commonly discussed in terms of the intrinsic or contact activation pathway (shown in blue), the extrinsic pathway, initiated by tissue factor (shown in green). The intrinsic and extrinsic pathways converge at the activation of factor X in the common pathway (shown in orange), which ultimately leads to the formation of a crosslinked fibrin clot. Endogenous anticoagulants (shown in red) including protein C, activated protein C (APC), thrombomodulin (TM), antithrombin (AT), and tissue factor pathway inhibitor (TFPI), act at various points on the coagulation cascade to prevent inappropriate or excessive coagulation. The fibrinolytic pathway (shown in purple) is responsible for the degradation of the fibrin clot.

The extrinsic pathway is activated by the presence of tissue factor (TF) in the circulation. TF is a cell surface protein expressed on subendothelial cells and can therefore be exposed to the circulation following endothelial damage. Other conditions, including inflammation, can increase the expression of TF on intravascular cells. TF functions as a cell surface receptor for FVII and catalyzes the conversion of FVII to

FVIIa. The TF/FVIIa complex then catalyzes the conversion of FX into the active protease FXa.

The intrinsic pathway is initiated upon exposure of subendothelial collagen to the circulation by the formation of a complex of high molecular weight kininogen (HMWK), prekallekrein, and FXII on a collagen scaffold. This leads to the conversion of FXII to its active form FXIIa. The generation of FXIIa leads to an activation cascade, ultimately resulting in the generation of FXa.

The common pathway begins where the intrinsic and extrinsic pathways converge at the conversion of FX to FXa. FXa is responsible for the conversion of prothrombin (FII) to thrombin (FIIa). Thrombin is a serine protease with the ability to convert soluble fibrinogen into insoluble fibrin. Thrombin also exerts positive feedback on the coagulation cascade, activating factors XI, VII, V, and XIII. Factor XIII crosslinks fibrin strands, stabilizing the clot.

Endogenous anticoagulants, including antithrombin (AT), tissue factor pathway inhibitor (TFPI), and protein C, serve to prevent excessive or inappropriate coagulation. These anticoagulants act at different points throughout the coagulation cascade, preventing excessive coagulation initiated by either the intrinsic or extrinsic pathway. AT is an inhibitor of factors XIa, Xa, IXa, and IIa. TFPI inhibits the conversion of FVII to FVIIa as well as FXa. Protein C is an inhibitor of FVIIIa and FVa.

**Fibrinolysis.**

The fibrinolytic system consists of the enzymes responsible for clot dissolution. Like the coagulation factors, the main fibrinolytic enzyme, plasmin, is produced as a proenzyme, plasminogen, in the liver and activated when cleaved into its enzymatic form. The breakdown of a clot is a necessary part of the healing and tissue repair process. Plasmin cleaves the fibrin meshwork, degrading the clot. This forms fibrin split products, including D-Dimer, which can be detected in the blood as biomarkers of clot breakdown. Fibrinolysis is regulated by promotors such as tissue plasminogen activator (t-PA) and inhibitors including plasminogen activator inhibitor 1 (PAI-1), $\alpha_2$-antiplasmin, and thrombin activatable fibrinolysis inhibitor (TAFI).

**Platelets.**

Platelets are small, discoid, anucelear cells derived from megakaryocytes that react to subendothelial proteins and can aggregate to form a primary hemostatic plug. Following endothelial injury, platelets adhere to subendothelial collagen. vWF serves as a molecular bridge between the collagen and the platelet membrane receptor glycoprotein (GP)Ib. Adhesion via GPIb works to platelets from the circulation and slow their flow to allow for activation and firmer adhesion mediated by GPIIb/IIIa. Activated platelets release granules. Light (α) granules contain fibrinogen, fibronectin, coagulation factors (F)V, and FVIII, platelet factor 4 (PF-4), platelet derived growth factor (PDGF), and transforming growth factor β (TGFβ). Dark (β) granules contain ADP, ATP, $Ca^{2+}$, histamine, serotonin, and epinephrine. The release of granule contents leads to the aggregation and activation of additional platelets. In addition to forming the primary

hemostatic plug, the phospholipid surface of activated platelets provides a site for coagulation factors to combine with ionized calcium to activate the intrinsic pathway of the coagulation cascade.

**Endothelium.**

Under normal physiological conditions, the endothelium has anticoagulant properties. Endothelial cells secrete prostaglandins and nitric oxide (NO), both of which inhibit platelet aggregation. Additionally, endothelial cells produce endogenous anticoagulants, including protein C, antithrombin (AT), thrombomodulin, protein S, and tissue factor pathway inhibitor (TFPI). The endothelial cell layer also prevents the exposure of circulating blood to subendothelial collagen. When the endothelium is damaged, collagen is exposed and initiates platelet aggregation and coagulation. Endothelial cells also produce von Willebrand Factor (vWF), which acts as a molecular bridge between platelets and collagen. In addition to causing the loss of the anticoagulant properties of the endothelium, activation or damage can cause a procoagulant response, mediated through the expression of the coagulation activator tissue factor (TF). The endothelium is also responsible for the production of many of the regulators of fibrinolysis.

**White Blood Cells.**

White blood cells (WBCs), also known as leukocytes, are the chief mediators of the immune system. Several distinct types of leukocytes develop in the bone marrow from hematopoietic stem cells, each with a district role in immunity. The WBC count is measured clinically as part of the complete blood count (CBC). The normal range for

WBC in a CBC is approximately 4,000-10,000 cells/µl. An elevated WBC count, known as leukocytosis, can be indicative of inflammation or infection.

The immune response can be broadly divided into two categories; innate and acquired. Innate immunity is the set of nonspecific mechanisms that respond rapidly to pathogen exposure. Several cell types are involved in innate immunity, including natural killer cells, dendritic cells, basophils, eosinophils, mast cells, and the phagocytes; macrophages, monocytes, and neutrophils. Adaptive immunity develops throughout an individual's life based on exposure to pathogens. B and T lymphocytes are the primary mediators of adaptive immunity.

Neutrophils are of particular importance in the response to infections such as sepsis. Neutrophils are the most abundant circulating leukocyte and are the first responder in the case of bacterial or fungal infection. Neutrophils are often described as polymorphonuclear (PMN) leukocytes on the basis of their multilobed nucleus. In response to infection or inflammatory mediators such as interleukin 8 (IL-8), neutrophils migrate through the blood vessel wall towards the site of infection. At the site of infection, neutrophils release cytokines in order to recruit additional WBCs. Neutrophils also have several direct anti-infectious activities: phagocytosis, degranulation, and the release of neutrophil extracellular traps (NETs). In phagocytosis, neutrophils internalize and subsequently kill bacteria. Neutrophils can also degranulate, releasing a variety of antimicrobial proteins into the extracellular environment to kill or neutralize bacteria. NETosis, or the production of NETs, describes the process by which neutrophils can expel their nuclear contents, including chromosomal DNA, as well as antimicrobial

proteins, into the extracellular environment in order to trap or kill invading bacteria. As NETs have been shown to exhibit pro-inflammatory and pro-thrombotic properties, this process is particularly relevant to sepsis-associated coagulation dysfunction.

Monocytes are a type of leukocyte capable of differentiation into macrophages or dendritic cells. Macrophages are phagocytes located primarily in the tissue that consume pathogens and cellular debris. Macrophages can secrete both pro-inflammatory and anti-inflammatory cytokines in order to modulate the immune system. Macrophages and dendritic cells are also involved in the initiation of the adaptive immune response to a specific pathogen. In response to inflammation, monocytes and macrophages can also express TF, contributing to a procoagulant state.

**Abnormal Hemostasis.**

Numerous pathological conditions can result in abnormal hemostasis, leading to either excessive bleeding or pathological clot formation. Thrombosis is the pathological formation of a clot, known as a thrombus, within an intact blood vessel. A thrombus blocks blood flow through the affected vasculature. Additionally, a thrombus can break free from its location of origin through a process known as embolization and become lodged in a distant vessel. Embolization of a clot can be fatal, particularly if lodged in the lungs (pulmonary embolism), heart (myocardial infarction), or brain (thromboembolic stroke).

The conditions required for thrombosis to occur are described by Virchow's Triad, shown in Figure 2. Thrombosis occurs under conditions of endothelial damage,

stasis, and hypercoagulability. Endothelial damage or dysfunction results in the expression of TF, exposure of collagen, and the loss of endothelial anticoagulant properties. Low flow rates facilitate clot formation, and therefore coagulation is also favored in locations where blood is static in the vasculature. This commonly includes the atria of patients with atrial fibrillation or the veins of the lower legs, particularly in sedentary or immobilized individuals. Consequently, hospitalized patients often require prophylactic anticoagulation. Hypercoagulability can occur due to genetic factors, including mutations in coagulation factors or endogenous anticoagulants, or can be acquired due to situations including inflammation, smoking, oral contraceptive use, sepsis, cancer, or trauma. When endothelial damage, stasis, and hypocoagulability co-occur, there is potential for thrombus formation. Anticoagulant therapy is often indicated in these situations.

In addition to thrombosis, abnormal hemostasis can also result in bleeding. Thrombocytopenia can occur due to platelet consumption, platelet destruction, splenic sequestration, or reduced production due to hereditary or acquired alterations in bone marrow. Qualitative platelet disorders such as Glanzmann's thrombasthenia or Bernard-Soulier disease can also predispose patients to bleeding. Reductions in circulating coagulation factors, through excessive consumption, pharmacologic modulation, or genetic disorders such as hemophilia (deficiencies in FVIII or FIX), can also contribute to bleeding risk. Bleeding can also occur secondary to excessive activation of the fibrinolytic system due to decreased circulating fibrinogen and the anticoagulant effects of fibrinogen degradation products.

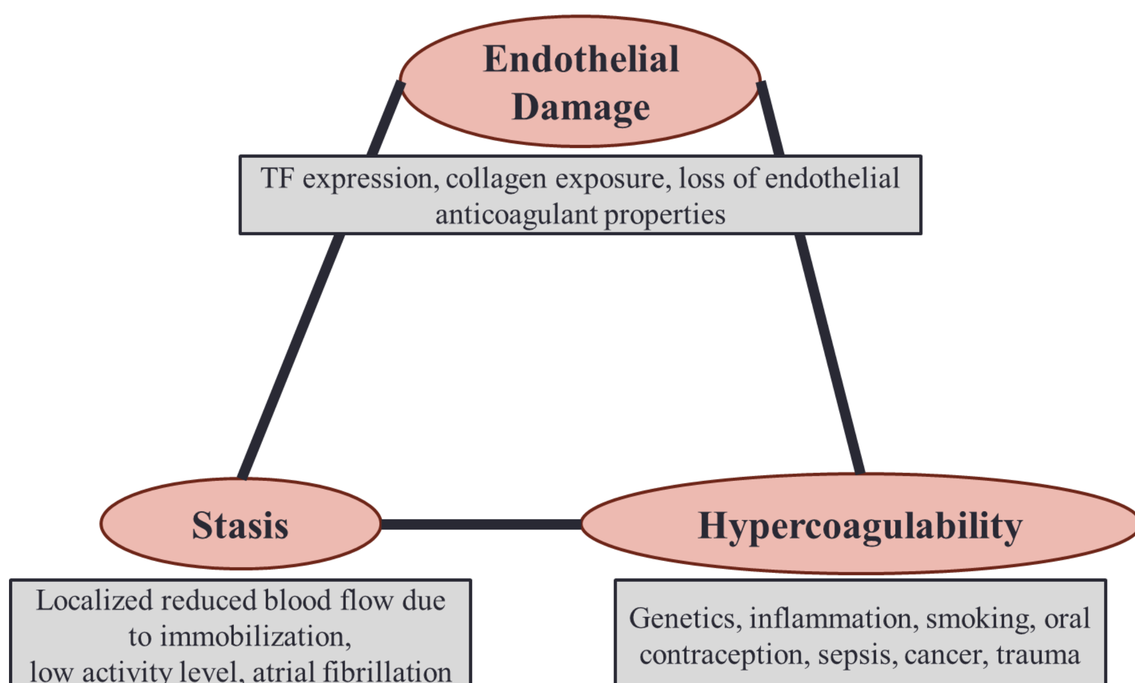


**Figure 2. Virchow's Triad.** Virchow's Triad describes the three conditions that must be met for thrombosis to occur.

**Inflammation.** A significant, bi-directional relationship exists between inflammation and thrombosis. Inflammation and inflammatory factors including the multi-protein inflammasome complex, have been implicated in the pathogenesis of many thrombotic disorders including sepsis, atherosclerosis, coronary artery disease, and atrial fibrillation. Several of the prototypical proinflammatory cytokines including IL-6, IL-1β, and TNFα induce the expression of TF on intravascular cells, particularly monocytes and endothelial cells, resulting in the development of a procoagulant state. Coagulation can also contribute to systemic inflammation. Thrombin and FXa activate protease activated receptors (PARs), G-protein coupled cell surface receptors, which have diverse effects including pro-inflammatory activities.

## Sepsis

Sepsis is a severe systemic response to infection characterized by an overwhelming inflammatory response, and is a complex and potentially devastating clinical scenario with high mortality. Sepsis often develops from the spread of a localized infection, such as pneumonia, urinary tract infection, or a skin or surgical site infection, but can also occur due to direct entry of bacteria into the bloodstream. The defining pathophysiologic feature of sepsis is the overwhelming host response to infection. Although a robust immune response is necessary to overcome infection, the immune response in sepsis can occur to excess. This leads to the derangement of multiple physiological processes and can cause dysfunction and failure of most major organ systems. The immune dysfunction observed in sepsis is not limited to the initial hyperinflammatory response, typically characterized by elevated plasma cytokine levels. Septic patients may experience qualitative defects in the function of neutrophils and other immune cells. Patients may also progress into a state of “immune paralysis” in which the resources of the immune system are exhausted and a patient can no longer mount an effective defense against infection. Clinical manifestations of sepsis include hypo- or hyperthermia, tachycardia, increased respiratory rate or reduced oxygen saturation, altered mental status, and hypotension. This hypotension may progress to shock and contribute to organ failure and death through impaired perfusion. However, numerous other processes, such as coagulation dysfunction, are involved in the development and progression of sepsis and contribute greatly to patient outcome.

**Clinical Definitions of Sepsis.**

For clinical and research purposes, scoring systems incorporating clinical and laboratory parameters are used to describe and diagnose sepsis patients. The most recent set of guidelines (SEPSIS-3) was published by the Society for Critical Care Medicine (SCCM) in 2016 (Singer2016). These guidelines, summarized in flow chart form in Figure 3, present a process to be used in the diagnosis of sepsis in patients with a documented or suspected source of infection. Using these guidelines, patients are first screened with the Quick Sequential Organ Failure Assessment (qSOFA) tool. Patients meeting qSOFA criteria, defined as two or more of: respiratory rate ≥ 22 breaths per minute, altered mentation, and systolic blood pressure ≤ 100 mmHg, are then evaluated with the Sequential Organ Failure Assessment (SOFA) score, shown in Table 1. Patients with a SOFA score of two or greater, or a change of 2 or greater from baseline status in the case of pre-existing organ dysfunction, are considered to have sepsis. Patients with sepsis are then further evaluated for septic shock on the basis of the requirement for vasopressors to maintain mean arterial pressure (MAP) ≥65 mmHg and serum lactate levels >2 mmol/L.

While new definitions for sepsis were published in 2016, the currently available literature describes sepsis according to the previous set of guidelines published in 1992 (Bone 1992) and updated in 2001 (Levy 2003). Understanding of these guidelines is necessary to facilitate understanding of previous research, and these guidelines are detailed in Table 2. Patient samples used in this dissertation were collected prior to the publication of the 2016 guidelines and therefore were collected on the basis of the 1992

and 2001 guidelines. These guidelines define a spectrum of severity of illness ranging from sepsis to multiple organ dysfunction syndrome (MODS). These guidelines also define systemic inflammatory response syndrome (SIRS) as a sepsis-like inflammatory response in the absence of an infection.

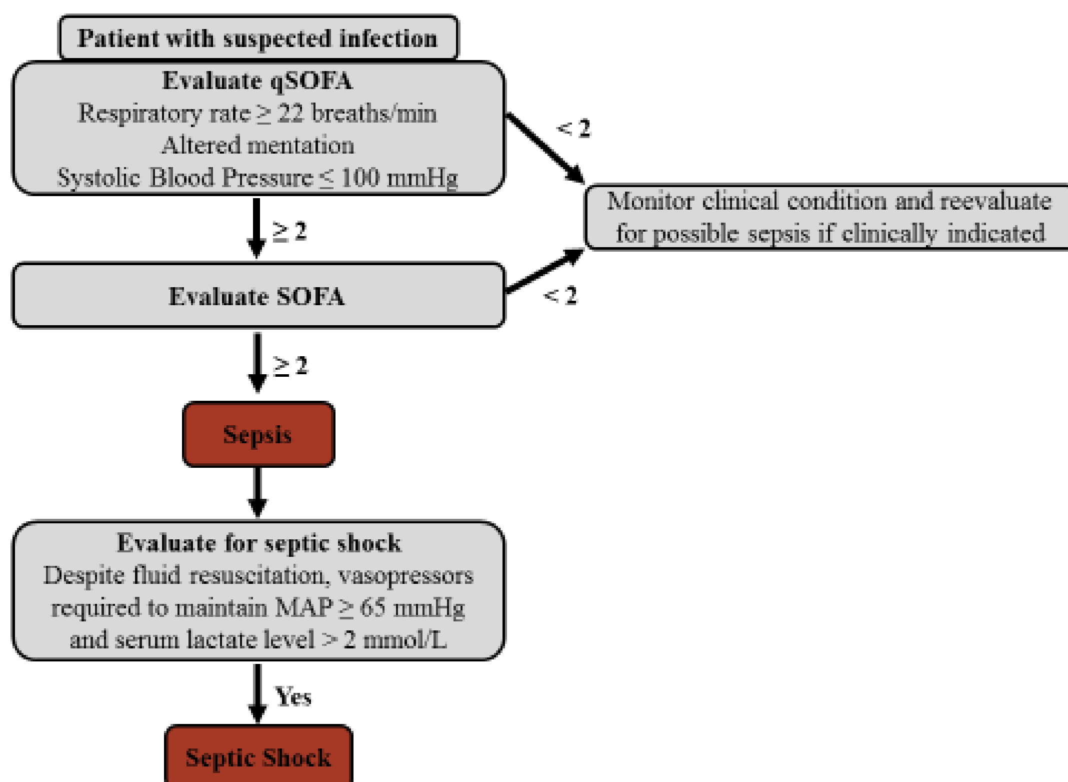


**Figure 3. Summary of SEPSIS-3 Guidelines for the Diagnosis of Sepsis**. Figure adapted from Singer et. al. 2016. In patients with confirmed or suspected sepsis, the qSOFA score is first evaluated as a screening mechanism. In patients with a qSOFA ≥ 2, the more extensive SOFA score is then evaluated. Patients with sepsis are then further evaluated for the presence of shock.

**Table 1. The Sequential Organ Failure Assessment (SOFA) Scoring System**

| SOFA Score | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| ***Respiration*** **$PaO_2/FiO_2$, mmHg** | <400 | <300 | <200 (with respiratory support) | <100 (with respiratory support) |
| ***Coagulation*** **Platelets, K/ml** | <150 | <100 | <50 | <20 |
| ***Liver*** **Bilirubin, mg/dl (μmol/l)** | 1.2-1.9 (20-32) | 2.0-5.9 (33-101) | 6.0-11.9 (102-204) | >12.0 (>204) |
| ***Cardiovascular*** **Hypotension (Doses given in μg/kg*min)** | MAP < 70 mmHg | Dopamine ≤ 5 Or dobutamine, any dose | Dopamine > 5 Or epinephrine ≤ 0.1 Or norepinephrine ≤ 0.1 | Dopamine > 15 Or epinephrine > 0.1 Or norepinephrine > 0.1 |
| ***Central nervous system*** **Glasgow Coma Score** | 13-14 | 10-12 | 6-9 | <6 |
| ***Renal*** **Creatinine, mg/dl (μmol/l) or urine output** | 1.2-1.9 (110-170) | 2.0-3.4 (171-299) | 3.5-4.9 (300-440) Or <500 ml/day | >5 (>440) Or <200 ml/day |

Table adapted from the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine (Singer 2016; (Vincent1996)

**Table 2. SCCM Definitions for Sepsis and Associated Conditions**

| Condition | Definition |
|---|---|
| **Bacteremia** | The presence of viable bacteria in the blood |
| **Systemic inflammatory response syndrome (SIRS)** | Systemic inflammatory response to a variety of severe clinical insults, manifested by two or more of the following conditions:<br>• Temperature >38ºC or < 36ºC<br>• Heart rate >90 BPM<br>• Respiratory rate >20 breaths per minute or $PaCO_2$ <32 mmHg<br>• White blood cell count >12,000/cu mm, <4,000/cu mm, or >10% immature forms |
| **Sepsis** | The systemic response to infection, manifested by two or more of the following as a result of the infection:<br>• Temperature >38ºC or < 36ºC<br>• Heart rate >90 BPM<br>• Respirator rate >30 breaths per minute or $PaCO_2$ <32 mmHg<br>• White blood cell count >12,000/cu mm, <4,000/cu mm, or >10% immature (band) forms |
| **Severe sepsis** | Sepsis associated with organ dysfunction, hypoperfusion, or hypotension, including but not limited to lactic acidosis, oliguria, or acute alteration in mental status |
| **Septic shock** | Sepsis-induced hypotension despite adequate fluid resuscitation along with perfusion abnormalities including but not limited to lactic acidosis, oliguria, or acute alteration in mental status. Patients receiving inotropic or vasopressive agents may not be hypotensive at the time perfusion abnormalities are measured |
| **Sepsis-induced hypotension** | Systolic blood pressure <90 mmHg or a reduction of ≥40 mmHg from baseline in the absence of other causes of hypotension |
| **Multiple organ dysfunction syndrome (MODS)** | Presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention |

Table adapted from the consensus guidelines set forth by the ACCP/SCCM consensus conference committee (Bone1992; Levy2003)

**Epidemiology of Sepsis.**

Although it is clear that sepsis is a critical clinical condition with high mortality, precise estimation of the incidence of sepsis and associated mortality is difficult due to disease heterogeneity. However, it is clear that sepsis and associated illnesses are major causes of death in the United States and around the world. In 2010, "septicemia" was listed by the Centers for Disease Control (CDC) as the 11th most common cause of death (Murphy 2013). In that same year, more than 132,000 hospitalized patients in the US died with a first-listed diagnosis of sepsis (Hall 2013), and between 1999 and 2016, 2,470,666 deaths (6% of total deaths in the United States) listed sepsis among the causes of death (Epstein 2016). Over 1 million cases of sepsis are estimated to occur in US hospital patients annually, with 2% of all hospitalized patients and 6-30% of ICU patients experiencing sepsis (Martin 2012). A 2016 meta-analysis estimated that 31.5 million cases of sepsis occur annually around the globe, including 19.4 million cases of severe sepsis and accounting for 5.3 million deaths (Fleischmann 2016).

In addition to occurring with a high incidence worldwide, sepsis is associated with a high short-term mortality rate. The same 2016 analysis estimated in-hospital mortality in high-income countries at 17% for sepsis and 6% for severe sepsis (Fleischmann 2016). A separate study also published in 2016 reported the incidence of sepsis in the US in 2012 as 436 cases per 100,000 individuals, with an overall mortality of 17.3% (Stoller 2016). Using the 1992 definitions, mortality has been estimated at 10-20% for sepsis, 20-50% for severe sepsis, and 40-80% for septic shock (Martin 2012). Other estimates for mortality for severe sepsis range from 14.7-29.9% (Gaieski 2013;

Hawiger 2015). Less data is available with regards to patient outcome using the 2016 sepsis definitions. However, a French study published in 2017 reported 24% mortality for patients presenting to the emergency department with suspected infection and a qSOFA of ≥ 2 (Freund 2017). A retrospective analysis of 844 patients in the placebo arm of a large clinical trial in severe sepsis patients reported that those who die within the first 5 days in the ICU are most likely to die of refractory shock, while those who die after more than 5 ICU days are more likely to die of respiratory failure (Macias 2004).

The consequences of sepsis extend beyond short term in-hospital mortality. A study of 3195 patients with severe sepsis in 42 ICUs throughout Japan found a 26.4% 28 day mortality rate but a 43.7% 90 day mortality rate (Hayakawa 2016). A Taiwanese study demonstrated that patients who recover from sepsis experience higher rates of severe adverse events, including: ischemic or hemorrhagic stroke, myocardial infarction, heart failure, and sudden cardiac death (Ou 2016). These patients also experienced higher all-cause mortality compared to age and gender matched non-septic hospitalized or healthy controls (Ou 2016). Even when patients survive, sepsis still places a significant financial burden. The median cost of a hospital stay for a patient with severe sepsis in 2012 was estimated at $55,749 (Stoller 2016). In 2011 it was estimated that the annual cost of sepsis in the US is greater than $20 billion (Hawiger 2015).

## Disseminated Intravascular Coagulation

Disseminated intravascular coagulation (DIC) is an acquired coagulation disorder with high mortality that is characterized by both thrombotic and bleeding complications. In DIC, widespread activation of the coagulation cascade occurs, leading to fibrin deposition in the microvasculature and subsequent ischemia and organ failure. This inappropriate coagulation activity consumes platelets and coagulation proteins, leading to an elevated bleeding risk. Consequently, patients with DIC typically present with symptoms of both thrombosis, including organ dysfunction due to microthrombi in the vasculature, as well as bleeding, ranging from low-grade oozing from vascular access sites or surgical sites to potentially massive hemorrhage (Hunt 2014).

DIC does not develop spontaneously but rather occurs as a complication of a number of predisposing conditions. DIC occurs most commonly in association with sepsis or trauma (Gando 2016), but can also occur in conjunction with cancer, obstetrical complications, vascular disorders, toxin exposure, aneurysm, liver disease, and immunological disorders (Levi 1999; Taylor 2001; Wada 2013). Although DIC secondary to each of these conditions may exhibit certain similarities, there are notable differences in DIC pathophysiology based on the underlying condition. For the purposes of this dissertation, discussion of DIC will be limited to cases occurring secondary to sepsis.

**Pathophysiology of DIC.**

The pathophysiology of DIC is complex and involves multidirectional interactions between the coagulation cascade, platelets, the vascular endothelium, and the inflammatory and immune response. Excessive inflammation, dysregulation of the endogenous anticoagulant system, activation of platelets and endothelial cells, and bacterial and antibacterial processes contribute to a systemic prothrombotic state in DIC patients. Ongoing coagulation can lead to further dysregulation of these processes, amplifying disease development.

Extensive cross-talk between inflammation and coagulation has been noted in DIC as well as in other clinical scenarios characterized by inflammation. In sepsis, bacterial components, particularly lipopolysaccharide (LPS), elicit a vigorous inflammatory response. This includes production of high levels of inflammatory cytokines such as interleukin (IL)-6, IL-1β, and tumor necrosis factor α (TNFα). These factors induce the expression of tissue factor (TF) on the surface of intravascular cells including monocytes and endothelial cells. The presence of high levels of TF in the circulation activates the extrinsic pathway of the coagulation cascade and is generally considered to be a major initiator of coagulation in sepsis-associated DIC (Esmon 2011; Gando2016; Levi 2017).

Under homeostatic conditions, excessive coagulation is prevented by a set of endogenous anticoagulants, including thrombomodulin (TM), antithrombin (AT), activated protein C (APC), and tissue factor pathway inhibitor (TFPI) (Gando 2016; Ikezoe 2015). The endogenous anticoagulant system becomes dysregulated in DIC,

allowing inappropriate coagulation to occur. In addition to increasing the levels of procoagulant factors, high levels of inflammatory cytokines also downregulate endogenous anticoagulants, including TM, protein C, and endothelial cell protein C receptor (Esmon 2004). The reduction in protein C levels in DIC patients has been shown to correlate with poor outcomes (Levi 2001; ten Cate 2000). In addition to its anticoagulant activity, APC also possesses anti-inflammatory properties; these are also lost in DIC, further contributing to disease (Gando 2016). Although circulating levels of TM may not be markedly reduced in DIC patients, this is not due to upregulation but rather to shedding from the damaged endothelium, which further contributes to dysregulation of the endogenous anticoagulant system. Levels of AT are typically reduced in DIC patients, due to both reduced synthesis in the liver and increased consumption to combat excessive coagulation activation.

Other factors that contribute to the pathophysiology of DIC include endothelial damage, platelet activation, and antibacterial response. In response to bacterial presence in the bloodstream, bactericidal factors such as neutrophil extracellular traps (NETs), composed of nuclear materials and bactericidal enzymes, are released into the bloodstream. While these components play a role in the eradication of bacteria, they are also prothrombotic and contribute to coagulation dysfunction and endothelial damage. Activation of the endothelium, including TF upregulation induced by inflammatory cytokines, as well as damage due to bactericidal factors provides favorable sites for thrombus initiation throughout the circulation. Inflammatory and bactericidal factors can also activate platelets, further perpetuating thrombus formation.

In order to fully understand the pathophysiology of DIC, it is important to appreciate the contribution of each of these dysregulated systems as well as their replication in animal models of DIC and modulation by therapeutic agents. Representative markers of these aspects of DIC will be discussed in an upcoming section.

**The Coagulation Cascade in DIC.** In the clinical setting, coagulation is assessed through a set of standardized coagulation tests. These tests are also used to monitor coagulation status and response to therapy in patients treated with anticoagulants. Patients with sepsis and DIC have abnormal results on these standard coagulation screening tests, with prolonged clotting times indicating a hypocoagulable state. However, patients with sepsis and DIC also experience a propensity for clotting, indicating that the appropriate interpretation of these tests may differ between DIC patients and anticoagulated patients.

The extrinsic pathway is monitored by prothrombin time (PT), and is commonly reported as international normalized ratio (INR), a standardized method to allow comparison of values between laboratories. INR is commonly elevated in sepsis patients and is used in the diagnosis of DIC. PT/INR is also used to monitor coagulation in patients taking warfarin, which reduces the synthesis of the vitamin K dependent factors II, VII, IX and X. A normal INR value is 1, and the therapeutic target range for patients taking warfarin is between 2 and 3. Functional and antigenic levels of FVII can also be measured to evaluate the function of the extrinsic pathway.

The activated partial thromboplastin time (aPTT) is used to monitor the function of the intrinsic pathway and to monitor coagulation in patients receiving heparin. Factor

II, VI, IX, X, and XII antigenic and functional levels can be measured to assess this arm of the coagulation cascade.

Fibrinogen, the soluble precursor to fibrin, can also be measured clinically. Reduced fibrinogen levels due to consumption is a component of the DIC scoring system. Fibrinogen is produced in the liver as an acute phase reactant. Accordingly, fibrinogen can also be elevated in DIC, particularly in the earlier stages. Factor X antigenic and functional levels can be measured as an alternative assessment of the function of the coagulation cascade

The coagulation anomalies observed in sepsis patients range from slight perturbations in laboratory values to severe overt DIC. A study of 38 patients with severe sepsis reported an abnormal standard coagulation screen, including PT and aPTT, in 95% of patients (Collins 2006). As INR is one of the key parameters used clinically to identify patients with sepsis-associated coagulopathy, it is important to understand the appropriate interpretation of this measure in this specific patient population. Prolonged PT or elevated INR is generally indicative of a hypocoagulable state; however, patients presenting with SAC with an elevated INR are at risk of complications due to both thrombosis and bleeding.

Elevated PT or INR is reported in 90% or more of sepsis patients with severe disease (Collins 2006; Kinasewitz 2004; Koami 2015). Prolonged PT and elevated INR are associated with increased mortality and poor clinical outcome in sepsis patients (Dhainaut 2005; Kinasewitz 2004) as well as other critically ill or injured patient populations (MacLeod 2003; Walsh 2010). Elevated PT-INR (typically INR $\geq 1.2$) is

often a component of the inclusion criteria for clinical trials in patients with sepsis-associated coagulation disorders (Abraham 2003; Vincent 2013). The majority of the elevated INRs within this patient population have been reported to fall into the range of 1.6 to 2.5 (Walsh 2010). Other changes in global coagulation parameters, including aPTT (Bakhtiari 2004; Collins 2006; Daudel 2009; Johansson 2010; Kinasewitz 2004; Koami 2015) and whole blood clotting ability as measured by thromboelastography (Daudel 2009; Johansson 2010; Koami 2015), are also often reported in sepsis patients as well as in other critically ill patient populations.

Despite clear evidence that significant changes to the overall coagulation profile occur in sepsis, changes in the levels of individual coagulation factors in sepsis and DIC patients are less well established. Reduced levels of coagulation factors including factors II, V, VII, X, and XII relative to those in normal individuals have been reported in sepsis-associated coagulopathy (Collins 2006). However, these results demonstrated no discernible relationship to standard coagulation tests and are highly variable between studies (Collins 2006; Daudel 2009; Johansson 2010).

PT/INR was designed to monitor the anticoagulation status in patients treated with warfarin and is widely used clinically for this purpose. Warfarin treated patients are typically considered appropriately anticoagulated with an INR of between 2 and 3, and regular adjustments to drug dosage are made to maintain the INR within this range. A study of the relationship of serial INR levels to severe bleeding in patients receiving warfarin anticoagulation found that warfarin patients hospitalized with severe bleeding

showed an elevated INR compared to non-bleeding patients (5.9 ± 5.9 vs. 2.3 ± 0.7) as well as higher INRs before the event of the bleed (3.0 ±1.2 vs. 2.1 ± 0.8) (Kucher 2004)

The difference in INR levels at which bleeding occurs in warfarin treated and DIC patients as well as the fact that DIC patients with an elevated INR indicative of hypocoagulability experience both thrombotic and bleeding complications suggests that the information provided by this common laboratory test may be significantly different in these two patient populations. Accordingly, studies are required to compare the relationship of laboratory coagulation tests and levels of individual coagulation factors with INR in patients with DIC to the relationships observed in warfarin treated patients.

**Diagnosis of DIC.**

DIC is diagnosed through the application of a scoring system incorporating clinically available laboratory parameters describing coagulation dysfunction. For the purposes of this project, the International Society of Thrombosis and Hemostasis (ISTH) scoring system for overt DIC, shown in Table 3, will be used (Taylor 2001); however, the Japanese Association for Acute Medicine (JAAM) definition for DIC (Gando 2006), which incorporates a similar set of parameters, is used in some literature and is shown in Table 81 in Appendix C. The presence of a condition associated with DIC, including sepsis, trauma, organ destruction, malignancy, obstetrical calamity, vascular abnormality, severe hepatic failure, and severe toxic or immunologic reaction is a prerequisite for the application of this scoring system. For the purposes of this proposal, discussion of DIC is limited to cases associated with sepsis.

**Table 3. ISTH Scoring System for DIC**

| Variable | Value | Points |
|---|---|---|
| **Platelets (K/µl)** | >100 | 0 |
| | 50-100 | 1 |
| | <50 | 2 |
| **INR** | <1.3 | 0 |
| | 1.3-1.7 | 1 |
| | >1.7 | 2 |
| **D-Dimer (ng/ml)** | <400 | 0 |
| | 400-4000 | 2 |
| | >4000 | 3 |
| **Fibrinogen (mg/dl)** | >100 | 0 |
| | <100 | 1 |

Following the diagnosis of a condition associated with DIC, points are assigned based on clinical test results according to the above scoring system. Platelet count is typically reduced in DIC due to consumption. The international normalized ratio (INR), a standardized method of reporting the prothrombin time (PT), which is reflective of the status of the extrinsic coagulation pathway, is prolonged due to coagulation factor consumption. D-Dimer, a clinically validated marker of thrombosis, is elevated due to thrombus formation in the microvasculature. Fibrinogen, the soluble precursor of insoluble fibrin, is typically reduced in DIC due to consumption. However, in some patients, fibrinogen may be elevated due to its production in the liver as an acute phase reactant.

Using this scoring system, a score of 5 or higher is indicative of overt or severe DIC. A score of 3-4 indicates the presence of an intermediate phenotype, typically referred to as non-overt DIC. A score of 0-2 indicates that DIC is not present.

DIC may be present in patients at the time of presentation to the emergency room or may develop in already hospitalized sepsis patients. In a study of 259 patients with septic shock actively monitored for DIC, 61 patients had DIC at admission, and 32 additional patients developed DIC within 24 hours following admission despite the initiation of antibiotics and other therapies (Delabranche 2016).

**Epidemiology of DIC.**

Studies of DIC epidemiology and outcomes are complicated by the general heterogeneity of this disease as well as variants in the predisposing condition, diagnostic criteria used, and local standard of care. Coagulopathy is common in patients with sepsis. It has been estimated that changes in hemostasis become clinically significant in 50 to 70% of sepsis patients, and that 35% of patients with sepsis will meet the criteria for DIC (Levi 2017). Another study identified DIC in 27% of patients with thrombocytopenia following diagnosis with severe sepsis or septic shock (Hawiger 2015). In a study of 77 Japanese patients admitted to the ICU with a diagnosis of sepsis, 48.1% developed overt DIC according to the ISTH scoring system (Koyama 2014). Furthermore, these patients also experienced more severe illness, as quantified by higher Acute Physiology and Chronic Health Evaluation (APACHE) II and SOFA scores (Koyama 2014). In a study of 259 patients with septic shock, 93 developed DIC as diagnosed by the ISTH score (Delabranche 2016). These patients had elevated mortality compared to those who did not develop DIC (45.2% vs. 28.3%) as well as elevated SOFA scores, incidence of acute kidney injury and hepatic failure, and increased requirement for renal replacement therapy, vasopressors, and blood products (Delabranche 2016). A study evaluating

consecutive critically ill patients with DIC diagnosed according to the ISTH criteria at Mayo Clinic found an incidence rate of 18.6 per 100,000 person years in 2010, with higher rates in men and the elderly (Singh 2013). Sepsis was a predisposing factor in 59.7% of cases, and the overall in-hospital mortality ranged from 39%-58% in the years included in this study (Singh 2013)

Development of DIC in a patient with sepsis is an independent predictor of mortality (Bakhtiari 2004; Levi 1999; Ogura 2014), and may double the risk of sepsis-associated death (Levi 1999). Numerous studies have reported mortality due to DIC at or above 40% (Cauchie 2006; Delabranche 2016; Gando 2016; Gando 2008). Specifically, mortality has been reported at 56% in patients with overt and non-overt DIC by the ISTH diagnostic criteria (Cauchie 2006) or 21.9% in patients diagnosed by the JAAM criteria (Gando 2008).

## Biomarkers in DIC

The scoring algorithms used to describe DIC incorporate only clinically available coagulation parameters. While this may effectively describe a developed coagulopathy, it does not provide significant insight into the underlying pathophysiology of DIC. In order to address this shortcoming, over 160 biomarkers have been assessed for relevance to sepsis (Pierrakos 2010), with many also assessed for their relevance to DIC, but no single marker has been established as diagnostic or prognostic for either condition (Annane 2005; Bakhtiari 2004; Koyama 2014; Pierrakos 2010; Sims 2016; Wacker, 2013; Wada 2013). Many studies have evaluated biomarkers of a single system, such as inflammation

or coagulation, or have not established relationships between biomarker levels and well-defined coagulopathy.

The studies outlined in this dissertation include profiling of an array of biomarkers representative of multiple facets of the pathophysiology of sepsis-associated DIC in a cohort of patients with well-defined illness. These markers will be used in combination to gather information about the underlying pathophysiology and its relationship to outcome. Additionally, use of these markers will be validated in an animal model and the response of these factors to treatment will be assessed. Hemostatic biomarkers analyzed include D-Dimer, Prothrombin Fragment 1.2 (F1.2), and Plasminogen Activator Inhibitor 1 (PAI-1). Inflammatory and infection biomarkers analyzed include nucleosomes, High Mobility Group Box 1 (HMGB-1), procalcitonin (PCT), the interleukins (IL) IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10, Tumor Necrosis Factor α (TNFα), Interferon γ (IFNγ), Monocyte Chemoattractant Protein 1 (MCP-1), Epidermal Growth Factor (EGF), and Vascular Endothelial Growth Factor (VEGF). Endothelial biomarkers analyzed include Tissue Factor Pathway Inhibitor (TFPI), Protein C, endocan, angiopoietin 2 (Ang-2), and von Willebrand factor (vWF). Platelet biomarkers analyzed include CD40L, platelet factor 4 (PF4), microparticles (MP), and microparticle-derived tissue factor (MP-TF). A description of each marker follows.

**Hemostatic Biomarkers.**

As DIC is a disorder defined by hemostatic dysfunction, evaluation of multiple hemostatic parameters is critical to the understanding of this disease. Aside from clinically used functional coagulation tests such as INR, the most commonly evaluated marker is D-Dimer, an indicator of thrombus breakdown. While this marker has been validated as an indicator of ongoing coagulopathy in patients with DIC, evaluation of other components of the coagulation system including the thrombin generation marker prothrombin fragment 1.2 (F1.2) fibrinolytic regulator plasminogen activator inhibitor 1 (PAI-1) may provide further insight into the coagulopathy occurring in DIC.

**D-Dimer.** D-Dimer is formed when the crosslinked fibrin mesh is cleaved by plasmin as a clot is degraded, and is therefore considered a fibrin degradation product. Structurally, D-Dimer is composed of 2 D domains, giving the factor its name, as well as a single E domain. Clinically, D-Dimer is used in the diagnosis of thrombotic conditions, such as deep vein thrombosis (DVT) or pulmonary embolism (PE), and is particularly useful in excluding thrombosis in situations with a low probability.

D-Dimer is well established as a potential marker for sepsis and DIC and is a component of the ISTH DIC scoring algorithm (Taylor 2001). D-Dimer is elevated in patients with sepsis and DIC due to the breakdown of pathologically formed thrombi (Ishikura 2014; Singh 2015; Taylor 2001). As D-Dimer is formed from the breakdown of already formed thrombi, it may not be an optimal marker for early diagnosis of DIC but rather better suited for late-stage DIC (Singh 2015).

**Prothrombin Fragment 1.2 (F1.2).** Prothrombin Fragment 1.2 (F1.2) is produced when prothrombin, the precursor of thrombin, is cleaved to form active thrombin and is therefore measured as a marker of thrombin generation. Due to ongoing thrombin generation and thrombus formation, F1.2 is expected to be increased in sepsis-associated DIC. F1.2 levels are also measured to assess coagulation activation in experimental settings, such as the administration of LPS to healthy volunteers (de Jonge 2000).

F1.2 levels may have prognostic implications for patients with sepsis-associated DIC. Elevated levels of F1.2 were observed in 77.5% of patients enrolled in the PROWESS study, a clinical trial of APC in patients with severe sepsis (Kinasewitz 2004). Furthermore, F1.2 levels were significantly lower in survivors than non-survivors over the first 5 days after study enrollment (Kinasewitz 2004). In the Phase 2b study for rTM (ART-123), F1.2 levels changed significantly in response to treatment, with a 16% decrease from baseline to day 7 in the rTM treated group compared to an 8% increase from baseline in the placebo group (Hoppensteadt 2014). Preliminary data from the ongoing Phase 3 trial for ART-123, which is designed to enroll patients with more severe DIC than the Phase 2b study, has demonstrated elevated F1.2 at baseline in the Phase 3 patients compared to the Phase 2b patients, indicating a correlation with disease severity (Hoppensteadt 2015). F1.2 has also been shown to be predictive of DIC development and thrombotic risk in patients with thermal burn injury (Kowal-Vern 2013).

**Plasminogen Activator Inhibitor 1 (PAI-1).** Plasminogen activator inhibitor 1 (PAI-1) is an endogenous suppressor of fibrinolysis, and increased PAI-1 levels can

result in impaired thrombus clearance (Esmon, 2004). Increased thrombin generation is the more common causative factor for DIC; however, impaired fibrinolysis is observed in some patients and contributes to disease pathophysiology. Patients with DIC with suppressed fibrinolysis often have increased PAI-1. These patients cannot break down the thrombi in their microvasculature, leading to disproportionately severe organ dysfunction compared to the thrombus load predicted by the D-Dimer level (Asakura 2003).

In the PROWESS trial, elevated PAI-1 was only observed in 44% of patients at baseline, indicating that impaired fibrinolysis is not a universal phenomenon in patients with DIC (Kinasewitz 2004). However, in this same study, elevated PAI-1 correlated significantly with APACHE score and was associated with reduced survival (Kinasewitz 2004). PAI-1 levels were also found to correlate with disease severity in a study of sepsis patients during evaluation in the emergency department (Shapiro 2010). A study examining fibrinolytic markers in 117 patients with sepsis-associated DIC demonstrated an inverse relationship between PAI-1 and D-Dimer levels and a direct relationship between PAI-1 and poor prognosis, elevated MODS score, and reduced survival (Madiowa 2006). A study of 77 patients admitted to the ICU with sepsis, including 37 with DIC found that PAI-1 was elevated at baseline in non-survivors compared to survivors and in those with overt DIC compared to non-DIC patients on ICU days 0 through 3 (Koyama 2014).

**Inflammatory and Infection Biomarkers.**

The hyper-inflammatory response is one of the hallmarks of sepsis and contributes significantly to the pathophysiology of both sepsis and associated

coagulopathy. In addition to contributing directly to organ failure and shock, many of the inflammatory mediators produced at high levels in sepsis contribute to the procoagulant state. The relationship between inflammation and coagulation is bidirectional; coagulation is capable of inducing increased inflammation through several pathways, further emphasizing the importance of inflammation in sepsis-associated DIC.

While inflammation certainly plays a role in the pathophysiology of sepsis-associated DIC, inflammatory cytokines are by no means specific to this disease. Markers of infection or infection response have recently emerged as important in sepsis and DIC as diagnostic or prognostic markers, components of DIC pathophysiology, and potential therapeutic targets. These factors include not only traditional markers of infection such as procalcitonin but also nuclear material present in the extracellular space.

Materials typically restricted to the cell nucleus, including nucleosomes, histones, cell-free DNA (cfDNA), and DNA associated proteins such as HMGB-1 have recently been detected at elevated levels in the blood of patients with diverse pathologies including cancer, trauma, thrombosis, and sepsis. This phenomenon is thought to be the result of the immune response, particularly the generation of neutrophil extracellular traps (NETs). NETs consist of chromosomal DNA, associated histones, and various bactericidal factors expelled into the extracellular space by neutrophils through an active form of cell death (NETosis) in order to trap and neutralize bacteria (Brinkmann & Zychlinsky, 2012; Gould, Lysov, & Liaw, 2015). Nuclear components, including histones, cfDNA, and nucleosomes as well as DNA-associated proteins are commonly measured as surrogate markers of NET formation (Araujo 2016; Yost 2016).

Interestingly, NETs may also contain TF, leading to more direct induction of thrombin generation (Kambas 2017). Nucleosomes and HMGB-1 will be discussed in further detail in subsequent sections.

NET formation and association with clot formation and vascular occlusion has been confirmed in sepsis using animal models. NETs, identified by the co-localization of histone H2 with thrombin, have been visualized using fluorescence microscopy in the microcirculation of the liver, lungs, spleen, and mesentery of mice challenged with LPS, *S. aureus*, or *E. coli* bacteria, has been visualized using fluorescence microscopy (McDonald 2017). In these mice, thrombin was located predominately in or immediately downstream of NET sites (McDonald 2017). In mice with LPS-induced sepsis, occlusion of hepatic sinusoids was visualized by intravital microscopy (McDonald 2017). In this study, $PAD4^{-/-}$ mice unable to form NETs as well as mice treated with DNase exhibited reduced microvascular occlusion (McDonald 2017). NET components, including DNA and histones have been detected via confocal microscopy in the vasculature of endotoxemic mice with kidney injury (Czaikoski 2016).

In human sepsis patients, a higher proportion of polymorphonuclear leukocytes produced NETs than in healthy controls (Kambas 2017). Furthermore, treatment of healthy donor cells with serum from sepsis patients induced NET release (Kambas 2017). Another study demonstrated that platelets activated with LPS, but not with other platelet activators such as thrombin, are capable of stimulating NET formation (Clark 2007). In this study, platelets from septic patients, but not healthy individuals, also induced platelet-neutrophil binding (Clark 2007). In a study of 20 patients with septic shock, 10

with DIC and 10 without DIC diagnosed by the JAAM DIC score, NETs (detected by ELISA measurement of DNA-bound MPO as well as by neutrophil fluorescence, which is indicative of chromatin decompaction) were significantly elevated in DIC-patients compared to non-DIC patients (Delabranche 2017). NET formation has also been demonstrated in response to LPS, *S. aureus*, and dengue virus (Yost 2016).

The formation of NETs in sepsis patients is an integral component of the host response and contributes to bacterial clearance (Araujo 2016). Conversely, inhibition of NET formation or degradation of NETs is associated with reduced bacterial clearance (Czaikoski 2016; Yost 2016). However, NET degradation may also lead to overall improved survival (Yost 2016). In particular, inhibition of NETosis may be beneficial in situations where bacteria can be controlled through antibiotic administration. In a mouse cecal ligation and puncture (CLP) model of sepsis, degradation of NETs by systemic administration of DNase did not lead to reduced liver or kidney damage and increased circulating bacteria; however, when administered in conjunction with systemic antibiotics, DNase treatment improved survival (Czaikoski 2016). Furthermore, in a mouse model of LPS-induced septic shock that did involve live bacteria, administration of DNase reduced organ damage and improved survival (Czaikoski 2016). This indicates that while NETs contribute to the clearance of bacteria, their presence may be mainly negative in situations in which bacterial growth can be controlled through other means.

Numerous substances secreted in NETs, including nuclear materials, may contribute to the link between inflammation and thrombosis. Additionally, the fibrous NET meshwork can induce platelet activation and aggregation (Fuchs 2010). This

interaction is not entirely harmful; formation of a clot around bacteria, a phenomenon known as “immunothrombosis”, can help to halt the spread of infection. However, this can also contribute to the development of DIC.

Individually, both DNA and histones contribute to the procoagulant state. Extracellular DNA is highly procoagulant and may incorporate into and strengthen fibrin clots as well as decrease the rate of fibrinolysis (Gould 2015). *In vitro* coagulation studies have demonstrated that histones dose-dependently decrease clotting time and enhance thrombin generation (Ammollo 2011). Histones have also been shown to enhance thrombin-thrombomodulin complex mediated APC generation both *in vitro* and in a mouse model (Kowalska 2014). Histones have been shown to promote thrombin generation in platelet rich plasma in the absence of a platelet agonist (Semeraro 2011; Xu 2011; Yang 2016) and to contribute to platelet activation and depletion (Esmon 2011; Fuchs 2010; Gould 2015) in a TLR-2 and TLR-4 dependent manner.

Histones can also contribute to the procoagulant state through less direct mechanisms. Histones H3 and H4 can also act through TLRs to promote the production of the pro-inflammatory cytokines TNFα and IL-6 (Ikezoe, 2015). Histones or histone-rich plasma is also toxic to endothelial cells and can induce a procoagulant endothelial phenotype (Abrams 2013; Daigo 2014; Gould 2015; Kim 2016; Yang 2016). Histones induce endothelial TF expression at both the mRNA and protein levels and reduce surface TM expression in a partially TLR-2 and TLR-4 dependent manner (Kim 2016). At an organismal level, histones have been shown to contribute to DIC-like symptoms. Injection of histones into mice at a dose of 75 mg/kg induced pulmonary hemorrhage and

death with increased vascular permeability and consumptive coagulopathy similar to that seen in in DIC (Abrams 2013; Nakahara 2013).

Elevated levels of histones and DNA have been detected and associated with disease severity and outcome in both human and animal studies. Elevated histone levels have been observed in a mouse *E. coli* injection model, in which anti-histone intervention also reduced the levels of cardiac injury markers (Alhamadi 2015; Xu 2009). In a study measuring plasma DNA in ICU patients, plasma DNA concentrations were highest in patients who ultimately developed sepsis and in non-survivors (Rhodes 2006). In a study of 31 patients admitted through the emergency department with sepsis, cfDNA was used as a surrogate marker for NET formation and correlated significantly with SOFA score as well as with lung injury, acute respiratory distress syndrome (ARDS), kidney injury, and elevated bilirubin (Czaikoski 2016). In a study of 43 sepsis patients, histone H3 levels were increased in non-survivors compared to survivors and had an inverse relationship with platelet counts and AT levels (Wildhagen 2015). Extracellular histones have also been shown to contribute to cell death in mouse models of inflammatory and chemical cellular injury (Xu 2011).

Extracellular nuclear material is important not only for its role in the pathophysiology of sepsis-associated DIC but also for its potential role in the response to treatment. Potential therapeutics for DIC, including rTM (Iba 2014; Nakahara 2013; Osada 2017), APC (Kutcher 2012; Xu 2009), and AT (Iba 2017) may exert some of their therapeutic effect by dampening the production and effects of these materials, particularly histones. APC is capable of cleaving and thus inactivating histones H3 and H4 in the

extracellular environment and is protective in mice subjected to histone injection (Xu 2009). In a study of 132 critically ill trauma patients, increasing levels of histone over time was a predictor of mortality; however, concomitant increase in APC mitigated this effect (Kutcher 2012). *In vitro*, recombinant thrombomodulin inhibited histone-induced thrombin generation and cell death and promoted protein C dependent histone cleavage (Osada 2017). In addition to activating protein C and therefore inducing histone cleavage, rTM may inhibit NET formation and the release of nuclear material. LPS-induced NET formation can be inhibited by rTM at concentrations of 2, 10, or 50 μg/ml (Shimomura 2016). In a study of rTM administration to mice subjected to LPS challenge, administration of rTM at a dose of 3 mg/kg reduced the levels of circulating HMGB-1 and nucleosomes as well as the inflammatory mediators IL-6, MCP-1, and TNFα (Takehara 2017). AT has also been shown to ameliorate some histone-mediated damage; treatment of endothelial cells treated with histone H4 with 300 μg/ml AT led to a reduction in histone-induced morphological changes including intracellular junction disruption and lactate dehydrogenase production (Iba 2017). However, treatment of the endothelial cells with lower doses of AT did not elicit this effect. Further research is necessary to determine how potential therapeutics for sepsis-associated DIC interact with extracellular nuclear material.

**Nucleosomes.** Nucleosomes are the basic physical unit of DNA organization, consisting of chromosomal DNA wrapped around a histone protein core. Like their component parts, nucleosomes are strongly pro-inflammatory and pro-thrombotic and may thus provide a significant link between infection, inflammation, and coagulopathy in patients with sepsis-associated DIC.

In a study of 199 patients with sepsis-associated DIC, including 53 patients with overt DIC, high levels of nucleosomes were associated with poor prognosis and death, with better predictive values for death than conventional markers such as platelets or D-Dimer (Kim 2015). In a study of 165 patients in medical and surgical ICUs, nucleosomes were found to be significantly elevated in septic versus non-septic ICU patients, with ability to differentiate sepsis with 86% sensitivity and 52% specificity (Chen 2012). Nucleosomes were also elevated in DIC patients compared to the non-DIC patients and showed a strong correlation with other indicators of NET formation (Delabranche 2017).

**High Mobility Group Box 1 (HMGB-1).** High Mobility Group Box 1 Protein (HMGB-1) is a nuclear-associated protein with a physiological role in the physical organization of DNA. When detected in the extracellularly in sepsis, HMGB-1 exhibits pro-inflammatory and pro-thrombotic properties and is considered a late-phase mediator of sepsis.

*In vitro*, HMGB-1 released from both endothelial cells (Bae 2011) and peritoneal macrophages (Kim 2009) has been reported in response to LPS exposure. Elevated circulating HMGB-1 levels have been reported in disease states including not only sepsis but also in other inflammatory diseases including chronic kidney disease (Bruchfeld

2008), acute appendicitis (Albayrak 2011), and heat stroke (Hagiwara 2010). Elevated HMGB-1 levels have been demonstrated in both LPS and CLP induced sepsis models in mice and rats (Li 2007; Nagato 2009; Yang 2004; Yin 2005). In a mouse model of CLP-induced sepsis, HMGB-1 was not detectable until 18 hours following surgery and peaked after 24 hours, supporting the idea of HMGB-1 as a late-phase mediator (Yang 2004).

Elevated HMGB-1 levels and their association with outcome and severity of illness has been reported in patients with sepsis, DIC-and related conditions. In a study of 201 patients with suspected DIC of varied etiologies including but not limited to sepsis, plasma levels of HMGB-1 were significantly elevated in patients with DIC compared to those without DIC and correlated with DIC score (Hatada 2005). Elevated HMGB-1 was also associated with organ failure and non-survival (Hatada 2005). In a study of 122 patients with community-acquired pneumonia, HMGB-1 levels were elevated compared to healthy controls, and were significantly elevated in survivors compared to non-survivors, although no differences were seen in HMGB-1 levels between those who ultimately developed severe sepsis and those who did not (Angus 2007). In a study of 42 patents with septic shock, baseline HMGB-1 levels were not significantly different between survivors and non-survivors but correlated positively with SOFA score, lactate, and procalcitonin (Gibot 2007). In this population, HMGB-1 levels held steady in non-survivors and declined in survivors; on day 3, HMGB-1 levels were able to discriminate survivors from non-survivors with 66% sensitivity and 67% specificity (Gibot 2007). In a study of 26 patients with severe sepsis, 33 patients with sepsis shock, and 5 patients with

sepsis, HMGB-1 levels were elevated in all patients with no relationship with severity of sepsis (Sunden-Cullberg 2005).

HMGB-1 contributes indirectly to the development of a pro-thrombotic state through increases in inflammation as well as synergistic interactions with thrombin. In a mouse stasis thrombosis model, HMGB-1 contributed to the pathogenesis of venous thromboembolism (VTE) as well as promoted NET formation, leading to further thrombosis and increases in HMGB-1 levels (Stark 2016). *In vitro*, HMGB-1 has been shown to induce monocyte cell surface TF expression (Ito 2006). In a rat model of thrombin-induced DIC, administration of HMGB-1 acted synergistically with thrombin to induce production of the proinflammatory cytokines IL-6 and TNFα (Ito 2006). Co-administration of HMGB-1 with thrombin increased thrombin-induced mortality, fibrin deposition in the glomeruli, and alveolar hemorrhage as well as prolonged PT and aPTT compared to administration of thrombin alone, whereas treatment with HMGB-1 alone did not produce these effects (Ito 2006).

HMGB-1 is of interest in sepsis-associated DIC not only as a pathophysiological mediator and biomarker but also as a therapeutic target. HMGB-1 neutralizing antibodies have been shown to increase survival in both rat (Suda 2006) and mouse (Yang 2004) models of CLP-induced sepsis. HMGB-1 may also interact with endogenous anticoagulants and therefore with therapeutics targeting these pathways. In an *in vitro* study, HMGB-1 did not modulate AT function, but concentration-dependently inhibited TM-mediated protein C activation (Ito 2006). In a separate *in vitro* study, APC inhibited LPS-mediated release of HMGB-1 from endothelial cells as well as HMGB-1 mediated

expression of endothelial cell adhesion molecules (Bae 2011). Reduction in circulating HMGB-1 levels in response to treatment with rTM has been demonstrated in rat models of both heat stroke (Hagiwara 2010) and LPS-induced sepsis (Nagato 2009). In a study of rTM administration to mice subjected to LPS challenge, administration of rTM at a dose of 3 mg/kg following LPS challenge reduced circulating HMGB-1 and nucleosome levels as well as IL-6, MCP-1, and TNFα (Takehara 2017).

**Procalcitonin (PCT).** Procalcitonin (PCT) is a 116 amino acid, 13 kDa polypeptide often cited as an indicator of bacterial infection. Under normal physiological circumstances, PCT is produced by the C-cells of the thyroid and subsequently cleaved to the active hormone calcitonin, which is involved in calcium homeostasis. Blood levels of PCT are typically very low in healthy individuals (<0.1 ng/ml). In infectious conditions, PCT is produced by non-thyroid tissue and released into the blood in response to bacterial mediators and the associated inflammatory response (Sims 2016). Many studies have investigated PCT for its ability to distinguish between sepsis and non-infectious inflammatory conditions, such as non-infectious sterile inflammatory response syndrome (SIRS) (Annane 2005; Biron 2015; Harbarth 2001; Livaditi 2006; Pierrakos & Vincent, 2010; Riedel 2011; Wacker 2013; Wunder 2004; Zakariah 2008). A 2013 meta-analysis on the ability of PCT to distinguish between sepsis and SIRS in 30 studies involving a total of 2344 patients demonstrated a sensitivity of 0.77 and a specificity of 0.78, with a recommended cutoff for the diagnosis of sepsis between 1 and 2 ng/ml (Wacker 2013). Studies have also shown that PCT is elevated in non-survivors compared to survivors in severe sepsis (Wunder 2004), predictive of death in SIRS and sepsis patients (Harbarth

2001), elevated in severe sepsis or septic shock compared to sepsis (Livaditi 2006), and higher in patients with positive blood cultures compared to those with negative blood cultures (Riedel 2011).

Although PCT is a marker for infection, it may still be useful to assess the presence of coagulopathy in sepsis patients or to predict mortality in patients with sepsis-associated DIC. In a study of 82 patients meeting SIRS criteria with suspected DIC, PCT had an area under the curve (AUC) of 0.904 for distinguishing sepsis from SIRS and an AUC of 0.785 for determining the presence of DIC (Ishikura 2014). In the ART-123 phase 2b study, a non-significant trend towards elevated PCT was observed in the overt DIC group compared to the non-overt DIC group (Hoppensteadt 2015).

**IL-1α and IL-1β.** Interleukins (IL) 1α and 1β are inflammatory cytokines that contribute to the development of a pro-coagulant state associated with severe inflammation. IL-α is produced largely by activated macrophages, neutrophils, epithelium, and endothelium. IL-1β is produced as a pro-protein by activated macrophages or activated platelets and is cleaved into its active form by caspase-1.

IL-1, particularly IL-1β, promotes a procoagulant endothelial phenotype by increasing TF expression and downregulating anticoagulants. Infusion of IL-1 into rabbits led to a 10-fold increase in TF expression accompanied by a significant decrease in protein C activation in aortic endothelium over a 3 hour time period (Nawroth, Handley, Esmon, & Stern, 1986). Treatment with human IL-1α and IL-1β induced dose-dependent increases in the procoagulant activity of human monocytes *in vitro* (Osnes, Westvik, Joo, Okkenhaug, & Kierulf, 1996). Additional studies have confirmed both the

IL-β-induced upregulation of TF expression (Abraham, 2000) and the downregulation of TM and endothelial cell protein C receptor gene activation (Esmon, 2004). Endothelial procoagulant response to IL-1 has been documented to have a rapid rise, peak activity at 4 hours, and a decline towards basal levels by 24 hours (Bevilacqua 1986). Exposure of whole blood drawn from healthy volunteers to IL-1β at a concentration of 20 pg/ml significantly altered the viscoelastic properties of the clots as measured by thromboelastography. Specifically, IL-1β lowered the maximum amplitude of the clot as well as maximum velocity to reach clot growth, indicating a reduction in clot stability (Bester & Pretorius, 2016). The procoagulant response to IL-1 may have implications for survival in DIC. In a re-analysis of an unsuccessful 1997 trial of an IL-1 receptor antagonist in patients with severe sepsis, when patients were re-classified based on DIC status, improved survival with IL-1 receptor blockade was observed in the subgroup of patients with DIC and hepatobiliary dysfunction (Shakoory 2016).

Previous work in our laboratory has demonstrated significant increases in IL-1α in patients with sepsis-associated DIC (Low 2016; Walborn 2017). However, 91% of patients in the PROWESS trial for APC had baseline levels of IL-1β below the threshold for detection (Kinasewitz 2004). IL-1β, along with TNFα, peaks early and transiently in response to infection and therefore may not be detectable in all sepsis patients (Kinasewitz 2004). The correlation of IL-1β with outcome in sepsis-associated DIC is also unclear. A study of 65 patients admitted to an intermediate care unit with sepsis showed no significant differences in IL-1β levels between survivors and non-survivors (Gogos 2000). In contrast, a study of 60 patients admitted to the ICU with SIRS criteria

and an obvious focus of infection found IL-1β to be significantly increased in septic shock compared with sepsis, positively correlated with SOFA score, and higher in non-survivors than survivors (Bozza 2007).

**IL-2.** IL-2 is an inflammatory cytokine involved in the differentiation of T cells. Although IL-2 is less commonly discussed in the sepsis and DIC literature, research from the 1990s investigating the use of IL-2 immunotherapy saw DIC or DIC-like syndromes, commonly known as “vascular leak syndrome”, as a consequence of high-dose IL-2 therapy. IL-2 infusion into 7 cancer patients led to thrombocytopenia, elevated D-Dimer, and decreased plasminogen levels, without changes in PT, aPTT, or Factors VI or VII (Fleischmann 1991). IL-2 infusion into 9 tumor patients led to endothelial activation contributing to DIC development, indicated by elevated circulating levels of adhesion molecules, accompanied by increases in TPA and PAI-1, thrombocytopenia, increase in fibrin degradation products, prolonged aPTT, and decreased fibrinogen (Locker 1999). Previous work in our laboratory demonstrated significant elevation in IL-2 in patients with sepsis-associated DIC (Low 2016; Walborn 2017).

**IL-4.** IL-4 is an anti-inflammatory cytokine involved in the development of TH2 cells. IL-4 treatment has been shown to decrease the IL-1α, IL-1β, and LPS-induced procoagulant activity of human monocytes, including TF mRNA levels (Osnes 1996) and surface TF expression (Lindmark 1998). Another study demonstrated reduced TF expression on the surface of IL-1β, TNFα, or LPS-treated adult bovine aortic endothelial cell in response to IL-4 treatment (Herbert 1993). In a study of 60 patients admitted to the ICU with SIRS criteria and an obvious focus of infection, IL-4 was significantly elevated

in non-survivors compared to survivors and predictive of death within 48 hours (Bozza 2007). Previous work in our laboratory has demonstrated increased levels of IL-4 in patients with sepsis-associated DIC (Low 2016; Walborn 2017).

**IL-6.** IL-6 is the prototypical pro-inflammatory cytokine. It is necessary to mount a robust immune response, and to stimulate the production of acute phase reactants and inflammatory factors. However, excessively high levels of IL-6, such as those seen in sepsis, can injure the host through mechanisms including increased vascular leakage (Hawiger 2015) and increased thrombotic potential. IL-6 has been shown to induce mononuclear cell TF expression both *in vitro* and *in vivo* (Levi 2004). IL-6 is released in response to LPS injection into healthy human volunteers (de Jonge 2000). In a study of LPS injection into chimpanzees, anti-IL-6 antibody administration reduced activation of coagulation measured by levels of F1.2 and AT but did not impact fibrinolysis or the overall inflammatory state (van der Poll 1994). Stimulation of whole blood from healthy volunteers with 15 pg/ml IL-6 significantly altered the viscoelastic properties of the clots as measured by thromboelastography; specifically, IL-6 lowered the maximum amplitude of the clot, indicating a reduction in clot stability (Bester & Pretorius, 2016).

The relationship between inflammation and coagulation is bidirectional, and coagulation can stimulate further IL-6 production. IL-6 expression can be induced through activation of the PAR-1 (Hawiger 2015) and PAR-2 (Levi 2004) receptors, which are activated by coagulation factor complexes and thrombin, as well as through treatment with histones H3 and H4 (Abrams 2013).

IL-6 is frequently measured in studies of sepsis and DIC patients and is well established to be significantly elevated in this patient population (Bozza 2007; Chen 2012; Gogos 2000; Harbarth 2001; Iba 2006; Ishikura 2014; Kinasewitz 2004; Livaditi 2006; Low 2016; Mauri 2010; Taniguchi 1999; Walborn 2017; Wunder 2004). Although IL-6 is a relatively non-specific inflammatory mediator, it may still provide information about disease state and prognosis. IL-6 rises rapidly in patients with sepsis and may correlate with disease severity, hypercoagulability, or non-survival (Iba 2006). In a study of 82 patients with SIRS criteria and suspected DIC, IL-6 had an AUC of 0.893 for distinguishing SIRS versus sepsis and an AUC of 0.765 for distinguishing between patients with and without DIC (Ishikura 2014). In a separate study of 78 patients with SIRS or sepsis, IL-6 had an AUC of 0.75 for distinguishing SIRS from infectious sepsis (Harbarth 2001) In a study of 60 patients admitted to the ICU with SIRS criteria and an obvious focus of infection, IL-6 was significantly increased in patients with septic shock compared to those with sepsis, correlated positively with SOFA score on day 0,was higher in non-survivors versus survivors, and was a good predictor of death within 48 hours (Bozza 2007). However, other studies have demonstrated no association between IL -6 levels and mortality in sepsis patients (Gogos 2000; Wunder 2004). IL-6 levels may reflect response to therapy. In a study of rTM administration to mice subjected to LPS challenge, administration of rTM at a dose of 3 mg/kg reduced the levels of IL-6 as well as MCP-1, HMGB-1, nucleosomes, and TNFα (Takehara 2017).

**IL-8.** IL-8, known as neutrophil chemotactic factor, is an inflammatory cytokine that is significantly elevated in sepsis (Bozza 2007; Claushuis 2016; Harbarth 2001;

Kinasewitz 2004; Livaditi 2006; Low 2016) and may have diagnostic or prognostic value. IL-8 interacts with coagulation in a somewhat different way than other commonly investigated inflammatory cytokines. Exposure of whole blood from healthy human volunteers to 40 pg/ml IL-8 altered clot viscoelastic properties as measured by thromboelastography to a greater extent than IL-1β or IL-6. Specifically, IL-8 decreased the R time, angle, maximum amplitude, MTRG, TMRTG, and TTG and increased the K time, indicating that IL-8 caused a rapid formation of an unstable clot (Bester 2016). IL-8 expression may also be induced by coagulation. Administration of recombinant FVIIa to healthy volunteers produced small but statistically significant increases in plasma concentrations of IL-8 and IL-6 (de Jonge 2003). FVIIa, but not thrombin, was also shown to stimulate the production of IL-8 *in vitro* in a TF-expressing breast carcinoma cell line in a manner inhibited by PAR-2 blocking antibodies (Hjortoe 2004).

IL-8 is elevated in sepsis and may have moderate diagnostic or prognostic value. In a study of 78 patients with SIRS or sepsis, IL-8 had an AUC of 0.71 for distinguishing SIRS from sepsis (Harbarth 2001). In a study of 60 patients admitted to the ICU with SIRS criteria and an obvious focus of infection, IL-8 was significantly elevated in septic shock compared to sepsis. In these patients, IL-8 correlated with SOFA score on day 0, was higher in non-survivors than survivors, and was a good predictor of death within 48 hours (Bozza 2007). In a study of 47 patients with sepsis, significant differences were observed at baseline between patients with sepsis, severe sepsis, and septic shock, and IL-8 had an AUC of 0.73 for the prediction of 28 day mortality (Livaditi 2006). In a study stratifying sepsis patients by platelet count upon ICU admission, patients with platelets

less than 100 K/μl showed significant elevation in IL-8 compared to those with higher platelet counts (Claushuis 2016).

**IL-10.** IL-10 is an anti-inflammatory cytokine that acts in opposition to the pro-inflammatory and pro-coagulant effects of other factors, including IL-6. IL-10 is upregulated in patients with sepsis in response to the elevated inflammatory state (Claushuis 2016; Kinasewitz 2004; Low 2016). *In vitro,* IL-10 treatment caused concentration-dependent decreases in IL-1α, IL-1β, and LPS-induced procoagulant activity of human monocytes and lowered the amount of TF mRNA detectable in these cells (Osnes 1996). IL-10 also inhibited LPS-induced TF expression, mRNA, and procoagulant activity in a whole blood *in vitro* stimulation protocol (Lindmark 1998). In a human model of mild DIC induced by LPS injection, administration of IL-10 reduced the activation of the coagulation, assessed using F1.2 and AT levels (Pajkrt 1997). In mouse models, IL-10 production has been detected in the liver as soon as 1 hour after CLP, and anti-IL-10 antibody administration resulted in higher TNF levels and mortality following CLP (T van der Poll 1995). IL-10 may also be upregulated through the interaction of thrombin with the PAR-1 receptor on monocytes (Naldini 2005).

A study of 33 patients admitted to the ICU with severe sepsis found significantly higher levels of IL-10 in non-survivors than survivors on ICU days 1 and 2 but significantly higher IL-10 levels in survivors than non-survivors on day 3, suggesting that the time course of IL-10 in patients with sepsis is of particular importance (Wunder 2004). IL-10 levels were also found to be elevated in non-survivors compared to survivors in a study of 65 patients with sepsis who were admitted to the intermediate care

unit (Gogos 2000). The IL-6:IL-10 ratio, representing the balance between pro- and anti-inflammatory processes, has also been studied as a potential diagnostic or prognostic marker for patients with sepsis and DIC (Kellum 2007; Wunder 2004). In a study of 25 patients meeting SIRS criteria, an increase of the ratio of IL-6/IL-10 over time due to a lack of decline in IL-6 coupled with a gradual decrease in IL-10 was associated with increased mortality (Taniguchi 1999).

**Tumor Necrosis Factor α (TNFα).** Tumor Necrosis Factor (TNF) α is a pro-inflammatory cytokine that rises rapidly in sepsis, with peak levels observed as soon as 2 hours following LPS injection into rats (Kaspereit, Doerr, & Dickneite, 2004) or 1 hour following LPS challenge in mice (Remick 1990). TNFα contributes significantly to the procoagulant state both *in vitro* and *in vivo*. At an organismal level, administration of TNFα to healthy human volunteers induced a procoagulant state, measured by increased FX activity followed by increased F1.2 levels (van der Poll 1990) as well as inhibited fibrinolysis. Specifically, TNFα induces vascular TF expression, particularly by monocytes and endothelial cells (Abraham 2000; Bevilacqua 1986; Esmon 2004; Hezi-Yamit 2005). TNFα has also been shown to downregulate thrombomodulin and endothelial cell protein C receptor gene expression (Esmon, 2004) and to increase levels of the inhibitor of fibrinolysis PAI-1 (Abraham, 2000; Ikezoe, 2015). TNFα may contribute significantly to DIC pathophysiology and response to therapy. Although a trial of anti-TNFα monoclonal antibody in patients with sepsis found no reduction in 28-day mortality, antibody-treated patients experienced more rapid reversal of septic shock and a delay in time to onset of first organ failure (Cohen & Carlet, 1996). In a study of rTM

administration to mice subjected to LPS challenge, administration of rTM at a dose of 3 mg/kg suppressed TNFα production (Takehara 2017).

TNFα is generally found to be elevated in sepsis and associated conditions (Low 2016); however, analysis of severe sepsis patient samples from the PROWESS trial for APC could not detect TNFα in 47% of patients in this population. TNFα levels may be low in some sepsis patients due to the rapid and transient TNFα response in sepsis, with falling or normalized levels by the time of study enrollment (Kinasewitz 2004).

**Interferon γ (IFNγ).** Interferon (IFN) γ is an activator of macrophages and an inducer of MHC class II expression. Relative to other inflammatory mediators, limited information is available regarding the role of IFNγ in sepsis and DIC. However, administration of anti-IFNγ antibody to mice either before or 24 hours after CLP resulted in improved survival, reduced inflammation, and decreased peritoneal bacterial load (Marquez-Velasco 2011; Qiu 2001). In contrast, a separate study found that IFNγ receptor knockout mice had reduced survival in a colon ascendens stent peritonitis (CASP) model of sepsis (Zantl 1998).

**Monocyte Chemoattractant Protein 1 (MCP-1).** Monocyte Chemoattractant Protein 1 (MCP-1) is a potent chemotactic factor for monocytes and contributes to microvascular leakage through effects on endothelial cell tight junctions (Hawiger 2015). MCP-1 may be protective to the host in sepsis. In a mouse CLP model, blockade of MCP-1 decreased survival and increased bacterial presence in the peritoneum (Matsukawa 1999; Matsukawa 2000). Similar results were observed with the use of MCP-1 blocking antibodies in a mouse model of LPS injection, where administration of

exogenous MCP-1 was protective against endotoxin-induced lethality (Zisman 1997). In a study of rTM administration to mice subjected to LPS challenge, administration of rTM at a dose of 3 mg/kg reduced MCP-1 levels (Takehara 2017). Elevated levels of MCP-1 occurred within 24 hours of surgery in a rat CLP model of sepsis (Qiu 2001). In 60 patients admitted to the ICU with SIRS criteria and an obvious focus of infection, significant increases in MCP-1 were found, with greater elevation in non-survivors than in survivors (Bozza 2007).

**Epidermal Growth Factor (EGF).** Epidermal Growth Factor (EGF) is a cytoprotective factor. Minimal information is available regarding the role of EGF in sepsis and DIC. However, levels of intestinal EGF were found to be elevated in a mouse model of CLP-induced sepsis, and IP injection of 150 μg/kg/day of EGF led to a reduction in mortality from 60% to 30% (J. A. Clark, Clark, Hotchkiss, Buchman, & Coopersmith, 2008).

**Vascular Endothelial Growth Factor (VEGF).** Vascular Endothelial Growth Factor (VEGF) is a regulator of angiogenesis, neovascularization, and vascular permeability. In sepsis, VEGF may contribute to microvascular leakage through effects on tight junctions between endothelial cells (Hawiger 2015). Increased VEGF has also been observed in both CLP-induced and LPS-induced sepsis in mice and following LPS injection into human volunteers (Yano 2006). Potential mechanisms for increase in VEGF in DIC include release from activated platelets or LPS-induced production (Kim 2008).

Both elevated and reduced VEGF levels have been reported in sepsis and DIC. Decreased VEGF levels were observed in patients with overt DIC, and VEGF was shown to correlate positively with platelet counts and negatively with SOFA score (Jesmin 2012). A second study, including 57 severe trauma patients with or without DIC, also measured lower levels of VEGF in DIC patients (Wada 2012). In contrast, a study of 240 patients with suspected DIC found elevated levels of VEGF in patients with overt DIC compared to those without (Joo 2010). A small study of 18 patients with severe sepsis also found elevated VEGF in patients compared to healthy controls and an association between VEGF and organ dysfunction (van der Flier 2005).

**Endothelial Biomarkers.**

The endothelial cells lining the vasculature are in constant contact with the blood and are named in Virchow's Triad as one of the critical contributors to thrombosis. In DIC, damage to the endothelium as well as functional changes induced by high levels of inflammatory factors or bacterial components can contribute to the development of coagulopathy.

Under physiological conditions, the endothelium prevents inappropriate coagulation. Endothelial cells express or secrete an assortment of endogenous anticoagulants, including tissue factor pathway inhibitor (TFPI), protein C, thrombomodulin, and antithrombin. These molecules act at specific sites along the coagulation cascade to inhibit coagulation. The endogenous anticoagulant system is disrupted in DIC, and is a major focus of research both as a prognostic indicator and a therapeutic target.

In addition to endogenous anticoagulants, the endothelium is responsible for the production of an array of hemostatically active molecules, including von Willebrand Factor (vWF), TF, and PAI-1. Exposure to inflammatory mediators can modulate endothelial expression of these factors. Damage to the endothelium, such as that caused by exposure to histones, can also provide a site for clot initiation.

The role of the endothelium in sepsis, as reviewed by Ince et. al. in 2016 (Ince ., 2016), extends beyond coagulation. The endothelium produces vasoactive molecules, such as endothelin, which are involved in the regulation of vascular tone. These factors may be important in sepsis due to the role of shock and hypoperfusion in this disease process. The endothelium also produces and maintains the glycocalyx, a gel-like layer lining the vasculature. In conjunction with the endothelial cells themselves, the glycocalyx plays a role in the maintenance of the vascular barrier, hemostasis, adhesion, and anti-inflammatory effects. Inflammatory factors including TNFα can induce shedding of the glycocalyx, leading to loss of barrier function and subsequent edema. Other factors act on the junctions between endothelial cells themselves, also promoting vascular leakage. In addition to hemostatically active molecules, other indicators of general endothelial function such as endocan or angiopoietin 2 may provide insight into disease pathophysiology and prognosis.

**Tissue Factor Pathway Inhibitor (TFPI).** Tissue Factor Pathway Inhibitor (TFPI) is an endogenous anticoagulant present on the surface of endothelial cells and released into circulation following heparin exposure or platelet and monocyte activation (Abraham 2003; Gando 2016; Maroney 2008; Wood 2014). TFPI is a reversible inhibitor

of FXa, and is capable of inhibiting the FVIIa-TF complex when in complex with FXa. Through models including TF administration to rabbits, experimental bacteremia in baboons, and endotoxin-induced coagulation in healthy human volunteers, TFPI was identified as important in preventing excessive coagulation and promoting survival (Gando 2016). Subsequently, administration of exogenous TFPI was shown to reduce DIC symptoms such as platelet and fibrinogen consumption and thrombus number in a rat model of LPS-induced DIC (Elsayed 1996b), and to reduce thrombin generation in healthy human volunteers injected with LPS (de Jonge 2000). The OPTIMIST clinical trial, completed in 2003, evaluated the safety and efficacy of recombinant TFPI (tifacogin) in the treatment of DIC (Abraham 2003). This study found no reduction in mortality and increase in bleeding in patients with severe sepsis and INR $\leq$ 1.2 treated with tifacogin and therapeutic use of TFPI was not pursued further (Abraham 2003). Changes in TFPI levels in DIC are unclear. In a rat model of CLP-induced sepsis, TFPI activity was significantly reduced at 24 hours (Ravindranath 2007). However, human TFPI levels did not vary with administration of LPS to healthy volunteers (de Jonge 2000), in patients with trauma-associated DIC (Gando 2001), or in patients with DIC with or without INR greater than 1.2 (Abraham 2003).

**Protein C.** Protein C is an endogenous anticoagulant that is of significant interest in DIC as both a biomarker and a therapeutic target. Protein C is activated in the presence of thrombin with activation greatly enhanced by thrombomodulin or the endothelial cell protein C receptor (EPCR). Activated Protein C (APC) proteolytically inactivates coagulation factors Va and VIIa. Additionally, APC has anti-inflammatory and

cytoprotective effects (Jong-Sup Bae & Rezaie, 2011). Protein C levels are significantly reduced in patients with sepsis-associated DIC, and restoration of this pathway has been pursued as a therapeutic approach for DIC. APC, known in drug form as drotrecogin alfa (activated) or by the brand name Xigris was developed for use in patients with severe sepsis and was ultimately approved for use in this population (Bernard 2001). Post-approval data failed to show a reduction in mortality with APC treatment but did demonstrate an increase in bleeding, leading to the withdrawal of this drug from the market in 2011. Despite the lack of clinical success of APC as a therapeutic agent, the Protein C pathway is still of interest in DIC. Thrombomodulin, the activator of Protein C, is currently in clinical trials for DIC. Additionally, Protein C levels have the potential to be a strong diagnostic or prognostic marker for DIC.

Data analysis from the severe sepsis patients included in the PROWESS and ENHANCE trials for APC demonstrated that low levels of Protein C, particularly below 40% of normal levels, correlated with poor outcome (Macias 2004; Shorr 2008). Serial measurement of Protein C levels had better predictive ability for outcome throughout the hospitalization period than levels of IL-6 (Macias 2004). Furthermore, in a Phase 2 study for APC, increasing protein C levels with treatment was indicative of therapeutic efficacy (Shorr 2010). Other smaller studies, including a study of 80 ICU patients (Bouchard 2015), a study of coagulation function in 38 patients with severe sepsis (Collins 2006), and a study of patients with *B. pseudomallei* sepsis infections (LaRosa 2006) have also demonstrated reduced protein C levels in critically ill patients and an association between low Protein C levels and poor outcome. Protein C level may also be a viable marker for

use in assessing the response to treatment of patients treated with recombinant thrombomodulin (Iba 2016).

**Endocan.** Endocan was first described in 1996 as an endothelial cell factor, expressed in the lung and regulated by TNFα, IL-1β, and IFNγ (Lassalle 1996). Endocan levels also increase in response to LPS injection in healthy individuals, reaching average levels above 4 ng/ml (Cox 2015). Endocan was measured in 63 patients admitted to the ICU with sepsis as well as 20 healthy donors and 7 patients with SIRS, and was found to be elevated in sepsis with further elevation in patients with septic shock (Scherpereel 2006). In a study of 60 patients with sepsis, endocan was found to be significantly elevated in patients with organ failure and in non-survivors compared to survivors with an AUC of 0.71 for the prediction of mortality (Mihajlovic 2014). Postmortem serum endocan concentrations have also been evaluated and found to be elevated in patients who died of sepsis compared to patients with a non-infectious cause of death (Palmiere 2014). In a study of 175 patients with SIRS criteria and a known source of infection, endocan was the most effective marker investigated to evaluate worsening organ failure (AUC=0.77), defined by an increase in SOFA score of $\geq 2$ in a 24 hour period (Ioakeimidou 2017). Furthermore, endocan decreased in patients who showed clinical improvement and increased in patients showing clinical decline (Ioakeimidou 2017). A patent has been granted for a method using endocan levels to predict the risk of respiratory failure, renal failure, or thrombocytopenia in a septic patient using endocan (Lassalle, 2014).

**Angiopoietin 2 (Ang-2).** Under physiological conditions, Angiopoietin 2 (Ang-2) is involved in vasculogenesis and acts as an antagonist to Angiopoietin 1 (Ang-1) at the Tie2 receptor on the endothelial cell surface. Ang-1 promotes vascular stability, preserves cell-cell contacts, and has anti-inflammatory effects, while Ang-2 acts in opposition to these effects.

Ang-2 is relevant to the pathogenesis of sepsis for its role in the disruption of endothelial cell barrier function, which has been demonstrated both *in vitro* and *in vivo*. Treatment of endothelial cell monolayers with purified Ang-2 resulted in the development of stress fibers and intercellular gaps (Parikh 2006). In two separate *in vitro* studies, treatment of endothelial cell monolayers with serum from patients with elevated circulating Ang-2, but not patients with comparable pathological conditions and low Ang-2, led to increased intercellular gap formation and reduced endothelial barrier integrity (Gallagher 2008; Parikh 2006). These effects were reversed by co-treatment with Ang-1 (Gallagher 2008; Parikh 2006). In mice, administration of Ang-2 led to increased vascular permeability in the lung, liver, and intestine (Parikh 2006).

Although the precise mechanism is not well understood, Ang-2 is upregulated in sepsis and may be a component of the endothelial response to ongoing coagulation. Injection of LPS into 22 healthy volunteers led to elevated circulating Ang-2 levels, peaking 4.5 hours after LPS injection (Kumpers 2009). However, treatment with LPS, TNFα, or IL-6 reduced Ang-2 release from human lung microvascular endothelial cells, while LPS and TNFα, but not IL-6, stimulated Ang-2 release from bovine lung microvascular endothelial cells (Orfanos 2007). Serum from patients with septic shock

stimulated the release of Ang-2 from the peripheral blood monocytes of healthy volunteers whereas serum from patients with sepsis without shock did not (Kranidioti 2009). Neither stimulation of monocytes with LPS nor with serum from sepsis patient supplemented with LPS promoted Ang-2 release (Kranidioti 2009). In the absence of EPCR occupancy by protein C, stimulation of endothelial cell monolayers with thrombin led to increased Ang-2 and decreased Ang-1 and Tie2 production (Bae 2010). However, occupancy of the EPCR, even by catalytically inactive protein C mutants, led to Ang-1 upregulation and Ang-2 downregulation following thrombin stimulation (Bae 2010).

Elevated Ang-2 levels have been reported in septic patients as well as in patients with acute lung injury (ALI), ARDS (Gallagher 2008), or trauma (Ganter 2008). Ang-2 elevation shows a particularly strong association with pulmonary dysfunction (Gallagher 2008; Kumpers 2008; Lin 2015; Parikh 2006). A study of 341 patients with septic shock demonstrated associations between elevated Ang-2 and coagulation, hepatic and renal dysfunction, mortality, and levels of TNFα and IL-6 (Fisher 2016). Elevated Ang-2 has been demonstrated in severe sepsis patients compared to patients with mild sepsis or other hospitalized patients (Orfanos 2007; Parikh 2006; Siner 2009). Elevated Ang-2 has also been associated with impaired gas exchange (Parikh 2006), increased levels of inflammatory cytokines (Orfanos 2007; Siner 2009), increased organ failure (Davis 2010; Kranidioti 2009; Lin 2015; Orfanos 2007; Ricciuto 2011; Siner 2009), and poor outcome (Davis 2010; Kranidioti 2009; Lin 2015; Orfanos 2007; Ricciuto 2011; Siner 2009) in multiple cohorts of patients with sepsis and related conditions.

Although the association between Ang-2 and coagulopathy in sepsis has not been investigated, Ang-2 was associated with coagulopathy in a study of 208 adult trauma patients, where Ang-2 was significantly higher in patients with abnormal PT or aPTT than those without (Ganter 2008). Furthermore, D-Dimer increased with increasing Ang-2 while Protein C decreased with increasing Ang-2 (Ganter 2008).

**von Willebrand Factor (vWF).** Von Willebrand factor (vWF) is a glycoprotein produced in the endothelium as well as in megakaryocytes and the subendothelium. vWF is notable in hemostasis for its role in platelet adhesion as a link between the platelet receptor GPIb and the damaged endothelial wall. High or low molecular mass vWF multimers can be released into the circulation. Higher molecular weight vWF multimers are more prothrombotic than lower molecular weight multimers and can be secreted in response to inflammatory stimuli including TNFα, IL-6, or IL-8. Elevated circulating vWF or changes in vWF molecular weight profile in disease can indicate endothelial activation or damage.

vWF may contribute to the development of coagulopathy in septic patients. In a porcine model of septic shock, vWF-rich thrombi were abundant in the glomeruli of the septic pigs (Bockmeyer 2011). In a mouse model of CLP-induced sepsis, vWF knockout mice demonstrated improved survival compared to WT mice (Lerolle 2009).

Elevated vWF has been shown to correlate with severity of coagulopathy and mortality in septic patients. In a study of 40 patients with severe sepsis or septic shock, vWF activity and antigen levels were significantly elevated compared to age and gender matched healthy controls (Hovinga 2007). In a study of patients with severe sepsis or

septic shock, vWF levels were elevated in patients with a SOFA score ≥10 and in patients with an ISTH DIC score of ≥4 compared to those with lower scores (Claus 2009). vWF was also elevated in non-survivors compared to survivors (Claus 2009). vWF elevation also occurs in other illnesses characterized by endothelial activation or damage. vWF levels comparable to those seen in sepsis have been reported in patients with non-sepsis-associated organ failure (Martin 2007) as well as in patients with ALI or ARDS (Ware 2004).

The size distribution of vWF multimers and the vWF activity level may influence the contribution of vWF to sepsis-associated DIC. Larger vWF multimers are more potent inducers of platelet aggregation and may occur with greater frequency in disease. In a porcine model of septic shock, septic animals had a higher molecular weight distribution of vWF multimers than controls (Bockmeyer 2011). The major regulator of vWF multimers size, ADAMTS-13, has also been investigated in some studies of sepsis and DIC. Decreases in ADAMTS-13 have been reported in non-survivors of sepsis or DIC compared to survivors (Claus 2009; Hyun 2009). Furthermore, elevated vWF antigen to ADAMTS-13 activity ratio has been reported in non-survivors compared to survivors (Claus 2009), and ADAMTS-13 has been reported to have an inverse relationship with DIC score in patients with DIC of varied etiology (Hyun 2009).

**Platelet Biomarkers.**

Platelet abnormalities are almost always noted in DIC, as the development of thrombocytopenia due to consumption of platelets is one of the most readily available clinical indicators of DIC development. A study of 105 ICU patients revealed

thrombocytopenia in 53% of patients at the time of admission as well as an association between reduced platelet count and elevated mortality and a distinct cytokine profile including elevation of IL-8 and ICAM (Tsirigotis 2016). The role of platelets in DIC is not merely one of passive consumption. The primary function of platelets is hemostatic; platelets adhere to damaged endothelium, form the primary hemostatic plug to prevent blood loss, and ultimately contribute the formation of a stable clot. Platelets are also a crossroads between hemostasis, immunity, and inflammation. They are one of the first cell types to respond to compromised vasculature, invading pathogens, and sepsis. Studies have also shown that platelets may be involved in both the thrombotic and inflammatory aspects of disease through direct activity and the release of soluble mediators (de Stoppelaar 2014; Rondina 2011; Rondina 2012; Rondina 2015). In addition to responding to damaged endothelium, platelet aggregation can be induced by contact with a pathogen or by high levels of circulating inflammatory factors (Davis 2016). Platelets may also be activated through multiple mechanisms by NETs and their component parts (Davis 2016). Upon activation, platelets secrete their granule contents, releasing many factors into circulation, including insulin-like growth factor 1, platelet-derived growth factor (PDGF), transforming growth factor β (TGFβ), platelet factor 4 (PF-4), thrombospondin, fibronectin, Factor V, von Willebrand Factor (vWF), ATP, ADP, and serotonin. Accordingly, analysis of platelet function, including factors linking platelet function with inflammation and endothelial function, is relevant to the understanding of sepsis and DIC.

**CD40L.** CD40L (CD154) is a transmembrane protein expressed on the surface of CD4+ T cells and activated platelets. Platelets also release soluble CD40L into the circulation (Aukrust, Damas, & Solum, 2004). CD40L is a link between platelet and endothelial activation. Stimulation of endothelial cells with CD40L causes increased expression of adhesion molecules including as E-selectin, VCAM-1, and ICAM-1 on the endothelial cell surface as well as production of other inflammatory mediators including TF, IL-6, IL-8, and MCP-1 (de Stoppelaar 2014; Henn 1998; Semple 2011). CD40L also acts synergistically with FXa to induce endothelial TF expression (Hezi-Yamit 2005). Endothelial cell procoagulant activity can in turn lead to further CD40L production by inducing thrombin generation, which stimulates platelet CD40L production (Henn 1998). IL-1β activated human intestinal microvascular endothelial cells have also been shown to induce platelet CD40L expression and secretion (Danese 2003).

Soluble CD40L has been detected in the plasma of patients with inflammatory conditions, but specific studies related to sepsis-associated DIC are lacking. Elevated soluble CD40L was detected in a study of 63 children with meningococcal sepsis compared to age matched controls, but no relationship was observed between the levels of soluble CD40L and disease severity (Inwald 2006).

**Platelet Factor 4 (PF4).** Platelet factor 4 (PF4) is a chemokine released from α granules after platelet activation. PF4 binds to heparin and other glycosaminoglycans (GAGs) in a charge-dependent manner. This binding is notable for its role in heparin-induced thrombocytopenia (HIT), a condition in which antibodies are formed against the heparin-PF4 complex. PF4 has also been shown to bind to bacteria, including Gram

positive *S. aureus, S. pneumonia,* and *L. monocytogenes* and Gram-negative *E. coli* and *N. meningitides* (Krauel 2011). Interestingly, anti-heparin-PF4 antibodies from HIT patients were also shown to bind to PF4-coated bacteria and enhance neutrophil-mediated bacterial clearance (Krauel 2011).

In addition to binding to pathogens, PF4 may be relevant to DIC for its interaction with the APC system. In a purified system, PF4 enhanced histone-mediated APC generation in the presence of thrombin-thrombomodulin complexes (Kowalska 2014). Addition of heparin to this system reduced histone-mediated APC generation, but did not negate the ability of PF4 to induce APC generation (Kowalska 2014). PF4 was also shown to stimulate thrombin-induced APC generation *in vivo* (Kowalska, Mahmud, Lambert, Poncz, & Slungaard, 2007; Kowalska, Rauova, & Poncz, 2010), an effect which was negated by heparin administration (Kowalska 2010). PF4 injection in into mice was also shown to enhance histone and thrombin induced APC generation (Kowalska 2014). Whether through enhanced APC generation or another mechanism, PF4 may play a protective role in DIC. Mice with platelets overexpressing PF4 exhibited improved survival in an LPS-injection model of sepsis compared to both WT and PF4 knockout mice (Kowalska 2007). Furthermore, injection of platelets from PF4 knockout mice into WT mice with thrombocytopenia secondary to LPS challenge did not increase survival, whereas injection of platelets from mice overexpressing PF4 did (Kowalska 2007).

Elevated PF4 has been reported in animal models of sepsis. In a study involving LPS or thrombin injection into mice, PF4 levels in the lungs were significantly elevated 10 minutes after injection in both thrombin and LPS-treated mice compared to saline-

injected controls (Kowalska 2007). Elevated PF4 has also been reported following LPS injection into rats (Tang 2010).

**Microparticles (MP) and MP-Associated Tissue Factor (MP-TF).** Microparticles (MP) are small vesicles composed of cellular membrane and membrane proteins that are released into the circulation from numerous cell types, including endothelial cells, platelets, immune cells, and tumor cells. Based on their cellular origins, MPs have widely varied properties and thus play diverse roles in disease pathophysiology. Of greatest interest in DIC are the potential pro-coagulant properties of MP.

MP have intrinsic procoagulant properties due to their phospholipid surface, and MP isolated from the blood of healthy volunteers without ongoing coagulation disorders have been shown to support coagulation *in vitro* in a TF-independent manner (Berckmans 2001). It has been suggested that the role of these MP in healthy individuals is in fact anticoagulant, as the low-level thrombin generation promoted by these MP promotes protein C activation (Berckmans 2001). However, MP in disease states can also express abundant surface TF and thus exhibit significant procoagulant properties. Tumor cells are a notable source of circulating TF-bearing MP, and MP-associated TF was shown to be significantly elevated in patients with overt DIC secondary to malignancy (Langer 2008).

Elevated levels of MP of platelet (Nieuwland 2000; Ogura 2001; Soriano 2005), granulocyte (Nieuwland 2000), endothelial (Delabranche 2016; Matsumoto 2015; Soriano 2005), and leukocyte (Fujimi 2002) origin have been reported in sepsis and DIC patients compared to healthy individuals, although the precise MP profile associated with

sepsis is not well established (Joop 2001). Elevation of platelet and endothelial MP has been associated with non-survival in sepsis patients (Soriano 2005). In a study of 259 patients with septic shock with extensive follow-up to detect the development of DIC, procoagulant microparticles were elevated in all patients regardless of the ultimate development of DIC (Delabranche 2016). In this study, $CD105^+$ microparticles, in conjunction with platelet count and PT, were found to have strong ability to predict the development of DIC in patients with septic shock early in disease progression before clinically relevant hemostatic signs (Delabranche 2016). Levels of endothelial-derived MP have been shown to correlate with ISTH DIC score (Matsumoto 2015). In addition, EPCR-positive MP correlated significantly with APACHE II score and TF and EPCR-positive MP correlated with SOFA score (Matsumoto 2015).

Experimental conditions replicating sepsis also increase MP number. In a study of LPS infusion into healthy volunteers, platelet-derived and total MPs increased compared to baseline levels (Mooberry 2016). CLP-induced sepsis led to production of increased numbers of MP of platelet, endothelial, and monocyte origin in mice (Zafrani 2012).

In addition to changes in number, the procoagulant phenotype of MPs may be modified in disease states. A study of MP from patients with meningococcal sepsis found no absolute change in MP number but did observe alterations to the MP pattern of origin and procoagulant phenotype (Joop 2001). A second study of MP from patients with DIC secondary to meningococcal sepsis found that these MP supported *in vitro* thrombin generation more strongly than MP from healthy controls (Nieuwland 2000).

One of the main procoagulant mechanisms of MP is TF exposure. Procoagulant microparticles, defined by positive TF staining via flow cytometry, have been detected in the plasma of patients with sepsis and MODS (Delabranche 2013). TF-positive MP of endothelial origin also correlated significantly with levels of IL-6, soluble TF factor, D-Dimer, and decreased platelet count (Matsumoto 2015). MP from healthy human volunteers injected with LPS exhibited increased TF activity as well as reduced MP-dependent clotting time (Mooberry 2016). In a mouse model of LPS injection, injection of LPS led to a significant increase in total microparticle procoagulant activity, and the MP-TF activity correlated with levels of TAT in these mice (Wang 2009).

MP production and procoagulant activity may be detrimental to the host in sepsis. Mice with reduced MP production due to increased calpastatin expression showed improved survival in a CLP model as well as reduced kidney, liver, and lung dysfunction (Zafrani 2012). This reduction in MP number was also associated with delayed thrombin generation, reduced depletion of platelets and coagulation factors, and reduced DIC (Zafrani 2012).

## Animal Models of Sepsis and DIC

Sepsis and sepsis-associated DIC are complex clinical conditions with pathophysiology characterized by interactions between the immune system, blood-borne coagulation system components, and the endothelium. This pathophysiology cannot be effectively replicated by an *in vitro* system. Stimulation of whole blood with LPS (Lindmark 1998) or other factors such as histones can be performed to study the direct influence of these factors on coagulation; however, such approaches do not account for

the interactions with the endothelium or the mechanical influence of blood flow and cannot demonstrate the benefits of drugs that may occur through interactions with the endothelium. Although alternative *in vitro* systems are being developed to simulate DIC (Greineder 2015, 2016; Greineder 2017), animal model systems are the most viable methods to study DIC pathophysiology and drug mechanisms.

Several model systems are currently used for the study of sepsis, with the two most common being the infusion of exogenous toxins, typically LPS or more recently histones, or the induction of a polymicrobial infection through cecal ligations and puncture (CLP). These model systems may also induce coagulopathy and be appropriate for the study of DIC. Both model systems induce a sepsis-like inflammatory response and can lead to the development of coagulopathy. However, additional work is needed to determine how well these models truly replicate the DIC seen in human patients.

**Toxin Injection Models**

The LPS injection model is commonly used and presents minimal technical difficulties, simply involving the injection of LPS into a rat (Elsayed 1996a; Iba 2014; Iba 2014; Inoue 1991; Kaspereit 2004; Murakami 1996) or mouse (Abraham 1999; Standiford 1995; Wang 2009; Yano 2006). LPS injection has also been used to simulate sepsis or DIC in monkeys (van der Poll 1994) and in healthy human volunteers (de Jonge 2000; Pajkrt 1997). This creates a transient model of sepsis, including full activation of inflammatory cytokines, but does not replicate the later or prolonged stages of disease (Doi 2009) and results in an earlier and lower peak level of TNFα and IL-6 than is observed in human sepsis and in other models (Rittirsch 2007). The underlying

assumption of the LPS injection model is that the majority of the pathophysiology of sepsis is due to the host inflammatory response to the pathogen, primarily due to highly pro-inflammatory components such as LPS, rather than to the pathogen itself (Rittirsch 2007). Although this model is primarily used to study sepsis, development of symptoms consistent with DIC such as the formation of microthrombi in the organs, elevated markers of coagulation such as TAT and F1.2, and prolongations in PT or aPTT have been observed in the LPS injection model at doses ranging from 20 to 500 mg/kg in rats (Asakura 2003; Elsayed 1996a; Hasegawa 1996; Kaspereit 2004).

More recently, injection of histones has been used as an alternative model of DIC (Abrams 2013; Nakahara 2013). Injection of histones into mice at a dose of 75 mg/kg is sufficient to induce pulmonary hemorrhage and death accompanied by an elevation in TNFα, IL-6, and IL-10 and a consumptive coagulopathy similar to that seen in DIC (Abrams 2013; Nakahara 2013). Direct injection of a TF-containing reagent such as thromboplastin at a dose of 3.75 U/kg has also been used; however, this mimics cancer-associated DIC, not sepsis-associated DIC, and is thus beyond the scope of this project (Asakura 2003).

While toxin injection models of sepsis and DIC are useful for the examination of certain aspects of the pathophysiology of DIC, they cannot accurately replicate prolonged disease and are therefore less appropriate for the study of drug treatments. Models involving a true infection provide a more accurate representation of the conditions seen in human DIC and are more appropriate for this purpose.

**Cecal Ligation and Puncture**

The cecal ligation and puncture (CLP) model of sepsis is commonly used in both rats (Heuer 2004; Heur 2004; Hubbard 2005; Inoue 1991; Kim 2000; Laudes 2002; Otero-Anton 2001; Qiu 2001; Ravindranath 2007; Rittirsch 2007; Rittirsch 2008; Schabbauer 2012; Yang 1994; Yin 2005) and mice (Araujo 2016; Clark 2008; Cuenca 2010; Ganopolsky 2004; Hubbard 2005; Li 2007; Marquez-Velasco 2011; Ono 2001; Song 2013; Uolla 2002; van der Poll 1995; Wang 2004; H. Yang 2004; Yano 2006). Many slight variations on the CLP model exist and variations in the model allow for the fine tuning of disease severity. This protocol is well described by Rittirsch et. al. (Rittirsch 2007) and is widely accepted as a model for sepsis (Zanotti-Cavazzoni 2009).

In this surgical model, the cecum is ligated with a suture near to but not obstructing the ileocecal valve. The ligation of the cecum leads to ischemia and necrosis, adding a source of inflammation to the model (Schabbauer, 2012). Variations in the amount of the cecum ligated produce differing severities of sepsis and thus differing degrees of model lethality. Additionally, the cecum is punctured with a needle, allowing leakage of fecal matter into the peritoneum, creating a source of polymicrobial infection. Many variants on the needle puncture procedure exist, leading to varying severity of induced sepsis due to the number of punctures, size of feces droplet extruded, and gauge of the needle. 100% mortality has been reported with double puncture with an 18-gauge needle (Kim 2000), mid-grade sepsis with a survival rate of 40% with a single perforation with a 20-gauge needle (Laudes 2002), low grade sepsis with single perforation with an 18-gauge needle (Kim 2000), and confirmed DIC with 7 punctures made with an 18-

gauge needle with removal of the necrotic cecum after 12 hours (Inoue 1991). A single through-and-through puncture with an 18-gauge needle was used in this study to create sepsis and coagulopathy with low mortality to facilitate the study of drug mechanisms of action. This portion of the procedure has a high potential for inconsistency, and standardization of this method is necessary to obtain good experimental results.

Following puncture of the cecum and return to the peritoneal cavity, the incision is closed with clips or sutures. Fluid resuscitation practices are also highly variable and can have a significant impact on animal mortality (Kim 2000; Laudes 2002; Ravindranath 2007; Rittirsch 2008) ranging from no resuscitation to 40 (Kim 2000) or 50 (Rittirsch 2008) ml/kg of pre-warmed sterile normal saline injected subcutaneously immediately following surgery.

Although the CLP model is typically used to study sepsis, it leads to the development of coagulopathy and can therefore be used to study DIC. Thrombin generation, thrombus formation, decreased platelet count, and changes in global coagulation status have been reported in CLP models, suggesting that DIC does develop in this system (Heuer 2004; Inoue 1991; Laudes 2002; Song 2013). In a mouse model of CLP, reduction in platelet count, and elevated PT, aPTT, and D-Dimer were observed 6 hours following CLP, with extensive microthrombus formation within 12 hours (Song 2013).

The CLP model of sepsis provides several advantages for the study of the pathophysiology of DIC and modulation by therapeutic agents. This model replicates a common clinical scenario of polymicrobial infection with intestinal flora. Additionally,

CLP is adaptable and can be modified to induce sepsis and associated DIC along a spectrum of severity by altering the size or number of punctures made, amount of cecum ligated, and amount of fluid resuscitation used (Kim 2000; Rittirsch 2008).

**Alternative Approaches**

Although CLP or the injection of LPS are the most commonly used animal models for sepsis or DIC, several alternative models do exist. Several studies have used the direct injection of TF in order to model DIC (Asakura, 2014; Asakura2003). Differences in the nature of the coagulation dysfunction induced by LPS and TF injection have been described, and the direct injection of TF may be more appropriate for the study of cancer-associated DIC than sepsis-associated DIC. Direct injection of known quantities of viable live bacteria has also been used. Although these models do utilize live bacteria, they are often considered more similar to models of endotoxic shock than CLP models, as even high doses of bacteria may be rapidly destroyed and fail to establish lasting infection (Rittirsch 2007; Cross 1993; Lilley2015).

Two main alternatives to CLP exist: the implantation of a bacterial clot or fecal pellet into the abdomen, or the colon ascendens stent peritonitis (CASP) model. The implantation of a fibrin clot containing viable bacteria (Mathiak2000) or a fecal pellet (Rittirsch2007) into the abdomen of a rat is considered similar to the CLP model and is not commonly used (Rittirsch2007). The CASP model, in which a stent is inserted into the ascending colon, leading to continued leakage of fecal matter into the peritoneal cavity, may represent a viable alternative to the CLP model Schabbauer, 2012; Zanotti-Cavazzoni 2009; Zantl 1998). In this model, mortality is often controlled by removal of

the stent at a pre-determined time point. The added surgical complexity of this model was not warranted for these studies.

## Therapeutics for DIC

Current clinical practice for the treatment of DIC does not involve any coagulopathy-specific treatment but rather is focused on resolution of the underlying condition. For sepsis-associated DIC, this involves treatment of the underlying infection with pathogen-appropriate antibiotics accompanied by supportive interventions such as fluids, vasopressors, and mechanical ventilation. Although some instances of DIC, such as those induced by obstetric complications, may resolve rapidly with resolution of the underlying condition, sepsis-associated DIC often results in extended illness that contributes substantially to patient morbidity and mortality. Additionally, the organ failure induced by the coagulopathy in DIC can have long-lasting complications. There is an unmet therapeutic need for a drug to treat the coagulopathy in sepsis-associated DIC.

The development of therapies for DIC is complicated by the propensity for both bleeding and thrombosis in DIC patients. While treatment with a conventional anticoagulant such as heparin may prevent systemic coagulation, this therapeutic approach carries a risk of significant and potentially fatal bleeding. Bleeding risk is heightened in patients with DIC, where consumptive coagulopathy leads to a risk of bleeding even in the absence of anticoagulant therapy. Conversely, replacement of coagulation factors and platelets to prevent bleeding associated with DIC and correct laboratory coagulation parameters through the administration of blood products may add fuel to the fire of ongoing systemic thrombosis. In a 2016 meta-analysis of 24 clinical

trials involving 14,767 patients, including trials of multiple exogenous or endogenous anticoagulants, anticoagulant therapy led to a reduction in mortality in patients with sepsis-associated DIC but not in patients with less severe coagulopathy or with sepsis alone (Umemura 2016). Other studies of anticoagulant use in DIC patients have yielded similar results (Dhainaut 2004; Kienast 2006; Yamakawa 2016). This suggests that anticoagulation of patients with sepsis and severe DIC may be appropriate and beneficial whereas anticoagulant treatment of patients with less severe coagulopathy may in fact prove detrimental. Accordingly, improved approaches for determining if patients are appropriate candidates for anticoagulant therapy are needed. Additionally, methods to monitor treatment and assess a patient's response to therapy would also be beneficial.

A potential approach to DIC treatment is restoration of the endogenous anticoagulant system. Several endogenous anticoagulants have entered clinical trials for application in sepsis-associated DIC, including tissue factor pathway inhibitor (TFPI) (Abraham 2003; de Jonge 2000), activated protein C (APC) (Bernard 2004; J. Dhainaut 2004; J.-F. Dhainaut 2003; Ranieri 2012; Shorr 2010), antithrombin concentrate (AT) (Allingstrup 2016; Iba 2016; Iba 2012; Kienast 2006; Tagami 2015; Warren 2001), and recombinant thrombomodulin (rTM) (Hayakawa2016; Hoppensteadt 2014; Ito 2015; Saito 2007; Vincent 2013). Heparin, often in combination with another agent, has also been evaluated for use in sepsis-associated DIC (do Toit 1991; Jaimes 2009; Kienast 2006; Levi 2007; Pernerstorfer 1999). Treatment of DIC with TFPI was unsuccessful and did not lead to drug approval. APC, under the names drotrecogin alfa (activated) and Xigris, was successful in initial trials and was approved for use in patients with sepsis in

2001. However, post-approval studies showed low or no benefit to APC treatment coupled with an increased risk of severe bleeding, and APC was withdrawn from the market in 2011.

The current focus for endogenous anticoagulant treatment in sepsis-associated DIC is antithrombin (AT) and recombinant thrombomodulin (rTM). Both AT and rTM are approved for use in the treatment of DIC in Japan, and an international Phase III trial of rTM is ongoing (ClinicalTrials.gov identifier NCT01598831). Although AT and rTM are not currently used in the United States, they are widely used to treat sepsis-associated DIC in Japan, with a study of 3195 patients with severe sepsis in Japan between 2011 and 2013 reporting that 47% of these patients received treatment for DIC, with 31% receiving AT, 31% receiving rTM, and 16% receiving co-administration of the two agents (Hayakawa, Saito, 2016). In an analysis of anticoagulant therapy of 2663 sepsis patients in Japan, 1247 of whom received anticoagulants and 1416 of whom did not, administration of anticoagulants including AT, rTM, and heparin showed a benefit in those patients who were diagnosed with DIC, and a trend towards reduction in mortality in those with the highest SOFA scores (13-17) (Yamakawa 2016). However, this pattern was not observed in patients without DIC or with lower SOFA scores, underscoring the importance of targeting treatment to the most appropriate patients (Yamakawa 2016). In this cohort, bleeding risk, defined by the requirement for bleeding-related blood transfusions, ranged from 13-27% in the anticoagulant treated groups and from 6-10% in the control group (Yamakawa 2016).

Recombinant thrombomodulin, antithrombin, and heparin will be the focus of this research project. The following section provides a discussion of the mechanism of action and role of rTM, AT, and heparin in sepsis-associated DIC. Figure 4 illustrates the anticoagulant effects rTM, AT, and heparin. Table 4 summarizes the basic anticoagulant mechanisms and hypothesized additional activities of each agent with respect to their use in the treatment of sepsis-associated DIC.

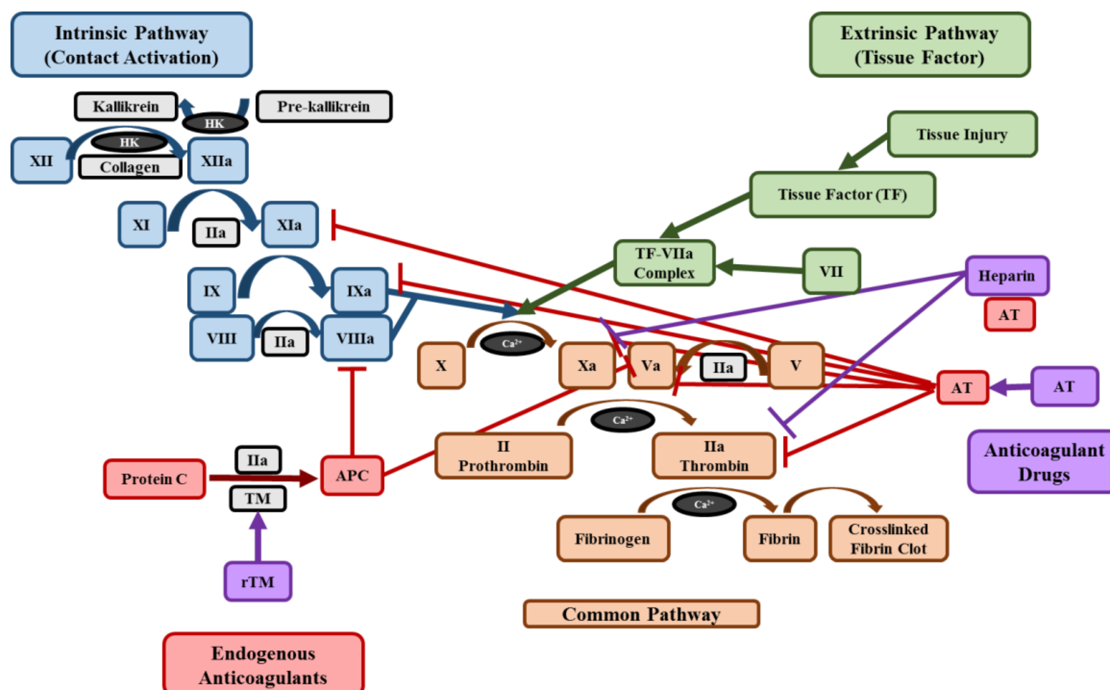


**Figure 4. Anticoagulant Actions of rTM, AT, and Heparin**. Coagulation cascade components are shown in blue (intrinsic pathway), green (extrinsic pathway), and orange (common pathway). The actions of endogenous anticoagulants including Protein C, activated Protein C (APC), thrombomodulin (TM), and antithrombin (AT) are shown in red. Anticoagulant agents with potential use in DIC include recombinant thrombomodulin (rTM), antithrombin (AT), and heparin, and are shown in purple. rTM serves as a replacement for endogenous TM and converts Protein C to APC, which subsequently inhibits FVIIIa and FVa. Exogenous AT serves as a replacement for depleted endogenous AT and inhibits multiple coagulation factors including FIIa, FVa, FXa, FIXa, and FXIa. Heparin is an AT-dependent factor Xa and IIa inhibitor. Heparin binds to AT and enhances the AT-mediated inhibition of these factors.

**Table 4. Summary of Mechanisms of Action of Recombinant Thrombomodulin, Antithrombin, and Heparin in DIC**

| Drug | Anticoagulant Mechanism | Hypothesized Additional Mechanisms |
|---|---|---|
| **Recombinant Thrombomodulin (rTM7; ART-123)** | Replacement for dysregulated endogenous anticoagulant system; activates Protein C, leading to inhibition of FVIIIa and FVa | • Direct anti-inflammatory effects including neutralization of LPS (Shi 2008)<br>• Prevention of damage caused by circulating histones and other nuclear material (Hagiwara 2010; Iba 2014; Iba 2014; Nagato 2009; Nakahara 2013; Osada 2017; Shimomura 2016; Takehara 2017; Tanaka 2013)<br>• Inhibition of NETosis (Shimomura 2016)<br>• Anti-inflammatory and cytoprotective effects mediated through TAFI (Colucci 2012; Tawara 2016) and APC (Bae 2011; Xu 2009) |
| **Antithrombin (AT)** | Replaces physiological anticoagulant that becomes depleted in DIC; inhibitor of thrombin, FXa, FVIIa, FIXa, and FXIa | • Reduction in inflammation through reduced coagulation (Iba 2014; Levy 2016)<br>• Preservation of the glycocalyx (Chappell 2009; Iba 2016) Increased prostacyclin synthesis and secretion, reduced neutrophil rolling and adhesion (Iba 2014; Levy 2015) |
| **Heparin (UFH)** | Exogenous anticoagulant; antithrombin-dependent inhibitor of FXa and FIIa | • Decreased TF expression (Ding 2011; Pernerstorfer 1999)<br>• Increased TFPI release (Pernerstorfer 1999)<br>• Reduced inflammation (Ding 2011)<br>• Anti-histone effects (Kowalska 2014)<br>• Reduced vascular permeability (Bentzer 2016) |

**The Protein C Pathway: Activated Protein C and Recombinant Thrombomodulin**

**Activated Protein C (APC).** Protein C is an endogenous anticoagulant that becomes depleted in DIC patients due to vascular leakage, reduced hepatic production, and excessive consumption. This acquired protein C deficiency is associated with hypercoagulability and increased mortality (Marcel Levi 2001; ten Cate, 2000). When converted to its active form, activated protein C (APC), APC proteolytically inactivates coagulation factors Va and VIIa in addition to exerting additional anti-inflammatory effects, potentially mediated through the cleavage of histones.

Direct replacement of APC, known in drug form as drotrecogin alfa (activated) or by the brand name Xigris, was pursued as a therapeutic approach in patients with severe sepsis and coagulopathy. APC was approved by the FDA in 2001 as the first drug for use in this indication following the PROWESS trial (Bernard 2001). However, subsequent clinical trials showed significant bleeding risk associated with APC treatment (Abraham 2005; Bernard 2004). Following the PROWESS-SHOCK study, which showed no reduction in mortality for patients treated with APC, the drug was ultimately removed from the market in 2011 (Ranieri 2012). Following the removal of APC from the market, drugs for the treatment of sepsis-associated coagulopathy represent an unmet medical need.

Despite the overall clinical failure of APC, some evidence suggested that APC might have benefits outweighing the bleeding risk in the population of patients with overt DIC, which composed only a small portion of the severe sepsis patients enrolled in these

large clinical trials, ultimately leading to the development of rTM as a therapeutic agent (Dhainaut 2004).

**Recombinant Thrombomodulin (rTM).** Recombinant thrombomodulin (rTM; also known as ART-123) was approved in Japan in 2008 for the treatment of sepsis-associated DIC (Saito 2007). rTM is a soluble form of the endogenous protein thrombomodulin (TM). TM is expressed on the surface of endothelial cells and has a high affinity for thrombin. Thrombin-thrombomodulin complex formation changes the specificity of thrombin from procoagulant substrates towards the conversion of Protein C to Activated Protein C (APC) (Adams & Huntington, 2006). Once Protein C is activated by the thrombin-thrombomodulin complex, it inhibits FVa and FVIIa, ultimately leading to reduced thrombin generation (Kisiel, 1979). The anticoagulant effects of TM, mediated through Protein C, are illustrated in Figure 5 (Ikezoe, 2015).

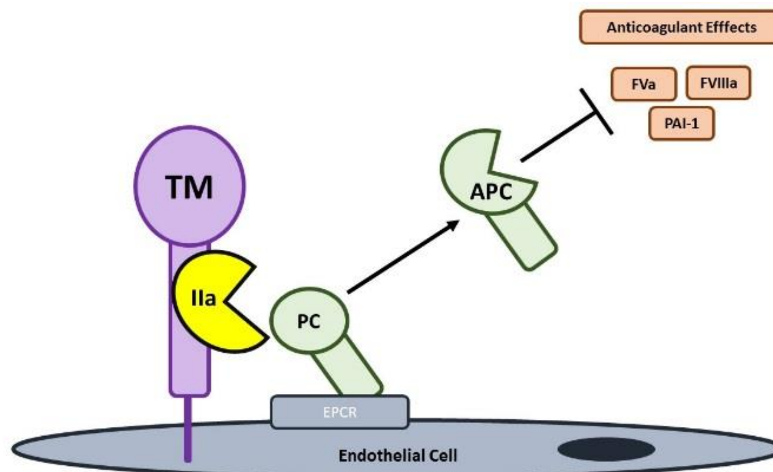


**Figure 5. Activity of Thrombomodulin on the Coagulation Cascade**. Adapted from Ikezoe et. al. 2015. Thrombomodulin exerts its anticoagulant effects through the activation of Protein C. Once activated, APC inactivates coagulation factors Va and VIIIa, leading to reduced thrombin generation (TM, thrombomodulin; IIa, thrombin; PC, Protein C; EPCR, endothelial protein C receptor; APC, activated protein C)

TM is dysregulated in DIC patients, which is hypothesized to contribute significantly to disease pathophysiology. Inflammation leads to reduced TM levels on endothelial cell surfaces (Moore, Andreoli, Esmon, Esmon, & Bang, 1987; Moore, Esmon, & Esmon, 1989). This in turn can lead to reduced activity of the Protein C system and increased coagulation as well as reduction in the non-anticoagulant effects of TM.

The effects of TM are not limited to actions on specific proteins in the coagulation cascade. Both TM and Protein C have direct anti-inflammatory and cytoprotective effects. rTM has been shown to bind to and neutralize LPS, ameliorating the LPS-induced inflammatory response (Shi 2008). The thrombin-thrombomodulin complex activates thrombin-activatable fibrinolysis inhibitor (TAFI), which itself has

antifibrinolytic and anti-inflammatory properties, including increased inactivation of complement factor C5a and decreased C5a-induced PMN migration (Colucci 2012; Tawara 2016).

Interestingly, rTM may also be protective against histone induced damage and death (Iba 2014; Nakahara 2013; Osada 2017; Shimomura 2016). *In vitro,* rTM was shown to inhibit histone-induced thrombin generation and endothelial cell death and promote Protein C-mediated histone cleavage (Osada 2017). In a mouse model of histone H3 induced lethal thromboembolism, rTM was shown to bind to extracellular histones, suppress histone-induced platelet aggregation, and protect mice from histone induced DIC (Nakahara 2013). Administration of rTM also decreased detectable levels of histone H3 from 17.0 pg/ml to 5 pg/ml in rats subjected to an LPS-induced model of sepsis (Iba 2014; Iba 2014). rTM at concentrations of 2, 10, or 50 μg/ml prevented LPS-induced NETosis in the presence of platelets (Shimomura 2016). APC was also capable of inhibiting histone-mediated damage through the cleavage of histone proteins H3 and H4 (Xu 2009). rTM (Hagiwara 2010; Nagato 2009; Tanaka 2013) and APC (Bae 2011) may also prevent damage caused by other nuclear materials in the extracellular environment, such as the chromatin associated protein HMGB-1. In mice subjected to LPS challenge, administration of 3 mg/kg rTM reduced levels of nucleosomes and HMGB-1 as well as IL-6, MCP-1, and TNFα (Takehara 2017). The non-anticoagulant properties of thrombomodulin and Protein C are summarized in Figure 6.

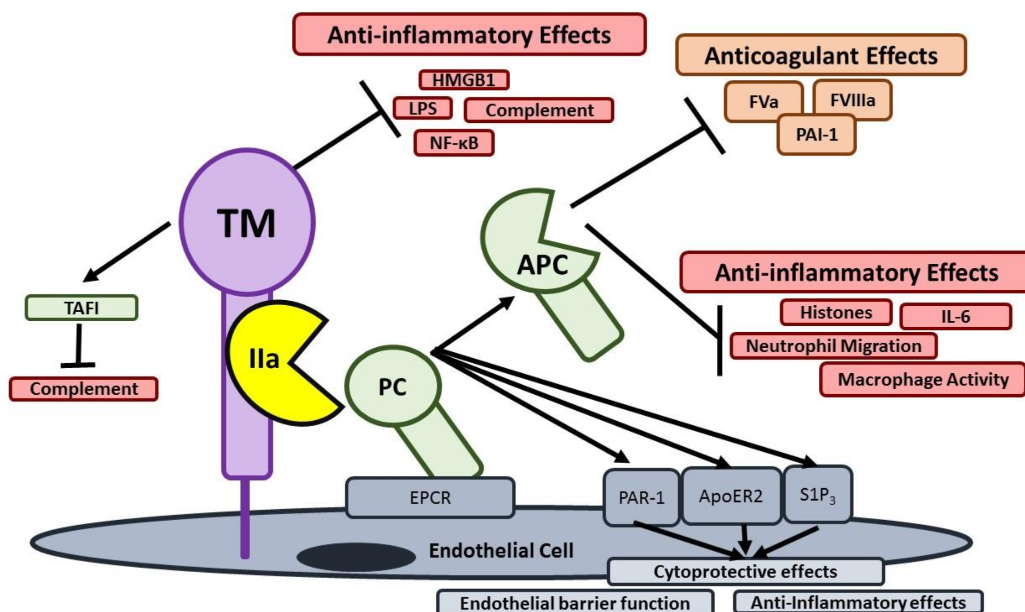


**Figure 6. Anti-Inflammatory and Cytoprotective Effects of Thrombomodulin**. Adapted from Ikezoe et. al. 2015. .In addition to Protein-C mediated anticoagulant effects, both thrombomodulin and Protein C exert other anti-inflammatory and cytoprotective effects that may be highly beneficial to patients with sepsis-associated DIC.

rTM was approved for use in patients with sepsis and DIC in Japan in 2008, and post-approval research has demonstrated a reduction in mortality without an increase in bleeding (Aota 2016; Hayakawa 2016; Ikezoe 2015; Ogawa 2011; Yamakawa 2015; Yamakawa 2011; Yamakawa 2013). Approval for rTM is currently being pursued in the United States and Europe, with a Phase II trial completed (Vincent 2013) and a Phase III trial is ongoing (ClinicalTrials.gov identifier NCT0158831).

As with any drug with anticoagulant properties, treatment associated bleeding risk is a significant concern. In patients treated with rTM, lower bleeding rates have been observed compared to heparin-treated patients (Saito 2007). Pre-clinical studies of rTM demonstrated reduced peak thrombin generation in a thrombin generation assay (Mohri

1999) as well as effects on conventional coagulation assays including aPTT, PT, and TT in the presence of Protein C (Mohri 1999). However, no significant increases in bleeding have been reported in several clinical trials for rTM in DIC patients, suggesting that rTM is safe for use in this patient population (Ogawa 2011; Saito 2007; Vincent 2013).

rTM is administered clinically at a dose of 60 µg/kg/day (Ogawa 2011; Saito 2007; Vincent 2013; Yamakawa 2015; Yamakawa 2011; Yamakawa 2013) (0.06 mg/kg/day) with a maximal dose of 6 mg per day (Vincent 2013). However, the IC50 value of rTM on thrombin generation is much higher in rat plasma than in human plasma, and significantly higher doses of rTM must be used in rats to achieve comparable effects. The original preclinical studies of rTM in both LPS and TF-induced DIC in rats used rTM at doses of 0.3, 1, and 3 mg/kg, with the greatest effects seen at 3 mg/kg (Gonda 1993; Mohri 1994). Experimentally, rTM is commonly administered to rats at doses of 0.25 mg/kg (Iba 2013; Iba 2014; Iba 2014; Iba 2009) or 1 mg/kg (Aoki 1994; Aoki 1994; Gonda 1993; Hagiwara 2010; Iba 2013; Mohri 1994; Nagato 2009), with higher doses used in numerous studies (Aoki 1994; Gonda 1993; Hasegawa 1996; Iba 2013; Mohri 1994; Tanaka 2013). rTM has also been co-administered with antithrombin in both animal (Iba 2014) and human (Iba 2009; Iba 2014; Iba 2016) studies, although combination approaches may result in increased bleeding.

**Antithrombin**

Antithrombin (AT) is a physiological anticoagulant capable of inhibiting thrombin, FVIIa, FIXa, FXa, FXIa, and FXIIa. Reduced AT levels have been observed in patients with sepsis-associated DIC and are correlated with increased mortality (Warren

2001). In sepsis, AT levels are reduced not only due to consumption but also due to leakage from the vasculature (Aibiki 2007; Iba 2016). AT has been pursued as therapy for patients with sepsis and coagulopathy, most notably through the large Phase III KyberSept trial, enrolling 2314 patients with severe sepsis (Warren 2001). In this trial, no overall reduction in 28-day mortality was observed, and an increase in clinically significant bleeding was observed in patients receiving AT and concomitant prophylactic doses of heparin (Warren 2001). Subsequent post-hoc analyses revealed that patients with confirmed DIC who did not receive concomitant heparin may in fact have benefitted from AT treatment; AT treatment in this population reduced mortality from 40% to 25.4% in comparison to placebo (Kienast 2006). Additional smaller studies have also supported the safety and efficacy of AT in sepsis patients with confirmed DIC (Iba, Gando, 2016; Iba 2012; Tagami 2015). AT is used clinically as a therapeutic for DIC in Japan at doses of 3000 or 1500 IU/day (Iba 2012). While AT may be beneficial in the DIC patient population, use of this agent is associated with an increased risk of clinically significant bleeding (Allingstrup 2016).

Although less commonly discussed, AT may have properties that extend beyond anticoagulation, including anti-inflammatory effects. AT may protect the endothelium by preserving the glycocalyx, which is essential for the regulation of endothelial permeability and leukocyte adhesion (Chappell 2009; Iba 2016). Other anti-inflammatory effects of AT are thought to be mediated through the inhibition of coagulation (Levy 2016). In particular, the neutralization of thrombin by AT leads to reduced signaling through the PAR-1 receptor, which contributes to inflammatory activation (Iba 2014).

Inhibition of FXa and FVIIa may also decrease the induction of inflammation by coagulation (Levy 2016). Additionally, AT may be able to increase prostacyclin synthesis and secretion and prevent neutrophil rolling and adhesion (Iba 2014; Levy 2015).

AT is typically administered to sepsis and DIC patients at doses of 3000 IU/day or 1500 IU/day (Allingstrup 2016; Iba 2016; Iba 2012), although higher doses have been used in some clinical trials (Warren 2001). Based on the 70 kg "standard man", this corresponds to a dose of 43 IU/kg/day or 21 IU/kg/day, with lower doses achieved in heavier patients. Some studies in rats have used AT at doses comparable to those used in humans, such as 50 IU/kg (Uchiba, Okajima, & Murakami, 1998) or 62.5 IU/kg (Iba 2009). However, while this dose may be sufficient to reverse coagulation abnormalities, higher doses are required to have additional anti-inflammatory effects (Uchiba 1998). Consequently, AT is commonly administered to rats at doses of 125 IU/kg or 250 IU/kg (Uchiba 1998; Yamashiro 2001; Yang 1994). AT has also been co-administered with rTM in both animal (Iba 2014) and human (Iba 2009; Iba 2014; Iba 2016) studies, although combination approaches may result in increased bleeding. The co-administration of AT with heparin in DIC patients has also been investigated as a therapeutic approach (Hoffmann 2002; Yang 1994), although co-administration of heparin may reduce the efficacy of AT supplementation.

**Heparin**

Heparin is an anticoagulant drug derived from porcine mucosa that is used in numerous medical and surgical applications. Heparin acts as an antithrombin-dependent inhibitor of factors Xa and IIa. The binding of heparin to AT induces a conformational

change in AT which increases the anticoagulant activities of AT more than 1,000 fold. As heparin requires AT to exert its anticoagulant effect, the effects of heparin will be reduced in a severely AT-deficient patient. In addition to its classical anticoagulant effects, heparin has additional mechanisms of action including induction of endothelial cell TFPI release.

Unfractionated heparin (UFH), low molecular weight heparin (LMWH), or other heparin-derived products such as pentasaccharide (fondaparinux) or heparinoids may be used depending on the clinical scenario and desired benefits. UFH has traditionally been the focus of study in sepsis and DIC (Jaimes 2009; Y. Li 2011; Liu 2014). Prophylactic doses of UFH are commonly administered to hospitalized patients, while LMWH is the drug of choice for other applications. However, the non-anticoagulant activity and bleeding risks associated with UFH, LMWH, and heparinoids in sepsis-associated DIC may be different (Derhaschnig 2003; Iba 2009; van Bruggen 1996). Non-anticoagulant heparins have also been developed and may have relevance to sepsis and DIC (Ammollo 2011; van Bruggen 1996; Wildhagen 2014; Zhang 2014).

UFH is administered intravenously and is used to prevent or treat thrombosis in hospitalized patients, with significantly lower doses used for prophylaxis than for treatment of an established thrombus. Administration of prophylactic doses of heparin to hospitalized or immobilized patients is a common clinical practice. In addition to its antithrombotic effects, heparin may have non-anticoagulant effects that are beneficial to DIC patients.

The use of heparin in DIC has been studied in both animal models (do Toit 1991; Yang 1994) and in humans (Derhaschnig 2003; Kienast 2006; Levi 2007; Liu 2014; Pernerstorfer 1999; Saito 2007; Yang 1994). Heparin has been shown to inhibit coagulation in models of DIC. In a study of LPS administration to healthy human volunteers, administration of either UFH or LMWH decreased activation of coagulation, measured by blunted increase in F1.2 (Pernerstorfer 1999). However, it is less clear how well this contributes to increased survival. In general, clinical trials have failed to show a decrease in mortality with prophylactic UFH administration in DIC patients (Jaimes 2009; Levi 2007). In a baboon model of thrombin-induced DIC, IV heparin administration at a low dose of 10 IU/kg/hour prevented the development of DIC and reduced mortality (do Toit 1991). In a study of administration of 70 IU/kg heparin per day to 37 sepsis patients, significant reduction in number of days in the ICU and days requiring a ventilator was observed, and the percentage of patients ultimately developing MODS or DIC were reduced in heparin-treated subjects compared to controls (Liu 2014). However, no reduction in mortality was observed (Liu 2014).

Heparin may have benefits beyond anticoagulation in the treatment of DIC. UFH has been shown to decrease monocyte TF expression in response to LPS as well as increase TFPI levels (Pernerstorfer 1999). UFH pretreatment of mice subjected to LPS injection reduced inflammation and procoagulant phenotype, quantified by reduced levels of IL-1β, TNFα, and TF mRNA in blood cells as well as thrombus formation and fibrin deposition in the liver (Ding 2011). In a mouse model of histone injection, injection of UFH at 50 mg/kg, a dose sufficient to increase aPTT to greater than 150s compared to a

normal value of 20s, reduced histone-mediated APC generation; however, UFH also protected mice from histone-induced death (Kowalska 2014). Heparin administration may also prevent increased vascular permeability induced by elevated levels of heparin binding protein, which is associated with severity of shock and hypoxemia in patients with sepsis (Bentzer 2016).

Heparin is used as part of the standard of care for DIC in Japan, and several animal studies and clinical trials for AT and rTM have used heparin as a comparator drug instead of placebo or have included heparin treatment concomitant with the investigational drug (Kienast 2006; Levi 2007; Saito 2007; Yang 1994). Despite theoretical mechanisms for benefit in DIC, clinical trials have failed to show a decrease in mortality with prophylactic UFH administration in DIC patients (Jaimes 2009; Levi 2007). Additionally, heparin carries a significant risk of treatment-associated bleeding. The co-administration of AT with heparin in DIC patients has also been investigated as a therapeutic approach (Hoffmann 2002; Yang 1994), although co-administration of heparin may reduce the efficacy of AT supplementation.

The doses of heparin used clinically are widely varied depending on the clinical scenario. Accordingly, the doses of heparin used in clinical trials and in animal models are highly variable (Li 2011). UFH has been administered to sepsis-associated DIC patients at doses of 5,000 U subcutaneously twice per day concomitant with APC (Levi 2007). In a study of UFH as a therapy for DIC, UFH was administered at a dose of 12,000 IU for a 24-hour period (Jaimes 2009). For a 70 kg standard man, a dose of 10,000 IU of UFH per day would correspond to a dose of 143 IU/kg. Multiple animal

studies of UFH in DIC have used UFH at doses in the range of 100-400 IU/kg/day (Gonda 1993; Iba 2009; Li 2011; Mohri 1994).

# CHAPTER TWO

## STATEMENT OF PURPOSE

Sepsis, defined by the Society of Critical Care Medicine in 2016 as "life threatening organ dysfunction due to a dysregulated host response to infection" (Singer 2016) is a severe clinical condition that contributes significantly to morbidity and mortality in the United States and worldwide. A significant fraction of patients hospitalized with sepsis develop coagulation anomalies, ranging from slight perturbations in laboratory values to a severe systemic coagulation disorder known as disseminated intravascular coagulation (DIC). DIC is characterized, paradoxically, by both bleeding and thrombosis. Inappropriate and widespread activation of the coagulation cascade leads to microvascular thrombosis, causing, vascular occlusion, ischemia, and ultimately resulting in multiple organ dysfunction. This inappropriate coagulation activity consumes platelets and coagulation factors through a process often referred to as "consumptive coagulopathy", placing patients at risk for severe and potentially fatal bleeding. Development of DIC leads to a significantly elevated risk of death in septic patients. The molecular pathophysiology of sepsis-associated DIC is complex, and much remains to be understood about the development and treatment of this disease.

Under normal physiologic conditions, blood flow and hemostasis is maintained though an intricate balance between the coagulation cascade, endogenous anticoagulants, and the fibrinolytic system. In highly pro-inflammatory states, such as sepsis, these

processes can become dysregulated through interactions with the host immune and inflammatory apparatus. Platelet activation and endothelial dysfunction activation or damage can also contribute to the development of a systemic prothrombotic state and subsequent DIC. Current practice for the diagnosis of DIC is based on scoring systems, most commonly the one defined by the International Society of Thrombosis and Hemostasis (ISTH) (Taylor, Toh, Hoots, Wada, & Levi, 2001), which incorporates the clinical laboratory parameters platelet count and prothrombin time (PT) or international normalized ratio (INR) as well as the thrombosis markers fibrinogen and D-Dimer. While this diagnostic approach utilizes readily available laboratory assays, it does not provide insight into the molecular aspects of sepsis-associated DIC. Factors including bacteria and bacterial virulence factors, host immune and inflammatory response, endothelial damage or activation, platelet activation, and the interactions of these processes with coagulation, fibrinolysis, and endogenous anticoagulants are all involved in the development of sepsis-associated DIC. **Assessment of biomarkers representative of the numerous processes underlying the development of DIC using plasma samples acquired from septic patients may provide greater insight into the molecular pathogenesis of DIC.** Development of a diagnostic or investigational test panel with parameters representative of the complex pathophysiology of DIC may provide improved diagnostic or prognostic information for patients. Additionally, this work will provide an improved method for the evaluation of animal models used to study DIC and potential therapeutic agents.

Current standard of care for the treatment of sepsis-associated DIC is focused on elimination of the underlying infection through antibiotic administration accompanied by supportive measures such as mechanical ventilation and vasopressor administration. No specific treatments are used for the coagulopathy in DIC. Development of therapies specific for DIC is made difficult by the dual risks for thrombosis and bleeding, both of which may be exacerbated by the administration of any hemostatically active substance.

While traditional anticoagulants are capable of inhibiting thrombosis in DIC patients, these drugs carry a significant risk of severe bleeding in this already susceptible patient population. Conversely, while replacement of depleted platelets and coagulation factors could prevent DIC-associated bleeding, this could also add fuel to the fire of ongoing thrombus formation. Due to the risk of bleeding associated with anticoagulant therapy, targeting of therapeutics to the patients with the greatest potential for benefit from treatment is a priority.

A potential therapeutic approach to preventing thrombosis without causing bleeding in DIC patients is replacement of endogenous anticoagulants, particularly antithrombin (AT) and thrombomodulin (TM), which become depleted during disease. Therapy with both AT (Allingstrup 2016; Gando 2006; Iba 2016; Iba 2012; Kienast 2006; Tagami 2015; Warren 2001) and recombinant TM (Hayakawa 2016; Hoppensteadt 2014; Ito 2015; Moll 2004; Ogawa 2011; Saito 2007; Takazono 2014; Vincent 2013; Yamakawa 2015; Yamakawa 2011; Yamakawa 2013) has been evaluated in clinical trials for this application. In addition to their function as endogenous anticoagulants, both AT and rTM have additional functions that are poorly understood but may be highly

beneficial to DIC patients (Hagiwara 2010; Iba 2014; Iba 2014; Nagato 2009; Nakahara 2013; Shi 2008; Shimomura 2016; Tawara 2016). Despite the risk of bleeding, heparin is also of interest for this application due to both its anticoagulant and non-anticoagulant properties (Ding 2011; do Toit 1991; Jaimes 2009; Kienast 2006; Pernerstorfer 1999; Yang 1994). **An understanding of the antithrombotic, anti-inflammatory, and other mechanisms by which rTM, AT, and heparin may modulate the pathogenesis of sepsis-associated DIC may improve the use of these therapeutic agents, including targeting to the appropriate patient population, as well as lay groundwork for design and testing of future therapeutics for sepsis-associated DIC.**

An improved understanding of the modulation of not only coagulation but also other processes such as inflammation, response to infection, and endothelial and platelet damage or activation, is necessary for optimal understanding, development and implementation of these agents. Assessment of drug impact on these factors through biomarkers selected based on human pathophysiology and validated in an animal model may be an important step in this direction.

**It is the purpose of this dissertation to identify biomarkers representative of multiple aspects of the molecular pathophysiology of DIC, validate the potential relevance of relevant markers through the use of animal models, and assess the response of these factors to treatment with recombinant thrombomodulin, antithrombin, and heparin in order to better understand the mechanism of action of these therapeutic agents.** Investigation of the molecular pathophysiology of sepsis-associated DIC in patient samples with respect to numerous factors involved in disease

development and subsequent validation of these findings in an experimental model will contribute to an improved understanding of the pathogenesis of this disease. Furthermore, this will improve the understanding of the full mechanism of therapeutic agents with potential application in DIC, leading to improved treatment outcomes for sepsis-associated DIC patients.

## Specific Aims

**Specific Aim 1:** To understand the molecular pathogenesis of sepsis-associated DIC by profiling plasma biomarkers of inflammation, infection, endothelial function, and platelet function as well as hemostatic dysregulation and assessing their relevance to disease progression and outcome.

**Aim 1A:** To assess the relationship between INR, aPTT, and coagulation factor levels in patients with sepsis-associated DIC and compare this relationship with that observed in patients receiving warfarin anticoagulation.

**Aim 1B:** To measure a panel of biomarkers in plasma from a cohort of patients with well-defined sepsis and DIC and determine the association of these markers with DIC scores and mortality.

**Aim 1C:** To develop an algorithm based on a combination of biomarkers to predict clinical outcome in patients with sepsis associated DIC.

**Specific Aim 2:** To modify and validate an *in vivo* animal model of sepsis-associated DIC to understand the pathophysiology and pharmacological modulation of this disease process.

**Specific Aim 3**: To assess the effects and mechanism of action of therapeutic modulation on the pathogenesis of sepsis and sepsis-associated DIC.

## Significance

Sepsis associated disseminated intravascular coagulation (DIC) is a severe clinical scenario with high prevalence and high mortality. The pathophysiology of sepsis-associated DIC is complex and involves dysregulation of multiple systems, which is not fully captured by current clinical diagnosis and evaluation protocols. Furthermore, therapeutic options for the treatment of DIC are limited, with no specific treatments currently approved in the United States. A comprehensive approach to the understanding of the molecular pathophysiology of sepsis-associated DIC, its replication in animal models, and its pharmacologic modulation will provide useful information for improved clinical management of this syndrome.

Although DIC is diagnosed on the basis of coagulation dysfunction, this disease involves numerous other processes including inflammation, immunity, platelet and endothelial dysfunction, and dysregulation of endogenous anticoagulants. These factors are not reflected in current assessments of sepsis-associated DIC. By assessing a combination of biomarkers representative of multiple aspects of the pathophysiology of sepsis-associated DIC in a cohort of patients with sepsis and well-characterized coagulopathy, this project will contribute to an improved understanding of this disease process. Additionally, this biomarker profile will also provide a framework for the evaluation of the relevance of animal models and the mechanism of investigational drugs.

Animal models for sepsis are widely used, and development of coagulopathy has been reported in several sepsis model systems. However, this coagulopathy is often poorly defined. Furthermore, the physiological relevance of these models in terms of not only coagulation or inflammation but also endogenous anticoagulants, endothelial damage, and other factors has not been comprehensively evaluated and compared to human patients. By comparing an animal model with a well-defined patient cohort, this project will provide improved validation of animal models for DIC.

Therapeutics for DIC represent a critical unmet medical need. As with all anticoagulant drugs, these therapies carry a risk of severe bleeding, and previous trials of anticoagulant agents in the sepsis population have proven largely unsuccessful, due in part to this elevated bleeding risk. However, analyses of subgroups of patients from larger trials have shown that the benefits of these agents may outweigh the risks in the population of patients with the most severe coagulopathy, suggesting that improved targeting of drugs to patients with specific pathologies may lead to improved outcomes. An improved understanding of the mechanisms of recombinant thrombomodulin, antithrombin, and heparin for the treatment of sepsis-associated DIC will be gained through analysis of the effects of these drugs on the molecular pathophysiology of the disease in a validated animal model. This will contribute to better application of therapy to the most appropriate patients, maximizing benefit while minimizing risk.

## Clinical Implications

The work presented in this dissertation has the potential to contribute to both the diagnosis and the treatment of patients with sepsis-associated DIC.

By examining the association of biomarkers with the severity of coagulopathy, this work may aid in the development of an improved diagnostic approach for DIC. Furthermore, this work includes a novel approach to the development of an algorithm for the prediction of outcome in this patient population. Improved diagnosis and prognostic prediction is important for the appropriate administration of care to these patients.

Currently, drugs for sepsis-associated DIC represent an unmet medical need. This dissertation includes studies on the mechanism of action of rTM, AT, and UFH as drugs with potential use in sepsis-associated DIC. This will contribute not only to an improved understanding of the optimal use of these agents, but also to the development of future treatments for this disease.

The improvement in diagnosis and prognostic prediction and the better understanding of the mechanism of action of potential therapies for sepsis-associated DIC are significant individually, but may have greater significance when combined. Previous clinical studies of treatments for DIC have shown that these potential therapeutic agents are not without risk and may have a favorable risk-benefit profile in some patients but not others. A combination of physiologically relevant approaches to patient identification with a better understanding of the mechanism of action of rTM, AT, and UFH will aid in the optimal administration of patients with the greatest potential to benefit from therapy.

# CHAPTER THREE

# MATERIALS AND METHODS

## Materials

### Coagulation Reagents

**Prothrombin Time (PT) and Fibrinogen.** Recombiplastin (Instrumentation Laboratory, Bedford, MA) was used for the measurement of PT and the calculation of fibrinogen level. This reagent contains lyophilized recombinant human tissue factor and synthetic phospholipids and has an IS value of 1.0.

**Activated Partial Thromboplastin Time (aPTT).** Platelin (Diagnostica Stago, Parsippany NJ) was used as the aPTT reagent. This reagent contains purified phospholipids and micronized silica as an activator. 0.025 M $CaCl_2$ was used to recalcify the citrated plasma.

**Thrombin Time (TT).** Human thrombin (Enzyme Research Laboratories, South Bend, IN) was diluted to 5 U/ml in 0.02 M $CaCl_2$ and was used in the Thrombin Time test.

**Thromboelastography (TEG).** TEG cups and pins were purchased from Haemonetics (Braintree, MA). 0.025 M $CaCl_2$ was used to recalcify samples and initiate clot formation.

## Drugs

**Recombinant Thrombomodulin.** Thrombomodulin is used clinically for the treatment of DIC in Japan and is currently in a global phase III clinical trial for this application. Recombinant human thrombomodulin (rTM or ART-123) was provided by Asahi Kasei Pharma (Tokyo, Japan). rTM was aliquoted at a concentration of 2.5 mg/ml and stored at -80ºC prior to use.

**Antithrombin.** Antithrombin concentrate is used clinically to treat patients with antithrombin deficiency and is approved in Japan for the treatment of patients with DIC. Antithrombin was purchased from Baxter Healthcare Corporation (Deerfield, IL). AT was reconstituted at a concentration of 125 U/ml, aliquoted, and stored at -80ºC prior to use.

**Heparin.** Unfractionated heparin (UFH) is a widely used anticoagulant with many clinical applications, including prevention and treatment of thrombosis and cardiovascular surgery. Heparin sodium for injection (lot 6012617, expiration date 8/2018) was purchased from Fresenius Kabi (Lake Zurich, IL). UFH was acquired in a 10 ml vial and stored at room temperature prior to use. The stock concentration of heparin was 1,000 U/ml, and heparin was diluted in saline to a concentration of 200 U/ml prior to injection.

## Plasma Samples

**Factor Deficient Plasmas.** Human plasma deficient in specific coagulation factors or associated proteins is commonly used as a reagent for specialized functional

coagulation testing. These plasmas are commercially available in lyophilized form. Lyophilized protein C deficient plasma was purchased from Diagnostica Stago (Parsippany, NJ). Lyophilized plasmas deficient in Factor VII, Factor IX, and Factor X, were purchased from Aniara (Westchester, Ohio). Plasmas were reconstituted according to the manufacturer's instruction for use in functional coagulation testing.

**Whole Blood.** Whole blood was drawn from apparently healthy volunteer donors under an IRB approved protocol (LU# 9191051098). Blood was drawn using standard phlebotomy technique into tubes containing 3.2% sodium citrate. All donors provided informed consent and a maximum of 40 ml of blood was drawn from each donor.

**Normal Human Plasma.** Pooled normal human plasma for use in coagulation assays was purchased from George King Biomedical (Overland, KS). Each pool contained citrated plasma from 30 or more donors and was certified to return normal values on standard coagulation tests including PT, aPTT, and fibrinogen and to have levels of coagulation Factors II, V, VII, VIII, IX, X, XI, and XII within the normal range. Plasma was aliquoted and stored at -80ºC prior to use.

**Individual Patient Samples.** Samples from individual patients or healthy volunteers were collected as described below.

***Individual healthy controls.*** Frozen, citrated plasma samples from apparently healthy individuals were purchased from George King Biomedical (Overland KS). These samples were drawn from 25 male and 25 female volunteers, ages 19-54, with a mean

age of 32. All volunteers were non-smokers, non-medicated, and of geographically diverse origins. Plasma was aliquoted and stored at -80ºC prior to use.

***De-identified patient samples.*** De-identified plasma samples were collected from the clinical laboratory at Loyola University Medical Center under an IRB approved protocol (LU #9192052016). Samples were collected from among specimens ready for discard and no modification was made to patient care due to this sample collection. Limited information was available to accompany each specimen including diagnosis and treatment.

De-identified, citrated plasma samples were collected from patients in the initial phase of warfarin therapy (n = 100) and from patients with diagnosed sepsis and suspected DIC (n = 78) using this protocol. Plasma was aliquoted and stored at -80ºC prior to use.

***Utah sepsis cohort plasma samples.*** Plasma samples from adult patients with sepsis and suspected DIC were collected between 2008 and 2012 under an IRB-approved protocol by Matthew Rondina, MD at the University of Utah Medical Center as described in the literature (Rondina 2011; Rondina 2012; Rondina 2015). Samples were collected from adult patients in the intensive care unit (ICU) at the University of Utah Hospital or an associated community hospital at ICU admission as well as on ICU days 4 and 8 for patients remaining in the ICU at those times. Sample collection was approved by the Internal Review Board (IRB) at the University of Utah (IRB_0029495), and all patients enrolled in the study provided informed consent.

In order to qualify for enrollment in this study, patients were required to meet the criteria for SIRS and have an identified focus of infection. SIRS was defined as the presence of 2 or more of the following: (1) temperature $< 36$ºC or $> 38$ºC, (2) heart rate $>$ 90 beats per minute, (3) respiratory rate $> 20$ breaths per minute or $PaCO_2 < 32$ mmHg, (4) white blood cell count $\geq 12{,}000$ or $\leq 4{,}000$ cells/$mm^3$ or $> 10\%$ bands.

Patients were excluded from the study if they had received a blood transfusion within the past 4 months, platelet transfusion within the past 14 days, or platelet count of less than 20 K/µl. Patients were also excluded from this study if they had a pre-existing disorder affecting platelet number or function, including idiopathic thrombocytopenic purpura, thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, end-stage liver disease, myeloproliferative disorders, multiple myeloma, Waldenstrom's macroglobulinemia, end-stage renal disease requiring hemodialysis, or inherited platelet disorders such as Bernard-Soulier syndrome, gray platelet syndrome, May-Hegglin anomaly, Wiskott-Aldrich syndrome, Glanzmann thrombasthenia, Chediak-Higashi syndrome, Hermansky-Pudlak syndrome, or thrombocytopenia-absent radius syndrome.

Blood was collected into 3.2% sodium citrate and centrifuged to prepare platelet poor plasma. Plasma was collected, aliquoted, and stored at -80ºC prior to analysis. Transfer of samples and accompanying de-identified clinical information to Loyola was approved by the Loyola University Chicago IRB (LU Number 207958). Samples were shipped to Loyola University Chicago on dry ice and stored at -80ºC prior to analysis.

Baseline (day 0) samples and accompanying data were available from 103 patients. 57 patients had day 4 samples and data available and 30 had day 8 samples and data available.

## Assays for Biomarker Analysis

**Assays for Human Proteins.** Commercially available enzyme linked immunosorbent assays (ELISAs) were used to quantitate the levels of proteins in human plasma samples. All assays were specific for human proteins and were performed according to the manufacturer's instructions. Assays used and their manufacturers are listed in Table 5.

**Table 5: Sources of Assay Kits for Human Plasma Proteins**

| Marker | Manufacturer |
|---|---|
| D-Dimer<br>Microparticle Tissue Factor (MP-TF)<br>Microparticles (MP)<br>Platelet Factor 4 (PF4)<br>von Willebrand Factor (vWF)<br>Tissue Factor (TF)<br>Factor VII (Zymutest)<br>Factor IX (Zymutest)<br>Factor X (Zymutest) | Hyphen BioMed<br>(Neuville-Sur-Oise, France) |
| PAI-1<br>Tissue Factor Pathway Inhibitor (TFPI) | Stago Asserachrom<br>(Asnieres-Sur-Sene, France) |
| CD40L<br>Angiopoietin 2 (Ang-2) | R&D Systems<br>(Minneapolis, MN) |
| Endocan | Lunginnov<br>(Lille, France) |
| High Mobility Group Box 1 Protein (HMGB-1) | LifeSpan BioSciences<br>(Seattle, WA) |
| Nucleosomes (Cell Death Assay) | Roche Diagnostics<br>(Indianapolis, IN) |
| Procalcitonin | Abcam<br>(Cambridge, United Kingdom) |
| Prothrombin Fragment F1.2 | Dade Behring-Siemens<br>(Erlangen, Germany) |
| IL-2<br>IL-4<br>IL-6<br>IL-8<br>IL-10<br>VEGF<br>IFNγ<br>TNFα<br>IL-1α<br>IL-1β<br>MCP-1<br>EGF | Randox<br>(Antrim, UK)<br>Cytokine High Sensitivity Assay Kit |

**Assays for Rat Proteins.** Commercially available ELISAs specific for rat proteins were used to quantitate protein levels in rat plasma. Assays and their manufacturers are listed in Table 6.

**Table 6. Sources of ELISA Kits for Rat Plasma Proteins**

| Assay | Manufacturer |
|---|---|
| Rat Histone H3 ELISA Kit (Sandwich ELISA)<br>Rat Procalcitonin ELISA Kit | LifeSpan BioSciences<br>(Seattle, WA) |
| Rat IL-10 Quantikine ELISA Kit | R&D Systems<br>(Minneapolis, MN) |
| ZYMUTEST Rat PAI-1 Antigen | Hyphen BioMed<br>(Neuville-Sur-Oise, France) |
| Rat IL-6 ELISA | Abcam<br>(Cambridge, United Kingdom) |
| Nucleosomes (Cell Death Assay) | Roche Diagnostics<br>(Indianapolis, IN) |

**Instruments**

**ACL-Elite.** An ACL-ELITE coagulation analyzer (Instrumentation Laboratories, Bedford, MA) was used for standardized clinical coagulation tests including PT, aPTT, and fibrinogen measurements. This instrument uses an optical method to detect clot formation in a plasma sample. The required materials for use of this instrument, including rotors, sample cups, reference emulsion, and cleaning solution, were also purchased from Instrumentation Laboratories.

**ST-4.** An ST-4 Coagulation Analyzer (Diagnostica Stago, Parsippany, NJ) was used for specialized coagulation tests, including determination of Protein C, Factor VII, Factor IX, and Factor X activity levels and measurement of PT and aPTT in whole blood or rat plasma. This instrument uses a mechanical method to evaluate clot formation in a

plasma or whole blood sample. The required materials for use of this instrument, including cuvettes and stir balls, were also purchased from Diagnostica Stago

**SpectraMax Plus.** A SpectraMax Plus Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA), which was used in conjunction with SoftMax Pro software for measurement of optical density for ELISA assays. A Randox Evidence Investigator (Randox, London, UK), was used for biochip analysis.

## Animals

Male Sprague-Dawley rats (275-500g, Charles River Laboratories, Wilmington, MA) were used in the rat cecal ligation and puncture (CLP) model of sepsis. Prior to participation in experiments, rats were allowed an acclimation period of at least 72 hours following arrival into the animal care facility. Rats were pair housed using standard rodent husbandry procedures in the Comparative Medicine Facility (CMF) at Loyola University Medical Center. Rats received unrestricted access to water and a standard rodent diet.

Studies were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) (IACUC #2017009, LU #209143, 175 rats approved for use). All animal studies were carried out in compliance with the guidelines set forth by the IACUC at the Loyola University Medical Center and the *Principles of Laboratory Animal Care* (National Institutes of Health, 1985).

## Methods

### Global Clotting Assays

**Prothrombin Time (PT).** Prothrombin time (PT) is a commonly performed clinical laboratory test which measures the overall functionality of factors involved in the extrinsic pathway of coagulation, particularly Factor VIIa and Factor Xa. PT is based on the time to clot after recalcified plasma has been activated by tissue factor. PT is often reported as International Normalized Ratio (INR), which relates the patient's PT to the standard PT measured using a given laboratory's specific instrument and reagent. This test is commonly used clinically to monitor warfarin therapy as well as to assess the global function of the coagulation cascade.

In human plasma, PT was measured using standard operating protocols on an ACL-ELITE coagulation analyzer (Instrumentation Laboratory, Bedford, MA). Recombiplastin (Instrumentation Laboratory, Bedford, MA) was used as the PT reagent. This instrument uses an automated optical method to detect clot formation in a plasma sample. The maximal clotting time detectable using this instrument was 300 seconds. This instrument performed automated calculation of INR.

In whole blood and rat plasma, PT was measured using standard operating procedures on an ST-4 Coagulation Analyzer (Diagnostica Stago, Parsippany, NJ). This device uses a mechanical method to determine the time required for clot formation. 100µl of whole blood or 50 µl of plasma was warmed to 37º in a cuvette with a metal mixing ball for 100 seconds. 100 µl of Dade Innovin PT reagent (Siemens Healthcare Diagnostics, Newark, DE) was added and the time to clot development was recorded.

**Activated Partial Thromboplastin Time (aPTT).** The activated partial thromboplastin time (aPTT) is a standard clinical laboratory test that assesses the function of the intrinsic pathway of the coagulation cascade (Factors I, II, V, VIII, IX, X, XI and XII). aPTT is based on the time to clot after recalcified plasma has been activated by a platelet substitute consisting of purified phospholipids as well as a micronized silica activator. This test is commonly used to monitor heparin therapy as well as to evaluate global function of the coagulation cascade.

In human plasma, aPTT was measured using standard operating protocols on an ACL-ELITE coagulation analyzer (Instrumentation Laboratory, Bedford, MA). This instrument uses an optical method to detect clot formation in a plasma sample. Either Platelin (Stago, Parsippany, NJ) or Triniclot (Diagnostica Stago, Parsippany, NJ) was used as the aPTT reagent along with 0.025 M $CaCl_2$ in order to recalcify the citrated plasma.

In whole blood, aPTT was measured using standard operating procedures on an ST-4 Coagulation Analyzer (Diagnostica Stago, Parsippany, NJ). This device uses a mechanical method to determine the time required for clot formation. For aPTT, 50 µl of sample was incubated with 50µl of Triniclot aPTT reagent (Diagnostica Stago, Parsippany, NJ) for 300 seconds at 37ºC. 50µl of $CaCl_2$ was added to initiate coagulation and the time to clot formation was recorded.

**Thrombin Time (TT).** Thrombin (TT) is a clot based assay in which the time required for the conversion of fibrinogen to fibrin following the addition of a known amount of thrombin to plasma is recorded.

In human plasma, TT was measured using standard operating protocols on an ACL-ELITE coagulation analyzer (Instrumentation Laboratory, Bedford, MA). This instrument uses an optical method to detect clot formation in a plasma sample. Thrombin was used at a concentration of 5 U/ml diluted in 0.02 M $CaCl_2$.

In whole blood, TT was measured using standard operating procedures on an ST-4 Coagulation Analyzer (Diagnostica Stago, Parsippany, NJ), which uses a mechanical method to determine the time required for clot formation. 100µl of whole blood was incubated with a metal stir bar for 100 seconds at 37ºC. 50µl of thrombin at 200 U/ml diluted in 0.02 M $CaCl_2$ was then added and the time until clot formation recorded.

**Thromboelastography (TEG).** Thromboelastography (TEG) is used to evaluate coagulation in whole blood. In addition to assessing coagulation function, TEG assesses platelet function, clot strength, and fibrinolysis. Whole blood is placed into a cup that rotates slowly around a sensor pin, around which a clot forms. Multiple parameters describing clot formation are recorded. The reaction time (R time) represents the time from the initiation of the test until clot formation is first detected. The K time is representative of the speed of clot formation and is the time between the first detectable clot formation (R time) and until the tracing reaches a size of 20 mm. The angle, which is the tangent of the curve at the K time, provides similar information to the K time. The MA is the maximal amplitude, which is a measure of clot strength.

Thromboelastography was performed on whole blood using a TEG 5000 system. 304µl citrated blood and 36µl of drug was added to each sample cup. 20µl of 0.02M

$CaCl_2$ was then be added to recalcify the sample and initiate clotting. R time, K time, maximum amplitude (MA), and angle were recorded.

**Fibrinogen.** In human plasma, fibrinogen concentration was measured using standard operating protocols on an ACL-ELITE coagulation analyzer (Instrumentation Laboratory, Bedford, MA). This method computes a derived fibrinogen concentration based on the prothrombin time utilizing a calibration curve created from standards with known fibrinogen concentrations. Recombiplastin (Instrumentation Laboratory, Bedford, MA) was used as the PT reagent.

**Coagulation Factor Activity Levels.** Functional levels of individual coagulation factors were measured in plasma samples using an ST-4 coagulation analyzer as described below.

***Factor VII activity.*** Factor VII activity level was measured in plasma samples using a modified one-step PT assay. Clot formation in this test was evaluated mechanically using an ST4 Coagulation Analyzer (Diagnostica Stago, Parsippany, NJ).

Patient samples were diluted 1:10 in Owren's Veronal Buffer. 50µl of Factor VII deficient plasma (Aniara, Westchester, OH) and 50µl of diluted patient plasma sample were warmed to 37ºC in a cuvette with a metal mixing ball for 180 seconds. 100µl of Dade Innovin PT reagent (Siemens Healthcare Diagnostics, Newark, DE) was added and the time to clot development was recorded. Factor VII level was calculated in each sample relative to normal human plasma based on a standard curve.

***Factors IX and X Activity.*** Factor IX and X activity levels were measured in plasma samples using a modified one-step aPTT assay. Clot formation in this test was

evaluated mechanically using an ST4 Coagulation Analyzer (Diagnostica Stago, Parsippany, NJ).

Patient samples were diluted 1:20 in Owren's Veronal Buffer. 50µl of diluted sample, 50µl of aPTT reagent, and 50µl of Factor IX or X deficient plasma (Aniara, Westchester, OH) were warmed to 37ºC in a cuvette with a metal mixing ball for 5 minutes. 50µl of $CaCl_2$ was added and the time to clot development was recorded. Factor IX and X levels were calculated in each sample relative to normal human plasma using a standard curve.

***Protein C Activity.*** Functional levels of Protein C were measured using a clot-based assay performed using an ST4 coagulation analyzer (STACLOT, Diagnostica Stago, Parsippany, NJ). Patient and control plasmas were diluted 1:10 in Owren Koller Buffer. 50 µl of diluted sample, 50 µl of Protein C deficient plasma (Diagnostica Stago, Parsipanny, NJ) and 50 µl of Protein C activator (Diagnostica Stago, Parsipanny, NJ) were incubated in a sample cuvette with a metal mixing ball for 180 seconds at 37ºC. 50µl of 0.2 M $CaCl_2$ was added to each sample, initiating the clotting reaction. Time to clot formation was recorded as the time at which the metal ball was prevented from moving.

Protein C level, measured as percent of normal value, was calculated from the time to clot for each sample based on a standard curve. The standard curve consisted of dilutions of normal human pooled plasma at 100%, 75%, 50%, 25%, 12.5%, and 0%, diluted 1:10 in Owren Koller buffer. Clotting time had an inverse relationship with Protein C activity level.

**Biomarker Analysis**

**ELISA Assays.** Biomarker levels were measure in human and rat plasma samples using the commercially available enzyme linked immunosorbent assay (ELISA) kits listed in Table performed according to the manufacturer's instructions. An illustration of the general principle of an ELISA is shown in Figure 7.

Although the specific protocol varies for each assay, all ELISAs share a common principle. Each assay kit included a 96-well microtiter plate coated with an antibody specific to the desired analyte. Appropriately diluted sample was incubated in the plate and the analyte bound to the plate-bound antibody. The plate was washed to remove nonspecifically bound proteins. A secondary antibody against a distinct epitope on the analyte was then added. This secondary antibody was conjugated to an enzyme. Following additional washing, a substrate was added to the plate, producing a color change proportional to the amount of bound enzyme. The color change reaction was stopped through the addition of an acidic solution. Precise timing was maintained between the addition of the substrate and the addition of the stop solution using a stopwatch to ensure accuracy. Optical density at the specified wavelength was measured using a spectrophotometer and SoftMaxPro software (Molecular Devices, Sunnyvale, CA).

Each ELISA plate included a standard curve run in duplicate, generated using standards of known concentration included in the assay kit. This was used to generate a calibration curve relating measured optical density (OD) values to protein concentration using a linear equation. This curve was used to calculate the protein concentration in each

sample or control. In addition to internal controls included in each kit, pooled normal human plasma (NHP) and pooled pathological human plasma samples were included on each plate to monitor inter-assay variation. Patient and control samples were run in singlicate. Samples with an optical density exceeding that of the highest calibration point were diluted and rerun. Samples with an optical density value resulting in a calculated analyte concentration of less than zero were recorded as having a concentration of 0.

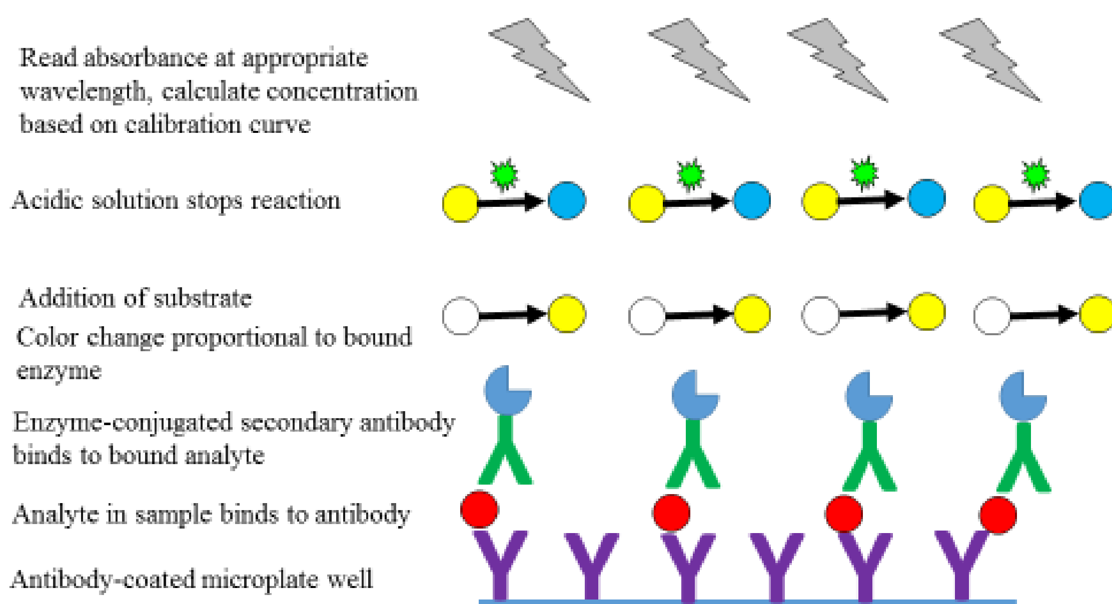


**Figure 7. Overview of the Principle of an ELISA Assay**. A plasma sample is added to an antibody-coated microplate well. The analyte in the sample then binds to the antibody, and an enzyme-conjugated secondary antibody binds to the bound analyte. The addition of a substrate leads to color change proportional to the amount of bound enzyme (shown as a change from white to yellow). The addition of an acidic solution stops this reaction (shown as a color change from yellow to blue). The absorbance is then red at the appropriate wavelength and the concentration of protein of interest is calculated based on calibration curve.

**Randox Biochip.** A Randox Cytokine and Growth Factors High-Sensitivity Array assay kit was used to quantify IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFNγ, TNFα IL-α, IL-1β MCP-1, and EGF (Randox, London, UK). This allowed quantification of all factors in a single patient sample simultaneously using a sandwich chemiluminescent immunoassay.

Each biochip provided in the kit contained 12 test regions, each with a different immobilized antibody specific to a different cytokine. The chip was incubated with 100µl of plasma sample. After washing, conjugate consisting of horse radish peroxidase-labeled, analyte-specific antibody was incubated with the chip. Increased level of a bound cytokine caused increased binding of conjugate and thus increased chemiluminescent signal emitted upon activation of the signal reagent. The luminescent signal generated in each region of the biochip was translated into analyte concentration by the Randox Evidence Investigator using a calibration curve generated based on controls of known concentration.

**Animal Models**

**Cecal Ligation and Puncture Model.** Cecal ligation and puncture (CLP) was performed in rats to model sepsis and associated DIC. This is useful for studies of treatments for sepsis and DIC as it creates a polymicrobial infection with significant inflammation, similar to many clinically observed scenarios. The severity of disease achieved through use of this model can be modified by altering the amount of the cecum ligated, the size and number of punctures, and the amount of fluid resuscitation given. Two variants of the CLP model were approved for use by the IACUC, one for the

production of moderate disease and on for the production of mild disease. Due to the success of the moderate disease protocol, the mild disease approach was not used.

The CLP protocol used in these studies was based on that published by Rittirsch et. al (Rittirsch 2008) and was similar to that used and described by others (Cuenca 2010; Heuer 2004; Heur 2004; Hubbard 2005; Inoue 1991; Kim 2000; Laudes 2002; Otero-Anton 2001; Qiu 2001; Ravindranath 2007; Rittirsch 2007; Rittirsch 2008; Schabbauer, 2012; Yang1994). Critical steps in the CLP procedure are shown in Figure 8. Male Sprague-Dawley rats were anesthetized by administration of 2-3% isoflurane. Anesthesia was initiated by placing rats in an anesthesia induction chamber and maintained throughout pre-operative procedures and surgery through the use of an individual nose cone. Once the rat was fully anesthetized, protective eye drops were applied, the rat was positioned on its back, the abdomen was shaved, and the skin was cleansed with alternating betadine and alcohol wipes (Figure 8a). SR-Buprenorphine was administered subcutaneously at a dose of 1 mg/kg in order to ensure adequate analgesia. Rats were kept on a heating pad or under a heat lamp to maintain appropriate body temperature during pre-surgical procedures, surgery, and during recovery. All surgical equipment was autoclaved prior to use. A separate surgical pack, sterile gloves, and sterile drape were used for each rat.

To perform the CLP procedure, a midline incision was made through the skin and muscle layers (Figure 8b) and the cecum exposed (Figure 8c). Moderate disease was produced by ligation of 50% of the cecum with a 2-0 silk suture (Figure 8d). The proximal portion of the cecum was returned to the abdomen and a single through-and-

through puncture with an 18 gauge needle was made through at the midpoint of the distal ligated portion of the cecum (Figure 8e). Appropriate anatomical landmarks were noted for each rat to ensure model consistency. Following puncture, a small droplet of feces was extruded from each puncture and the cecum was returned to the abdomen. For animals undergoing sham surgery, an incision through the skin and muscle layers was made, but no ligation or puncture of the cecum was performed. The peritoneum was closed using a 5-0 Vicryl monofilament suture and a simple interrupted stitch (Figure 8f) and the skin was closed using wound clips (Figure 8g).

Following surgery, rats were kept under a heating lamp and monitored during recovery from anesthesia. Rats also received 8ml saline each via subcutaneous injection. Appropriate analgesia was maintained post surgically by subcutaneous injection with SR-Buprenorphine at a dose of 1 mg/kg every 24 hours.

Rats were followed for up to 3 days (72 hours) following CLP procedure. Symptoms of sepsis manifested less than 24 hours following CLP. During the 72 hours post CLP, drugs were administered according to the experimental protocol. Blood was collected via cardiac puncture and rats were euthanized 72 hours following surgery.

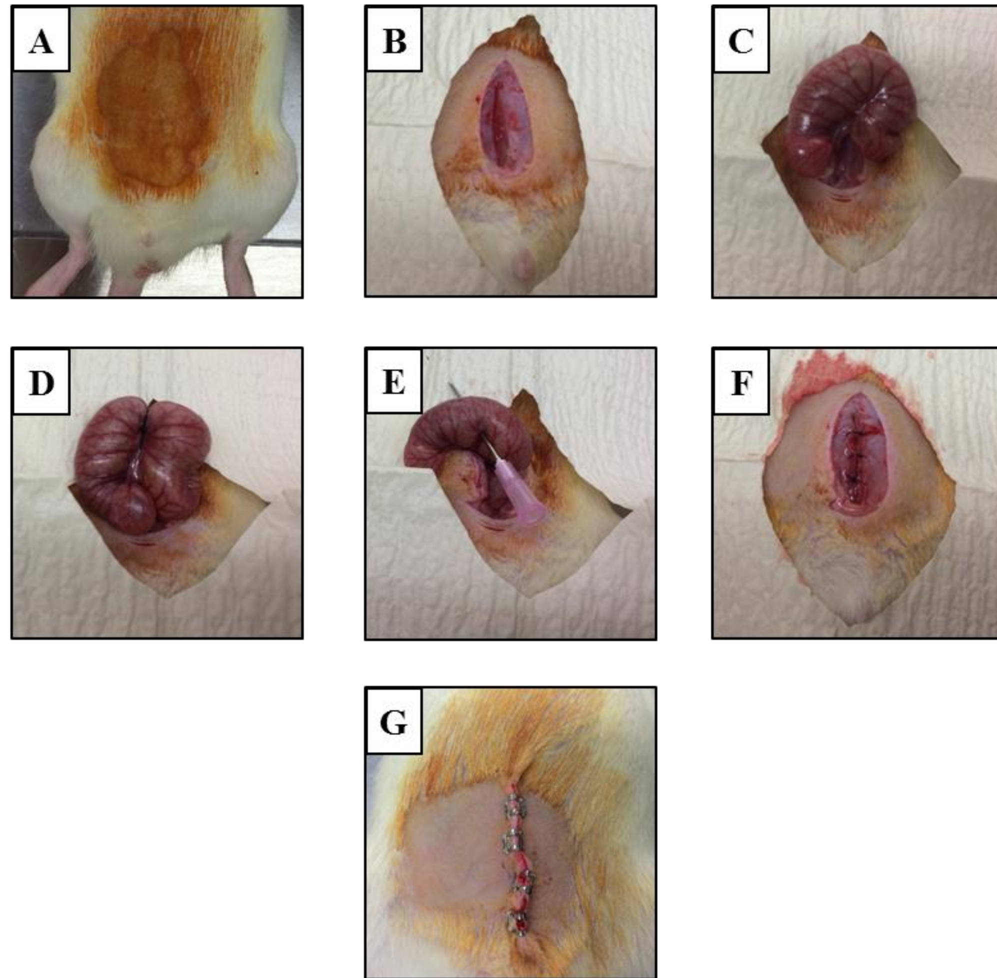


**Figure 8. Images of Critical Steps in the CLP Procedure**. (a) shaving and disinfection of the abdomen (b) midline incision through the skin and muscle layers (c) exteriorization of the cecum (d) ligation of 50% of the cecum (e) single through-and-through puncture of the cecum with an 18-gauge needle (f) closure of the muscle layer with simple interrupted sutures (g) closure of the skin with wound clips

**Drug Administration.** Drugs were administered to rats following induction of sepsis using the CLP procedure. Although drugs are often administered at the time of CLP in research settings, this does not best replicate a clinical scenario in which drugs are administered to treat disease that has already developed. An alternative approach, and the approach that was selected for these studies, is the administration of drug 24 hours

following surgery(Li 2007 ; Uolla 2002; Wang 2004; Yang 2004; Yin 2005), which allows adequate time for the development of sepsis.

24 hours following CLP surgery, rTM, AT, UFH, or saline were administered to septic rats intravenously via tail vein injection at clinically relevant doses, with an additional dose of drug administered 24 hours later. Rats were euthanized and blood was collected 72 hours following CLP. The experimental protocol is schematized in Figure 9 and drug doses and experimental groups are show in Table 7.

rTM is administered clinically at a dose of 60 μg/kg/day (Ogawa 2011; Saito 2007; Vincent 2013; Yamakawa 2015; Yamakawa 2011; Yamakawa 2013) (0.06 mg/kg/day). However, the IC50 value of rTM on thrombin generation is much higher in rat plasma than in human plasma, and significantly higher doses of rTM must be used in rats. 1mg/kg is a commonly used dose of rTM in rat studies (Aoki 1994; Aoki 1994; Gonda 1993; Hagiwara 2010; Iba 2013; Mohri 1994; Nagato 2009) and was the dose selected for use in this protocol.

AT is typically administered to sepsis and DIC patients at doses of 3000 IU/day or 1500 IU/day (Allingstrup 2016; Iba 2016; Iba 2012). Assuming a 70 kg “standard man”, this corresponds to a dose of 43 IU/kg/day or 21 IU/kg/day, with lower doses achieved in heavier patients. In these studies, AT was administered to rats with CLP-induced DIC at a dose of 50 IU/kg to model the effects of AT at clinically achievable levels.

Heparin is used clinically at a wide range of doses depending on the clinical scenario. Accordingly, the doses of heparin used in clinical trials and in animal models are highly variable(Li 2011). Dosages vary further depending on the type of heparin used

and the route of administration. For these studies, UFH was administered to rats at a dose of 25 IU/kg in order to achieve an effect with minimal risk of bleeding.

**Table 7. Experimental Groups for Rat CLP and Drug Administration**

| Procedure | Agent | Dose | N |
|---|---|---|---|
| None (control) | N/A | N/A | 10 |
| CLP | N/A | N/A | 22 |
| Sham | N/A | N/A | 6 |
| CLP | rTM | 1 mg/kg | 8 |
| CLP | Antithrombin | 50 IU/kg | 5 |
| CLP | Antithrombin | 125 IU/kg | 9 |
| CLP | Heparin | 70 IU/kg | 5 |
| CLP | Heparin | 25 U/kg | 10 |

The untreated CLP group includes rats that died within 24 hours of surgery and therefore did not receive drug treatment.

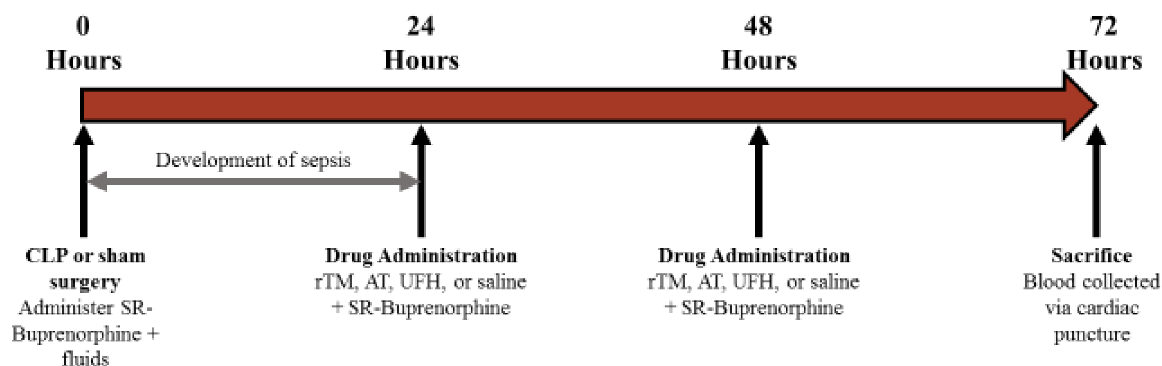


**Figure 9. Schematic of Experimental Protocol for Rat CLP and Drug Administration.** Drugs and analgesics are administered 24 and 48 hours following surgery, allowing time for the development of sepsis prior to drug administration. All animals were euthanized 72 hours following the surgical procedure, at which time blood was collected via cardiac puncture.

**Blood Collection via Cardiac Puncture.** At the time of sacrifice, rats were anesthetized via intraperitoneal administration of 90 mg/kg ketamine. Additional ketamine was administered as required to achieve complete anesthesia, with an average of 123 mg/kg total ketamine administration. Once anesthesia was confirmed, blood was collected via cardiac puncture. A needle was inserted immediately below the xiphoid process and advanced into the chambers of the heart. 5 ml of blood was collected into a syringe and placed immediately into siliconized glass tubes containing 3.8% sodium citrate at a ratio of 1 part citrate to 9 parts whole blood. Tubes were mixed well to prevent clotting. Rats were euthanized by intracardiac administration of 0.5 ml of Beuthanasia-D (390 mg/ml pentobarbital + 50 mg/ml phenytoin).

Samples were centrifuged at 3000g for 20 minutes, and plasma was aliquoted and frozen at -80ºC until analysis. Approximately 2-3 ml of plasma per rat was collected. PT was measured in each sample to assess the quality of blood draw. A normal rat PT is approximately 8-10 seconds.

**Platelet Counts in Rat Blood.** Platelets in whole blood collected from rats were counted manually using a hemocytometer. Using the capillary pipette provided in the LeukoCheck system, (Biomedical Polymers Inc., Gardner, MA), 20µl of whole blood was transferred into the reservoir containing red blood cell lysis buffer and allowed to stand for 20 minutes to allow complete lysis of erythrocytes. The lysed and diluted blood was then loaded into both sides of a Neubauer Bright-Line Hemocytometer and allowed to stand for 10 minutes. Platelets were counted at 40x magnification. Platelets were counted in 5 small squares and this number was multiplied by 5 to calculate the total

platelet count in whole blood in units of K/μl. Platelet counts were performed in duplicate and the average value was recorded.

## Data Processing and Statistical Analysis

**Stepwise Linear Regression Modeling.** Stepwise linear regression modeling was performed using MATLAB software (Mathworks, Natick, MA). Stepwise linear regression is a mathematical modeling approach in which a linear equation incorporating relevant predictor variables (i.e. biomarker levels) to predict the value of an output variable is developed using an iterative process to predict the value of a response variable (i.e. mortality) incorporating only data that significantly alters the model fit.

Two main approaches are possible for stepwise linear regression modeling. Using forward selection, also known as a constant model starting assumption, the initial model incorporates no predictor variables. In each iteration of the model, the variable that yields the greatest statistically significant improvement in model fit by its addition is added to the model. This process is repeated until no variable remains that improves model fit when added. Alternatively, backwards elimination, also known as a linear model starting assumption, can be used. Using this approach, all predictor variables are initially included in the model. With each model iteration, the variable that yields the least significant change to the model fit when removed from the model is removed. This process is repeated until no variables remain that do not statistically significantly worsen model fit when removed

MATLAB code is shown in Appendix D. Data tables including mortality as the response variable and biomarker levels as the predictor values were imported into

MATLAB from Microsoft Excel. Models were developed using the “stepwiselm” function. Both linear and constant model starting assumptions were used, as specified in the results. Model coefficients were recorded and model output value for each patient was calculated from the appropriate biomarker levels using Microsoft Excel. Model fit was evaluated using ROC analysis in GraphPad Prism using the area under the curve (AUC) as the descriptor of model quality.

**Statistical Analysis.** The experiments described in this dissertation represent multiple techniques, and appropriate data collection and statistical analysis were performed for each experiment. Data is presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) as specified throughout this document. $P < 0.05$ was used as the cutoff for statistical significance, and computed p values are present throughout this document. Results were tabulated and stored using Microsoft Excel (Microsoft Corporation, Redmond, WA). Statistical analysis was performed and graphs were generated using GraphPad Prism (GraphPad Inc., La Jolla, Ca).

Biomarker levels in patient populations are presented as mean ± SEM. Nonparametric statistical tests were used throughout as these tests are more appropriate for analysis of data sets with high variability than traditional parametric tests. Differences in biomarker levels between two patient groups (i.e. survivors and non-survivors) were analyzed using the Mann-Whitney test. Comparisons between three or more groups (i.e. sepsis + no DIC, sepsis + non-overt DIC, and sepsis + overt DIC) were analyzed using the Kruskal-Wallis one way analysis of variance (ANOVA) followed by Dunn’s multiple comparison test. Correlations between factors were analyzed using the Spearman

correlation coefficient. Predictive values were analyzed using receiver operator curve (ROC) analysis, with the main output for this being the area under the curve (AUC). When appropriate, the Chi Square test was also used.

# CHAPTER FOUR

# RESULTS

## Coagulation Profiling in DIC vs. Warfarin Treated Patients

INR is one of the key parameters used clinically to identify patients with sepsis-associated coagulopathy. In many settings, full diagnostic evaluation for DIC in septic patients is not performed, and patients are instead screened for coagulopathy on the basis of elevated INR and reduced platelet count. Accordingly, it is important to understand the appropriate interpretation of INR in this specific patient population. Prolonged PT or elevated INR is generally indicative of a hypocoagulable state; however, patients presenting with sepsis-associated DIC and an elevated INR are at risk of complications due to both thrombosis and bleeding.

Elevated PT or INR is often reported in 90% or more of sepsis patients with severe disease (Collins 2006; Kinasewitz 2004; Koami 2015). Prolonged PT and elevated INR are associated with increased mortality and poor clinical outcome in sepsis patients (Dhainaut 2005; Kinasewitz 2004) as well as in other critically ill or injured patient populations (MacLeod 2003; Walsh 2010). Elevated PT/INR, typically defined as INR ≥1.2, is often an inclusion criterion for clinical trials in patients with sepsis and coagulopathy (Abraham 2003; Vincent 2013). The majority of the elevated INRs within this patient population have been reported to fall into the range of 1.6 to 2.5

(Walsh 2010). Other changes in global coagulation parameters, including aPTT (Bakhtiari 2004; Collins 2006; Daudel 2009; Johansson 2010; Kinasewitz 2004; Koami 2015) and whole blood clotting ability as measured by thromboelastography (Daudel 2009; Johansson 2010; Koami 2015), are also often reported in sepsis patients as well as in other critically ill patient populations.

Despite the clear evidence that significant changes to the overall coagulation profile occur in sepsis, changes in the levels of individual coagulation factors in sepsis-associated DIC patients are less well established. Reduced levels of coagulation factors including factors II, V, VII, X, and XII compared to normal individuals have been reported in sepsis-associated DIC (Collins 2006). However, these results demonstrated no discernible relationship to standard coagulation tests and are highly variable between studies (Collins 2006; Daudel 2009; Johansson 2010).

PT/INR was designed to monitor the anticoagulation status in patients treated with warfarin and is widely used clinically for this purpose. Warfarin treated patients are typically considered appropriately anticoagulated with an INR of between 2 and 3, and regular adjustments to drug dosage are made to maintain the INR within this range. A study of the relationship of serial INR levels to severe bleeding in patients receiving warfarin anticoagulation found that warfarin patients hospitalized with severe bleeding showed an elevated INR compared to non-bleeding patients ($5.9 \pm 5.9$ vs. $2.3 \pm 0.7$) as well as higher INRs before the event of the bleed ($3.0 \pm 1.2$ vs. $2.1 \pm 0.8$) (Kucher 2004)

The difference in INR levels at which bleeding occurs in warfarin treated and sepsis-associated DIC patients as well as the fact that DIC patients with an elevated INR

indicative of hypocoagulability experience both thrombotic and bleeding complications suggests that the information provided by this common laboratory test may be significantly different in these two patient populations. The purpose of this study was to compare the relationship of laboratory coagulation tests and levels of individual coagulation factors with INR in patients with SAC to the relationships observed in warfarin treated patients.

Citrated, de-identified plasma samples were collected from the clinical laboratory under an IRB approved protocol. Samples were collected from among specimens ready for discard and no modification was made to patient care due to this sample collection. Limited information regarding diagnosis and treatment was available to accompany each sample.

Samples were collected from patients receiving warfarin anticoagulation (n=132) and patients with sepsis-associated DIC, defined as overt or non-overt DIC by the ISTH criteria (n=78). Frozen, citrated plasma samples from healthy individuals, ages 18-55, nonsmokers, with no known medical conditions, were purchased from George King Biomedical (Overland, KS) and stored at -80ºC prior to analysis.

PT, aPTT, and fibrinogen were measured in these samples using previously described methods. Additionally, protein and functional levels of coagulation factors VII, IX, and X were measured as described in the Materials and Methods section.

Statistical analysis was performed using GraphPad Prism software (La Jolla, CA). Patients were categorized into groups based on INR levels, with groups for INR of <1.5, 1.5-1.9, 2-2.9, ≥3. For both patient populations, variability of other factors based on INR

was assessed using the Kruskal-Wallis one-way ANOVA and Dunn's Multiple Comparison Test with $p<0.05$ as the cutoff for significance. Plots of test result or factor level versus INR were also created form both patient groups and trendline fit was assessed. Spearman correlation coefficients were determined for relationships between coagulation factor levels and coagulation test results for both patient groups.

**PT/INR Profiling**

Warfarin treated patients were screened for inclusion in this study on the basis of INR measurement to identify only patients compliant with warfarin therapy. Patients with an INR of ≥1.2 were included in the study (n=130). The INR range in this population was 1.2-6.6 with a mean value of 2.1 and a median value of 1.8. 78 patients with sepsis and overt or non-overt DIC according to the ISTH criteria were included in this study without a requirement for a minimum INR. The INR range in this patient population was 1-8.6 with a mean value of 1.6 and a median of 1.4. The mean INR was significantly higher in the warfarin treated patient population than in the DIC patients (Mann-Whitney test, $p<0.0001$). Scatter plots of INR values in both patient populations are shown in Figure 10.

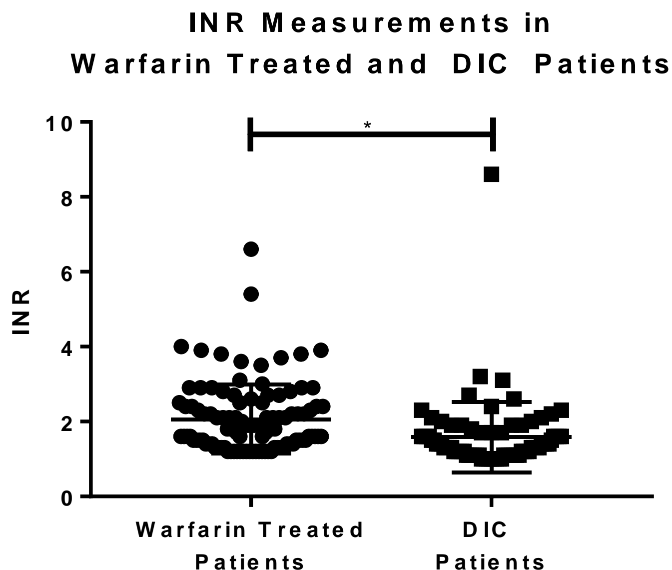


**Figure 10. Scatter Plot of INR Values in DIC Versus Warfarin Treated Patients**. INR was measured in 100 warfarin treated patients and 78 DIC patients. An INR of ≥ 1.2 was required for warfarin treated patients to be included in the study in order to include only patients actively taking warfarin therapy. DIC patients had sepsis with overt or non-overt DIC according to the ISTH criteria. Difference between the two groups was assessed using the Mann-Whitney test ($p < 0.0001$).

**aPTT**

Activated partial thromboplastin time (aPTT) was measured in warfarin treated patients and DIC patients using standard operating protocols on an ACL-ELITE coagulation analyzer, as shown in Table 8 and Figure 11. Overall, significant variation in aPTT based on INR was observed in both warfarin treated patients ($p=0.019$) and DIC patients ($p<0.0001$). For warfarin treated patients, a significant difference was observed between patients with a subtherapeutic INR of 1.5-1.9 and patients with a supertherapeutic INR ≥3 ($p=0.034$). In DIC patients, aPTT showed a stepwise increase

with increasing INR, although statistical significance was only reached between patients with an INR of <1.5 and patients with an INR of 1.5-1.9 ($p=0.0001$), 2-2.9 ($p<0.0001$), and ≥3 ($p=0.0011$). Although the aPTT values for patients with an INR of less than 2 were similar between the two patient populations, the maximum observed aPTTs were markedly higher in the DIC patient population than in warfarin treated patients.

Scatter plots of aPTT vs. INR were also generated for warfarin treated and DIC patients, and the fit of linear trendlines were evaluated. For warfarin treated patients, the trendline had a poor fit ($R^2=0.0046$) and the slope was not significantly non-zero ($p = 0.44$), indicating no consistent relationship between aPTT and INR. For DIC patients, the trendline had an $R^2$ of 0.68 and a significantly non-zero slope ($p<0.0001$), indicating that INR and aPTT are strongly related in DIC patients.

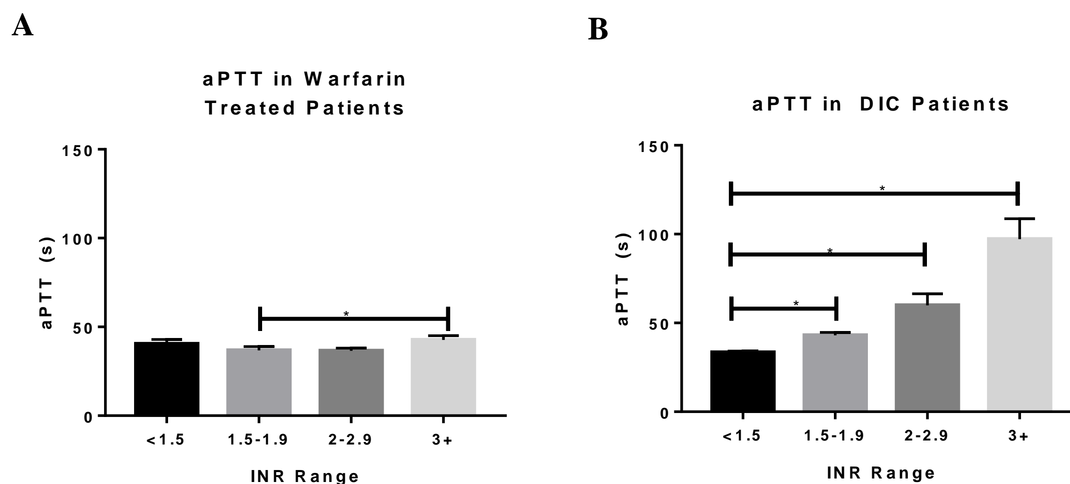


**Figure 11: Relationship of aPTT to INR in Warfarin Treated and DIC Patients**. aPTT in (a) warfarin treated and (b) DIC patients stratified by INR group. Comparison was made using the Kruskal-Wallis one-way ANOVA and Dunn's multiple comparison test with $\alpha = 0.05$ as the cutoff for significance (indicated by asterisk).

**Table 8. Relationship of aPTT to INR in Warfarin Treated and DIC Patients**

| aPTT (s) | INR | Mean | Median | SD | SEM | Range | n |
|---|---|---|---|---|---|---|---|
| **Warfarin** | < 1.5 | 40.41 | 38.15 | 14.9 | 2.483 | 24.9-98.7 | 36 |
| | 1.5-1.9 | 36.79 | 33.5 | 13.25 | 2.179 | 22.1-91.1 | 37 |
| | 2-2.9 | 36.56 | 33.7 | 9.526 | 1.545 | 24.9-66.5 | 38 |
| | ≥ 3 | 42.74 | 40.8 | 10.31 | 2.365 | 30.6-66.1 | 19 |
| **DIC** | < 1.5 | 33.5 | 32.9 | 5.01 | 0.7387 | 20.6-44.4 | 46 |
| | 1.5-1.9 | 43.12 | 43.4 | 6.906 | 1.584 | 31.1-61.1 | 19 |
| | 2-2.9 | 59.95 | 55.1 | 20.52 | 6.49 | 41-115 | 10 |
| | ≥ 3 | 97.13 | 92.9 | 20.09 | 11.6 | 79.5-119 | 3 |

**Fibrinogen**

Fibrinogen was measured in 132 warfarin treated patients and 77 DIC patients, as shown in Figure 12 and Table 9. Overall, significant variation was observed in warfarin treated patients ($p=0.0005$) but not DIC patients ($p=0.075$). For warfarin treated patients, the difference was significant between patients with an INR of <1.5 and an INR of ≥3 ($p=0.04$), an INR of 1.5-1.9 versus an INR of 2-2.9 ($p=0.008$), and an INR of 1.5-1.9 versus an INR of ≥3 ($p = 0.0077$).

Scatter plots of fibrinogen vs. INR were also generated for warfarin treated and DIC patients, and the fit of linear trendlines were evaluated. The trendline fit was poor for both warfarin treated ($R^2=0.054$) and DIC ($R^2=0.084$) patients, although the slope was significantly non-zero for both patient groups ($p=0.0075$ for warfarin treated patients and 0.011 for DIC patients). This indicates that while INR is related to fibrinogen levels in both warfarin treated and DIC patients, the relationship is not strong and linear in either patient group.

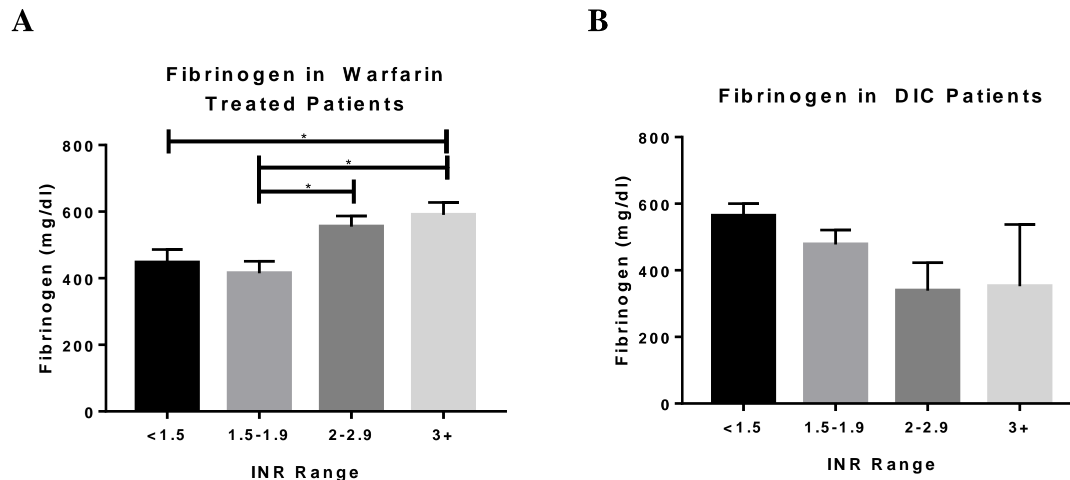


**Figure 12: Relationship of Fibrinogen to INR in Warfarin Treated and DIC Patients**. Fibrinogen in warfarin treated and DIC patients stratified by INR group. Comparison was made using the Kruskal-Wallis one-way ANOVA and Dunn's multiple comparison test with $\alpha = 0.05$ as the cutoff for significance.

**Table 9. Relationship of Fibrinogen to INR in Warfarin Treated and DIC Patients**

| Fibrinogen (mg/dl) | INR | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| Warfarin | < 1.5 | 447 | 420 | 237.9 | 39.1 | 97-999 |
| | 1.5-1.9 | 414.7 | 361 | 221.5 | 36.41 | 94-999 |
| | 2-2.9 | 554.7 | 582 | 200.7 | 32.13 | 124-999 |
| | ≥ 3 | 590.2 | 588 | 164.8 | 37.82 | 312-999 |
| DIC | < 1.5 | 563.9 | 516 | 246.3 | 36.31 | 159-999 |
| | 1.5-1.9 | 477.9 | 473 | 188.9 | 43.34 | 130-782 |
| | 2-2.9 | 338.7 | 223 | 253 | 84.35 | 62-692 |
| | ≥ 3 | 352.3 | 195 | 321.3 | 185.5 | 140-722 |

**Coagulation Factors**

The levels of coagulation factors VII, IX, and X were measured in warfarin treated and DIC patients as well as in a population of 50 healthy controls, as shown in Figure 13. Immunologic levels of all factors were determined using commercially available ELISA methods while functional levels were determined using clot-based methods. Coagulation factor levels in both the warfarin and DIC patient populations were compared to the levels in the healthy control group. Both functional and antigenic levels of all three factors were found to be significantly reduced in both warfarin treated and DIC patients compared to healthy controls.

The relationship of coagulation factor level to INR was assessed for each factor in both the DIC and warfarin treated patients. Differences in factor levels based on INR group were assessed using the Kruskal-Wallis ANOVA for non-parametric data with $\alpha=0.05$ as the cutoff for significance. Differences between individual groups were analyzed using Dunn's multiple comparison test.

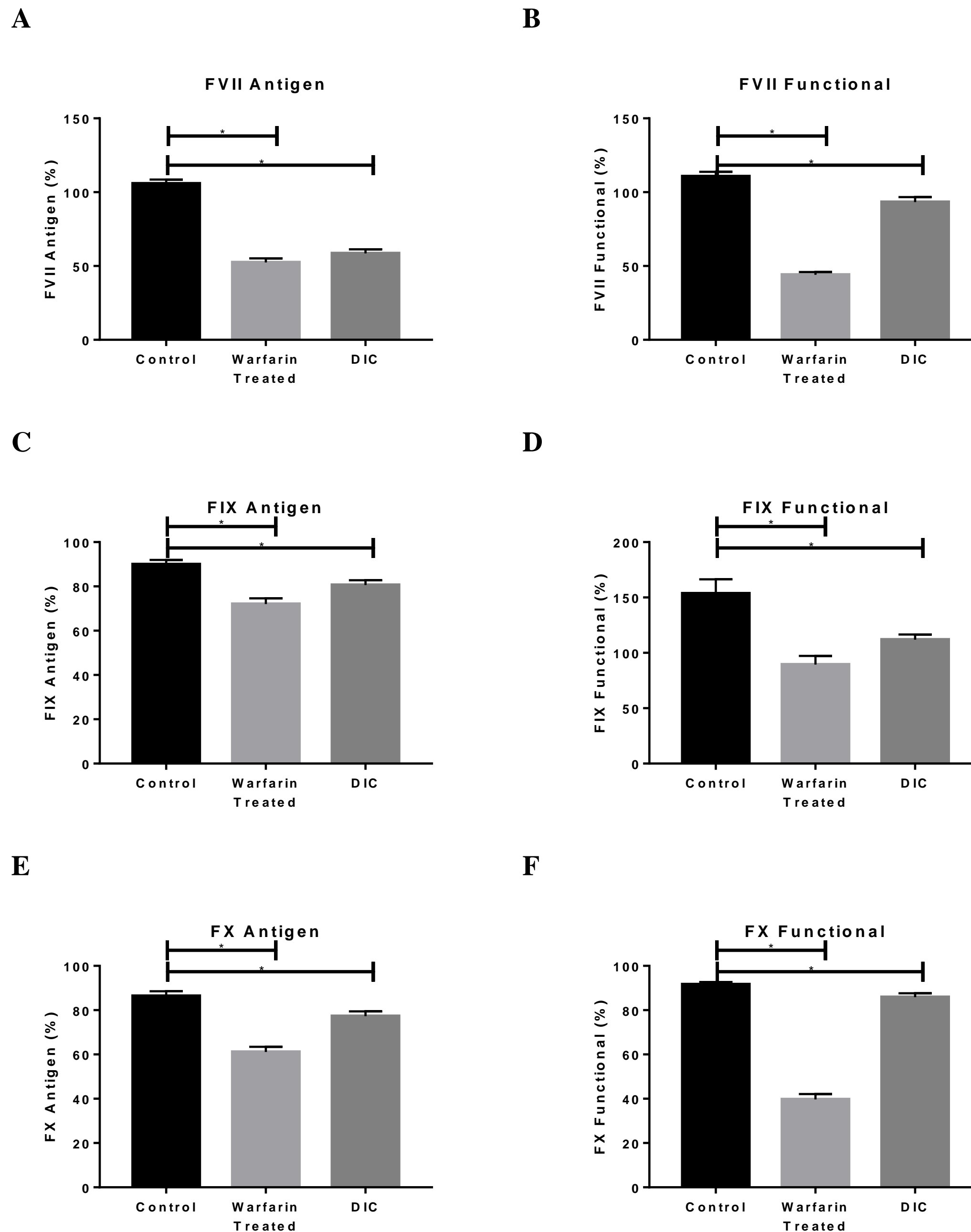


**Figure 13. Antigenically and Functionally Determined Levels of Coagulation Factors in Warfarin Treated and DIC Patients Compared to Healthy Controls**. For each factor, comparison was made between healthy controls and warfarin treated patients and healthy controls and DIC patients using the Mann-Whitney t test with $p < 0.05$ as the cutoff for significance (indicated by asterisk).

**Factor VII.** Statistical significance was not achieved for comparison of protein levels of Factor VII between INR groups for either patient group. Significant variation in functional Factor VII based on INR was seen in both patients treated with warfarin ($p<0.0001$) and DIC patients ($p=0.0004$) (Figure 14 and Table 10). For patients treated with warfarin, significant differences were observed for patients with an INR of <1.5 vs. 2-2.9 ($p<0.0001$), <1.5 vs. >3 ($p<0.0001$), 1.5-1.9 vs. 2-2.9 ($p=0.033$), and 1.5-1.9 vs. >3 ($p<0.0001$). For DIC patients, the difference was only significant for patients with INRs of < 1.5 vs. 1.5-1.9. The linear fit of FVII vs. INR was also evaluated. The fit was reasonably strong for warfarin treated patients ($R^2=0.42$) with a significantly non-zero slope ($p<0.0001$), suggesting a direct relationship between FVII and INR, but poor for SAC patients ($R^2=0.018$) with a not significantly non-zero slope ($p=0.84$), indicating no direct relationship of FVII to INR in this patient population.

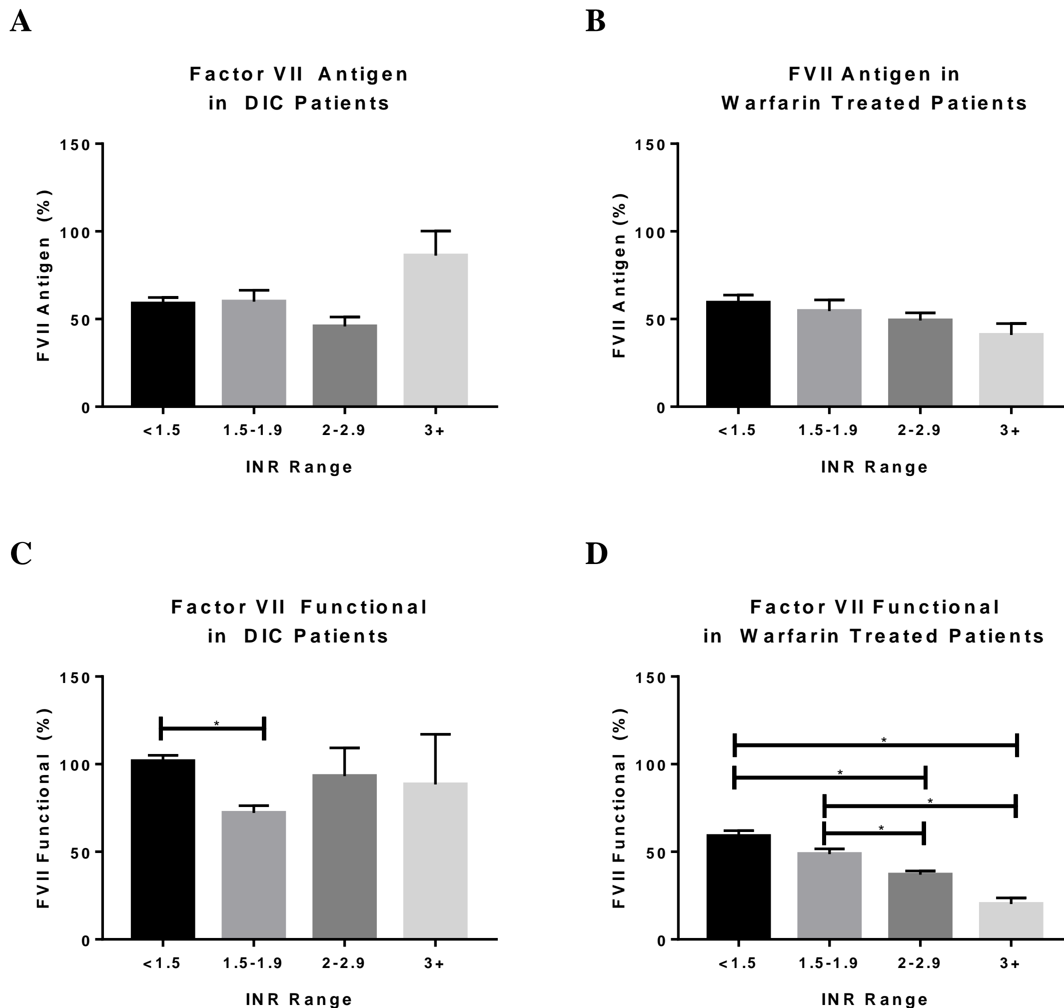


**Figure 14. Relationship of FVII levels to INR in Warfarin Treated and DIC Patients.** Protein (A and B) and functional (C and D) levels of FVII in warfarin treated and DIC patients stratified by INR group. Comparison was made using the Kruskal-Wallis one-way ANOVA and Dunn's multiple comparison test with α=0.05 as the cutoff for significance (indicated by *)

**Table 10. Relationship of Protein and Functional Levels of Factor VII to INR in Warfarin Treated and DIC Patients**

| | INR | Warfarin Treated Patients | | | DIC Patients | | |
|---|---|---|---|---|---|---|---|
| | | Mean | Median | SEM | Mean | Median | SEM |
| **Factor VII Antigen (%)** | < 1.5 | 59.4 | 60.3 | 4.4 | 58.9 | 57.4 | 3.5 |
| | 1.5-1.9 | 54.5 | 52.6 | 6.4 | 60.0 | 56.7 | 6.4 |
| | 2-2.9 | 49.2 | 47.8 | 4.3 | 45.9 | 47.8 | 5.4 |
| | ≥ 3 | 41.0 | 47.0 | 6.4 | 86.2 | 98.1 | 14.0 |
| **Factor VII Functional (%)** | < 1.5 | 59.0 | 59.3 | 3.1 | 101.7 | 104.3 | 3.4 |
| | 1.5-1.9 | 48.7 | 46.9 | 3.0 | 72.2 | 72.8 | 4.1 |
| | 2-2.9 | 37.0 | 34.8 | 2.1 | 93.2 | 85.9 | 16.1 |
| | ≥ 3 | 20.2 | 19.6 | 3.5 | 88.4 | 112.5 | 28.6 |

**Factor IX.** For Factor IX (Figure 15 and Table 11), comparable results were observed for both functional and antigenic factor levels. Significant variation in functional FIX based on INR was seen in warfarin treated ($p<0.0001$) but not DIC patients ($p=0.61$). For warfarin treated patients, significant differences in functional levels of Factor IX were observed for patients with an INR of <1.5 vs. 2-2.9 ($p=0.0008$), <1.5 vs. ≥3 ($p=0.0004$), 1.5-1.9 vs. 2-2.9 ($p=0.045$), and 1.5-1.9 vs. ≥3 ($p<0.0092$). The linear fit was reasonably poor for both warfarin treated patients ($R^2=0.17$) and DIC patients ($R^2=0.095$), with a significantly non-zero slope for both populations ($p<0.0001$ for warfarin treated patients and $p=0.006$ for DIC patients).

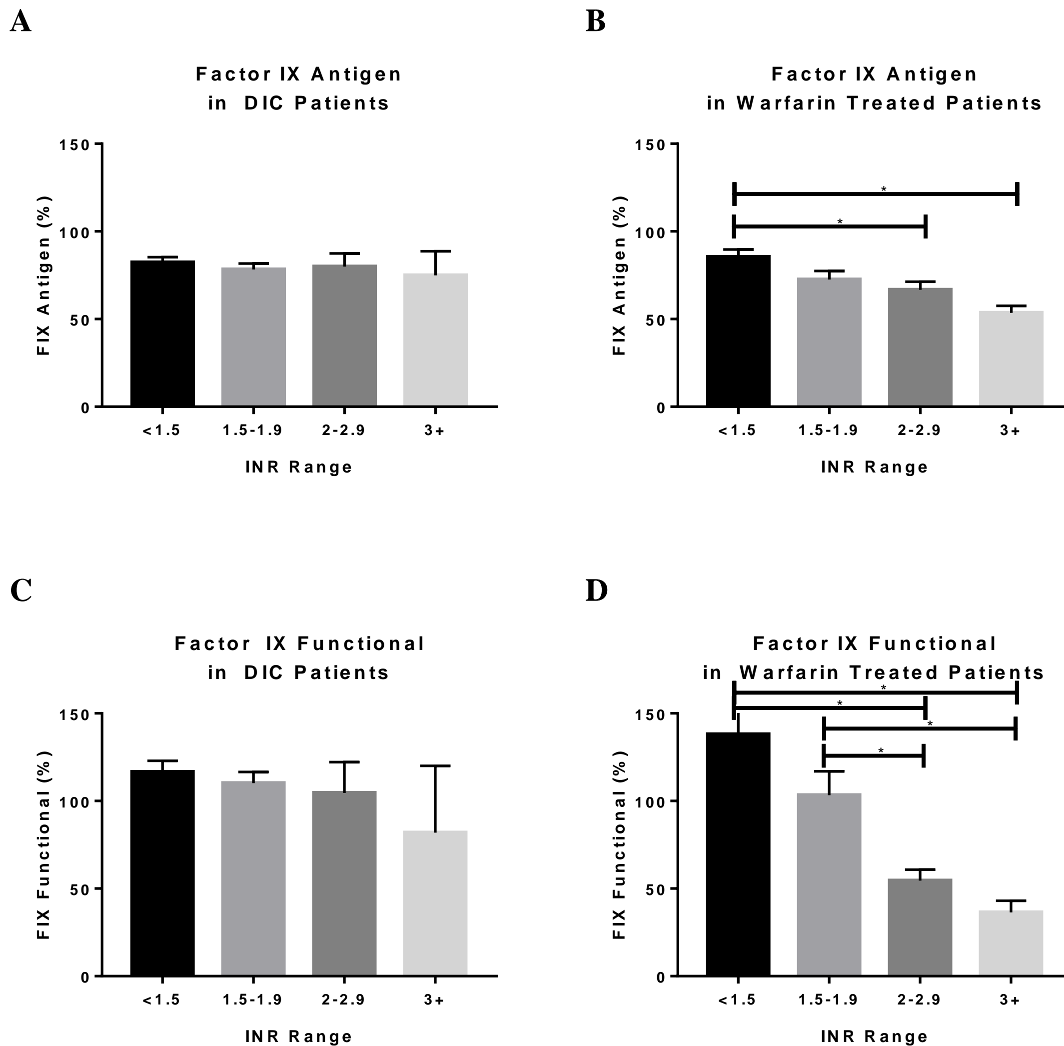


**Figure 15. Relationship of FIX levels to INR in Warfarin Treated and DIC Patients.** Protein (A and B) and functional (C and D) levels of FIX in warfarin treated and DIC patients stratified by INR group. Comparison was made using the Kruskal-Wallis one-way ANOVA and Dunn's multiple comparison test with α=0.05 as the cutoff for significance (indicated by *)

**Table 11. Relationship of Protein and Functional Levels of Factor IX to INR in Warfarin Treated and DIC Patients**

| | INR | Warfarin Treated Patients | | | DIC Patients | | |
|---|---|---|---|---|---|---|---|
| | | **Mean** | **Median** | **SEM** | **Mean** | **Median** | **SEM** |
| **Factor IX Antigen (%)** | < 1.5 | 85.4 | 88.6 | 4.2 | 82.3 | 84.8 | 3.1 |
| | 1.5-1.9 | 72.6 | 73.8 | 4.9 | 72.3 | 77.0 | 3.4 |
| | 2-2.9 | 66.7 | 70.8 | 4.7 | 80.0 | 80.2 | 7.4 |
| | ≥ 3 | 53.5 | 55.6 | 4.1 | 74.9 | 81.9 | 13.8 |
| **Factor IX Functional (%)** | < 1.5 | 85.4 | 88.6 | 4.2 | 82.3 | 84.8 | 3.1 |
| | 1.5-1.9 | 72.6 | 73.77 | 4.9 | 78.3 | 77.0 | 3.4 |
| | 2-2.9 | 66.4 | 70.8 | 4.7 | 80.0 | 80.2 | 7.4 |
| | ≥ 3 | 53.5 | 55.6 | 4.1 | 74.8 | 81.9 | 13.8 |

**Factor X.** As with Factor IX, comparable results were observed for the functional and antigenic levels of Factor X (Figure 16 and Table 12). Significant variation in functional FX based on INR was seen in both warfarin treated patients ($p<0.0001$) and DIC patients ($p=0.0003$). For warfarin treated patients, significant differences in functional Factor X levels were observed for patients with an INR of <1.5 vs. 2-2.9 ($p<0.0001$), <1.5 vs. >3 ($p<0.0001$), 1.5-1.9 vs. 2-2.9 ($p=0.0005$), and 1.5-1.9 vs. >3 ($p<0.0001$). For DIC patients, significant differences in functional Factor X levels were observed for patients with an INR <1.5 vs. 1.5-1.9 ($p=0.002$) and < 1.5 vs. >3 ($p=0.036$). The linear fit was reasonably good for warfarin treated patients ($R^2=0.51$), suggesting a direct relationship between FX and INR but poor for SAC patients ($R^2=0.14$), indicating no direct relationship between these factors, with a significantly non-zero slope for both populations ($p<0.0001$ for warfarin treated patients and $p=0.0007$ for DIC patients).

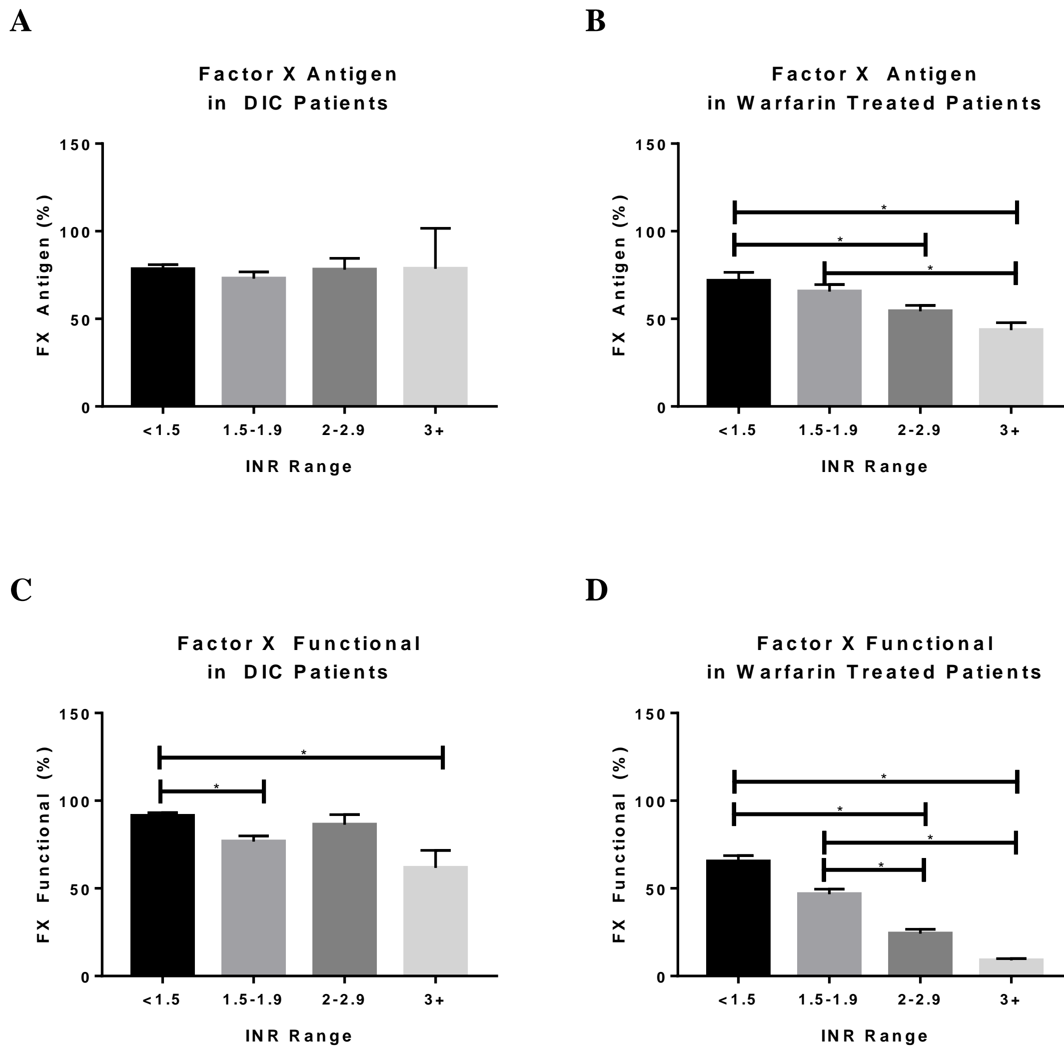


**Figure 16. Relationship of FX Levels to INR in Warfarin Treated and DIC Patients.** Protein (A and B) and functional (C and D) levels of FX in warfarin treated and DIC patients stratified by INR group. Comparison was made using the Kruskal-Wallis one-way ANOVA and Dunn's multiple comparison test with $\alpha$=0.05 as the cutoff for significance (indicated by *)

**Table 12. Relationship of Protein and Functional Levels of Factor X to INR in Warfarin Treated and DIC Patients**

| | INR | Warfarin Treated Patients | | | DIC Patients | | |
|---|---|---|---|---|---|---|---|
| | | **Mean** | **Median** | **SEM** | **Mean** | **Median** | **SEM** |
| **Factor X Antigen (%)** | < 1.5 | 71.7 | 78.8 | 4.9 | 78.3 | 76.5 | 2.6 |
| | 1.5-1.9 | 65.6 | 63.5 | 3.9 | 72.9 | 76.0 | 4.0 |
| | 2-2.9 | 54.3 | 57.7 | 3.3 | 78.1 | 84.7 | 6.5 |
| | ≥ 3 | 43.5 | 38.8 | 4.3 | 78.5 | 91.4 | 23.2 |
| **Factor X Functional (%)** | < 1.5 | 65.4 | 71.6 | 3.3 | 91.3 | 92.1 | 1.8 |
| | 1.5-1.9 | 46.7 | 47.1 | 2.9 | 76.7 | 80.0 | 3.3 |
| | 2-2.9 | 24.2 | 20.4 | 2.5 | 86.3 | 84.3 | 5.8 |
| | ≥ 3 | 9.0 | 10.4 | 0.9 | 61.7 | 53.1 | 9.9 |

**Correlations Between Factor Levels and Global Coagulation Tests**

Correlations between levels of all factors were analyzed for both the warfarin treated and DIC patient groups. Spearman correlation coefficients were analyzed with $\alpha=0.05$ as the cutoff for significance. Correlation coefficients for warfarin treated patients are shown in Table 13 and coefficients for DIC patients are shown in Table 14. Non-significant correlations are indicated as NS, and correlation coefficients are listed and highlighted in light blue for all significant correlations. Strong correlations (Spearman $r \geq 0.4$) are indicated with bold text and dark blue highlighting.

The observed patterns of correlations were markedly different for warfarin treated patients and DIC patients. For warfarin treated patients, significant and a strong correlation was observed between INR and both functional and antigenic levels of the coagulation factors. Correlations were stronger with functional factor levels than with antigenic factor levels. In this patient population, the levels of Factors VII, IX, and IX were highly correlated with each other as well. Strong correlations were also seen between antigenic and functional levels of all three coagulation factors. In contrast, fewer correlations were observed overall in DIC patients, and the observed correlations were overall weaker. A correlation was observed between INR and aPTT in the DIC patients, whereas no correlation was observed in the warfarin treated patients. In the DIC patients, both INR and aPTT correlated significantly with the functional, but not antigenic, levels of Factors VII, IX, and X, with the strongest correlations observed with functional Factor X for both tests. Fewer correlations between levels of coagulation factors were observed in DIC patients than in warfarin treated patients. The only strong correlation observed

between coagulation factors in DIC patients was between the functional and antigenic levels of Factor X. Functional and antigenic levels of FVII and FIX showed no correlations with each other.

The results of the comparison between patients with DIC and patients receiving warfarin therapy demonstrated that the same INR value describes distinct scenarios of coagulation dysfunction in these two patient populations. INR is one of the key parameters used to identify patients with DIC. In studies that do not utilize the full definition of DIC, identification of patients with sepsis-associated coagulopathy is often made solely on the basis of elevated INR and reduced platelet count. However, INR was not associated with a consistent pattern of underlying coagulopathy in patients with sepsis-associated DIC. Therefore, other tests or biomarkers may provide a more accurate description of the coagulopathy in DIC patients than INR.

**Table 13. Correlations among Coagulation Factors in Warfarin Treated Patients**

| | INR | FIB | aPTT | FVII Ant. | FVII Func. | FIX Ant. | FIX Func. | FX Ant. | FX Func. |
|---|---|---|---|---|---|---|---|---|---|
| **INR** | | 0.32 | NS | -0.22 | -0.66 | -0.39 | -0.50 | -0.46 | -0.85 |
| **FIB** | 0.32 | | NS | NS | NS | 0.30 | NS | NS | -0.23 |
| **aPTT** | NS | NS | | -0.27 | -0.22 | -0.31 | -0.40 | NS | NS |
| **FVII Ant.** | -0.22 | NS | NS | | 0.62 | 0.48 | 0.29 | 0.49 | NS |
| **FVII Func.** | -0.66 | NS | -0.22 | 0.62 | | 0.54 | 0.47 | 0.50 | 0.46 |
| **FIX Ant.** | -0.39 | 0.30 | -0.31 | 0.48 | 0.54 | | 0.52 | 0.57 | 0.37 |
| **FIX Func.** | -0.50 | NS | -0.40 | 0.29 | 0.47 | 0.52 | | 0.35 | 0.34 |
| **FX Ant.** | -0.46 | NS | NS | 0.49 | 0.50 | 0.57 | 0.35 | | 0.48 |
| **FX Func.** | -0.85 | -0.23 | NS | NS | 0.46 | 0.37 | 0.34 | 0.48 | |

**Table 14. Correlations among Coagulation Factors in DIC Patients**

| | INR | FIB | aPTT | FVII Ant. | FVII Func. | FIX Ant. | FIX Func. | FX Ant. | FX Func. |
|---|---|---|---|---|---|---|---|---|---|
| **INR** | | -0.28 | 0.84 | NS | -0.39 | NS | -0.22 | NS | -0.50 |
| **FIB** | -0.28 | | -0.30 | NS | NS | NS | NS | NS | NS |
| **aPTT** | 0.84 | -0.30 | | NS | -0.26 | NS | -0.34 | NS | -0.41 |
| **FVII Ant.** | NS | NS | NS | | NS | -0.32 | NS | NS | NS |
| **FVII Func.** | -0.39 | NS | -0.26 | NS | | 0.26 | NS | NS | 0.39 |
| **FIX Ant.** | NS | NS | NS | -0.32 | 0.26 | | NS | 0.30 | 0.23 |
| **FIX Func.** | -0.22 | NS | -0.34 | NS | NS | NS | | NS | 0.26 |
| **FX Ant.** | NS | NS | NS | NS | NS | 0.30 | NS | | 0.54 |
| **FX Func.** | -0.50 | NS | -0.41 | NS | 0.39 | NS | 0.26 | 0.54 | |

## Biomarker Profiling of Utah Cohort Patient Plasma Samples

In order to gain a better understanding of the pathophysiology underlying the coagulation dysfunction in patients with sepsis-associated DIC, biomarkers representative of hemostasis, infection, inflammation, endothelial function, and platelet function were measured in a cohort of patients with sepsis and well-defined DIC of variable severity. These biomarkers were assessed for association with both the severity of coagulation dysfunction and mortality as well as for the ability to predict outcome alone or in combination with other biomarkers. Based on the lack of association of INR with a predictable pattern of coagulation dysfunction, it was hypothesized that biomarkers other than commonly measured hemostatic parameters would provide insight into the underlying pathophysiology of DIC and describe the severity of ongoing coagulation dysfunction. Due to the complex pathophysiology of sepsis-associated DIC, it was hypothesized that a combination of biomarkers would provide superior descriptive or predictive ability than a single biomarker.

### Patient Cohort Baseline Characteristics

Plasma samples were collected from Utah cohort patients according to the protocols detailed in the Materials and Methods section of this dissertation. Samples were collected at the University of Utah Hospital and an associated community hospital and transferred to Loyola University Chicago under IRB approved protocols. Patient treatment was not altered as a result of participation in this study, and all patients provided informed consent.

Plasma samples were collected from 103 patients with sepsis, defined as meeting SIRS criteria with an identified focus of infection, at the time of ICU admission. Subsequent samples were collected from 46 patients remaining in the ICU on day 4 and 21 patients remaining in the ICU on day 8. Basic demographic information for this patient cohort, including known comorbidities, is shown in Table 15.

The basic demographics of this patient cohort are typical and appropriate for patients with sepsis. Sepsis is a disease with many causes that can affect patients of any age. This is reflected in the broad age range of patients, ranging from 18 to 90. The mean age of 57.1 ± 18.6 years describes a predominantly middle aged population and is typical of sepsis cohorts in the literature. The mean BMI (31.2) describes an obese patient, and 76% of patients are classified as either overweight or obese (BMI ≥25). The cohort is split fairly evenly between males and females (53.4% male vs. 46.6% female), and racial and ethnic makeup of this cohort (84.5% white) is typical of the geographic area in which these samples were collected.

Comorbidities analyzed in this patient cohort include conditions that may affect coagulation status and thus the development of DIC, particularly active cancer and cirrhosis. Both of these conditions occurred with low frequency (5.8%) in this patient population. The most prevalent recorded comorbidity in this patient cohort was hypertension, reported in 45.6% of patients.

Patient care was not modified as a part of this study, and patients received antibiotics as well as appropriate supportive treatments as deemed appropriate by the

medical team. Patients also received routine thromboprophylaxis at the discretion of the medical team, typically unfractionated heparin. Use of anticoagulants in addition to this routine thromboprophylaxis was minimal. Warfarin therapy was reported in 5.8% of patients. There was no reported use of direct thrombin inhibitors, direct anti-Xa agents, or low molecular weight heparin.

**Table 15. Baseline Characteristics of the Utah Patient Cohort**

| **Characteristic** | **Mean ± Standard Deviation** |
|---|---|
| Age (Years) | 57.1 ± 18.6 |
| Weight (kg) | 89.5 ± 27.4 |
| BMI | 31.2 ± 0.89 |
| **Characteristic** | **N (%)** |
| Gender | |
| Male | 48 (46.6%) |
| Female | 55 (53.4%) |
| Race | |
| White | 87 (84.5%) |
| Black | 2 (1.9%) |
| Hispanic | 9 (8.7%) |
| American Indian | 2 (1.9%) |
| Other | 1 (1%) |
| Cardiovascular Disease | 22 (21.4%) |
| Diabetes | 26 (25.2%) |
| Congestive Heart Failure | 9 (8.7%) |
| Cirrhosis | 6 (5.8%) |
| Hypertension | 47 (45.6%) |
| Pulmonary Disease | 17 (16.5%) |
| Recent or Active Cancer | 6 (5.8%) |
| Recent Surgery | 23 (22.3%) |
| Recent Transfusion | 7 (6.8%) |

### Disease Severity and Patient Outcomes

Outcome and disease severity information for the Utah patient cohort is shown in Table 16. The primary measure of outcome in this patient population was 28 day

mortality. This cohort was comprised of 88 survivors and 15 non-survivors, resulting in an overall 28-day mortality rate of 14.6%.

Severity of illness was further described through the requirement for vasopressors and mechanical ventilation as well as the Sequential Organ Failure Assessment (SOFA) and Acute Physiology and Chronic Health Evaluation (APACHE-II) scores at baseline. Septic shock, defined as the requirement for a vasopressor at baseline, was present in 46 patients (44.7%), and 48 patients (46.6%) required ventilator support.

The SOFA score describes organ failure in terms of both number of failing organ systems and severity of failure of each system. The SOFA scoring algorithm, included in Appendix C, assigns points based on clinically measurable values describing the dysfunction of organs including the lungs, liver, and kidneys, as well as the cardiovascular, hemostatic, and central nervous systems. An increasing score describes increasingly severe organ failure. A healthy individual with no underlying conditions leading to serious organ dysfunction is expected to have a SOFA score of zero. Elevated scores correlate with increasing ICU mortality, in-hospital mortality, and length of ICU stay. In this patient population, the SOFA score was 5.9 ± 3.7 (mean ± SD).

The APACHE-II score was computed in patients at the time of ICU admission. The APACHE II score is designed to be applied to patients within 24 hours of admission to the intensive care units, with higher scores in the range from 0 to 71 correlating with severe disease and elevated risk of death. The scoring algorithm for the APACHE II score is shown in Appendix C. In this patient cohort, the APACHE II score was 17.4 ± 7.3 (mean ± SD).

As a part of the study protocol under which these samples were collected, patients received additional monitoring for thrombosis throughout their hospital stay. In addition to recording symptomatic events including pulmonary embolism (PE), myocardial infarction (MI), or thromboembolic stroke, surviving patients received ultrasonographic assessment for asymptomatic DVT at the time of hospital discharge. No factors measured in this dissertation showed significant association with thrombosis diagnosed at discharge. However, the thrombi identified through this protocol may have developed significantly later in the course of hospitalization in the presence of risk factors other than sepsis. Without additional patient information, these thrombi cannot be said to be associated specifically with sepsis or DIC.

**Table 16. Outcome and Disease Severity Information**

| **Outcome** | **N (%)** |
|---|---|
| 28-Day Mortality | 15 (14.6%) |
| Septic Shock (Day 0) | 46 (44.7%) |
| Ventilator Use (Day 0) | 48 (46.6%) |
| In-Hospital Thrombosis (Total) | 25 (24.3%) |
| PE | 2 |
| MI | 1 |
| Stroke | 2 |
| DVT | 10 |
| Other/Unknown | 10 |
| **Clinical Disease Severity Score** | **Mean ± SD** |
| SOFA Score (Day 0) | 5.9 ± 3.7 |
| APACHE II Score | 17.4 ± 7.3 |

**DIC Score Stratification**

In this cohort, the DIC score was calculated for all patients using the ISTH scoring algorithm for DIC. This scoring system assigns points for abnormal values of

platelet count, INR, D-Dimer, and fibrinogen, and is shown both in Table 3 as well as in Appendix C. The presence of a predisposing condition for DIC, such as sepsis, cancer, trauma, or toxin exposure is a prerequisite for the use of this scoring algorithm. In this cohort, all patients were diagnosed with sepsis, fulfilling this requirement. Using this scoring system, a score of 5 or greater was classified as sepsis + overt DIC, a score of 3-4 was categorized as sepsis + non-overt DIC, and a score of 2 or lower was categorized as sepsis + no DIC.

Distribution of DIC scores is shown in Table 17. At baseline, 20 patients had sepsis + no DIC, 59 patients had sepsis + non-overt DIC, and 24 patients had sepsis + overt DIC. The relative prevalence of the degrees of DIC remained relatively constant between day 0 and day 4; however, on day 8, no patients remaining in the ICU had overt DIC.

**Table 17. DIC Score Distribution**

| | All Patients | Sepsis + No DIC | Sepsis + Non-Overt DIC | Sepsis + Overt DIC |
|---|---|---|---|---|
| **DIC Score** | Any | 0-2 | 3-4 | ≥5 |
| **Day 0 (n)** | 103 | 20 | 59 | 24 |
| **Day 4 (n)** | 57 | 11 | 36 | 10 |
| **Day 8 (n)** | 30 | 8 | 22 | 0 |

The association of mortality, shock and ventilator use with the severity of DIC was assessed using the Chi Square test. Although the prevalence of septic shock and mortality appeared to increase progressively with increasing severity of DIC, this difference was not statistically significant. Changes in SOFA and APACHE II scores based on DIC score category were evaluated using the Kruskal-Wallis One-Way

ANOVA. SOFA score showed significant variation based on DIC score category, indicating increased organ failure in patients with the most severe coagulopathy, while the APACHE II score did not. SOFA score describes organ failure in terms of both number of failing organ systems and degree of failure of each system. This score accounts for respiratory, hepatic, cardiovascular, central nervous system, and renal function as well as platelet count as a measure of coagulation function. The APACHE II score also includes similar parameters describing several physiological systems. However, platelet count is not included in the APACHE II score and coagulation function and liver function are not directly evaluated, which may contribute to the lack of association with DIC score. Outcome and illness severity information based on DIC score category is shown in Table 18.

**Table 18. Outcome and Severity of Illness Based on DIC Score Category**

| | All Patients | Sepsis + No DIC | Sepsis + Non-Overt DIC | Sepsis + Overt DIC | P Value |
|---|---|---|---|---|---|
| **Shock** | 44 | 5 | 28 | 13 | 0.12 |
| **Ventilator** | 48 | 10 | 25 | 13 | 0.59 |
| **Mortality** | 15 | 2 | 7 | 6 | 0.25 |
| **SOFA** Mean ± SD | 5.9 ± 3.7 | 4.2 ± 2.8 | 5.8 ± 3.9 | 7.5 ± 3.1 | 0.011* |
| **APACHE II** Mean ± SD | 17 ± 7.3 | 16 ± 6.3 | 17 ± 7.8 | 19 ± 6.8 | 0.37 |

**Association of Biomarker Levels with Severity of Illness**

The biomarkers measured in this dissertation can be divided into five general categories: hemostatic (platelets, INR, fibrinogen, D-Dimer, F1.2, and PAI-1), infection (nucleosomes, HMGB-1, and procalcitonin), inflammatory (IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFNγ, TNFα, IL-1α, IL-1β, MCP-1, EGF, and IL-6:IL-10 Ratio), endothelial

(TFPI, Protein C, endocan, Ang-2, and vWF), and platelet (CD40L, PF4, MP, and MP-TF).

In order to determine the relationships between biomarker levels and organ dysfunction, Spearman correlation coefficients were calculated between each biomarker and the SOFA and APACHE II scores with $p<0.05$ as the cutoff for significance.

The requirement for supportive therapies including a ventilator to maintain adequate gas exchange and vasopressors to maintain blood pressure are indicators of poor clinical status. Information regarding vasopressor type or dose was not available for most patients, and thus more in-depth analysis regarding the association of outcome or biomarkers with varied severity of shock could not be performed. Differences in baseline biomarker levels on the basis of ventilator or vasopressor use were assessed using the Mann-Whitney t-test with $p<0.05$ as the cutoff for significance.

**Hemostatic Biomarkers.** Baseline levels of hemostatic biomarkers indicating ongoing thrombosis or coagulation dysfunction showed significant association with organ failure. As shown in Table 19, SOFA score correlated significantly with platelet count ($p < 0.001$, $r = -0.36$), D-Dimer ($p = 0.035$, $r = 0.21$), and INR ($p = 0.043$, $r = 0.20$). The APACHE II score correlated significantly with platelet count ($p = 0.026$, $r = -0.22$).

No significant differences in platelet count, INR, fibrinogen, D-Dimer, F1.2, or PAI-1 levels at baseline were observed on the basis of ventilator use or shock, suggesting that while coagulation dysfunction may be involved in the development of global organ failure, it is not a major contributor to shock or respiratory dysfunction.

**Table 19. Association of Hemostatic Biomarkers with Severity of Illness**

| | Spearman Correlation Coefficients | | Mann-Whitney Test p Value | |
|---|---|---|---|---|
| | APACHE II Score | SOFA Score | Ventilator | Vasopressor |
| **D-Dimer** | 0.16 | **0.21** | 0.36 | 0.07 |
| **F1.2** | 0.13 | 0.12 | 0.19 | 0.61 |
| **PAI-1** | 0.09 | 0.04 | 0.11 | 0.10 |
| **INR** | 0.12 | **0.20** | 0.44 | 0.28 |
| **Platelets** | **-0.22** | **-0.36** | 0.16 | 0.08 |
| **Fibrinogen** | -0.03 | -0.02 | 0.26 | 0.71 |

For APACHE II and SOFA scores, Spearman correlation coefficients are shown. Significant correlations ($p<0.05$) are highlighted in blue. For ventilator and vasopressor use, Mann-Whitney Test p value is shown for comparison of biomarker levels between patients receiving or not receiving ventilator or vasopressor support. Light blue denotes significance ($p<0.05$).

**Infection and Inflammation Biomarkers.** As shown in Table 20, the presence of inflammation and infection was significantly associated with organ failure. SOFA score correlated significantly with IL-6 ($p = 0.008$, $r = 0.26$) IL-8, ($p = 0.001$, $r = 0.32$), IL-10 ($p = 0.022$, $r = 0.23$), MCP-1 ($p = 0.001$, $r = 0.23$), and TNFα ($p = 0.025$, $r = 0.22$). This highlights the association between widespread, generalized inflammation, and organ failure. In contrast, the only infection marker to show significant association with organ failure was procalcitonin ($p = 0.030$, $r = 0.28$).

Levels of MCP-1 and HMGB-1 were significantly elevated in patients requiring ventilator support at baseline. Procalcitonin, IL-6, IL-8, TNFα, and MCP-1 were significantly elevated in patients requiring vasopressors.

**Table 20. Association of Infection and Inflammation Biomarkers with Severity of Illness**

| | Spearman Correlation Coefficients | | Mann-Whitney Test p Value | |
|---|---|---|---|---|
| | APACHE II Score | SOFA Score | Ventilator | Vasopressor |
| **Nucleosomes** | 0.06 | -0.07 | 0.21 | 0.82 |
| **HMGB-1** | -0.08 | -0.07 | **0.05** | 0.29 |
| **Procalcitonin** | **0.21** | **0.28** | 0.84 | **<0.0001** |
| **IL-2** | 0.07 | 0.06 | 0.81 | 0.49 |
| **IL-4** | -0.02 | 0.01 | 0.99 | 0.21 |
| **IL-6** | 0.09 | **0.26** | 0.08 | **0.01** |
| **IL-8** | 0.19 | **0.32** | 0.05 | **0.04** |
| **IL-10** | 0.05 | **0.23** | 0.53 | 0.06 |
| **VEGF** | -0.04 | 0.00 | 0.07 | 0.12 |
| **IFNγ** | -0.06 | 0.01 | 0.34 | 0.54 |
| **TNFα** | 0.02 | **0.22** | 0.45 | **0.004** |
| **IL-1α** | 0.12 | 0.14 | 0.82 | 0.96 |
| **IL-1β** | 0.04 | 0.11 | 0.38 | 0.13 |
| **MCP-1** | 0.10 | **0.33** | **0.03** | **0.02** |
| **EGF** | -0.12 | -0.17 | 0.98 | 0.09 |
| **IL-6:IL-10** | 0.03 | 0.17 | 0.21 | 0.15 |

For APACHE II and SOFA scores, Spearman correlation coefficients are shown. Significant correlations ($p<0.05$) are highlighted in blue. For ventilator and vasopressor use, Mann-Whitney Test p value is shown for comparison of biomarker levels between patients receiving or not receiving ventilator or vasopressor support. Light blue denotes significance ($p<0.05$).

Somewhat surprisingly, MCP-1 showed the strongest association of any measured inflammatory marker with indicators of clinical status and organ failure, including SOFA score, as well as ventilator and vasopressor use. Although previous investigations of MCP-1 in sepsis and DIC have been limited, it has been suggested that elevated MCP-1

may contribute to the development of shock and organ dysfunction through increased vascular leakage.

**Endothelial Biomarkers.** As shown in Table 21, markers of endothelial function showed minimal associations with organ failure or disease severity. Statistically significant but weak correlation was seen between SOFA score and protein C ($p = 0.024$, $r = -0.22$). APACHE II score showed no significant correlation with any endothelial biomarker.

Minimal associations were observed between the endothelial markers and the presence of shock or ventilator use. Ang-2 was significantly elevated in patients requiring vasopressor support. vWF was significantly elevated in patients requiring mechanical ventilation. This low degree of association between endothelial markers and disease severity is somewhat surprising, as previous studies have demonstrated associations between endothelial damage and organ failure.

**Table 21. Association of Endothelial Biomarkers with Severity of Illness**

| | Spearman Correlation Coefficients | | Mann-Whitney Test p Value | |
|---|---|---|---|---|
| | APACHE II Score | SOFA Score | Ventilator | Vasopressor |
| **TFPI** | 0.10 | 0.00 | 0.45 | 0.55 |
| **Protein C** | -0.15 | **-0.22** | 0.11 | 0.25 |
| **Endocan** | 0.19 | -0.02 | 0.63 | 0.35 |
| **Ang-2** | 0.05 | 0.14 | 0.22 | **<0.0001** |
| **vWF** | -0.14 | -0.12 | **0.0006** | 0.22 |

For APACHE II and SOFA scores, Spearman correlation coefficients are shown. Significant correlations ($p<0.05$) are highlighted in blue. For ventilator and vasopressor use, Mann-Whitney Test p value is shown for comparison of biomarker levels between patients receiving or not receiving ventilator or vasopressor support. Light blue denotes significance ($p<0.05$).

**Platelet Biomarkers.** As shown in Table 22, multiple associations were observed between the levels of platelet function markers and organ failure. SOFA score correlated significantly with PF4 ($p < 0.001$, $r = -0.43$) and microparticles ($p = 0.011$, $r = -0.26$). APACHE II score correlated significantly with CD40L ($p = 0.003$, $r = -0.29$) and PF4 ($p < 0.001$, $r = -0.37$). Notably, PF4, vWF, MP, MP-TF, and CD40L were all elevated in sepsis and DIC patients compared to healthy controls despite the reduction in platelet count. However, negative correlations between platelet function markers and SOFA, MODS, and APACHE II scores were observed, indicating an inverse relationship between the levels of these markers and the degree of organ failure. PF4 was significantly elevated in patients requiring mechanical ventilation. No other differences in platelet markers were observed on the basis of vasopressor or ventilation status.

**Table 22. Association of Platelet Biomarkers with Severity of Illness**

| | Spearman Correlation Coefficients | | Mann-Whitney Test p Value | |
|---|---|---|---|---|
| | APACHE II Score | SOFA Score | Ventilator | Vasopressor |
| **CD40L** | **-0.29** | -0.18 | 0.44 | 0.51 |
| **MP** | -0.05 | **-0.26** | 0.81 | 0.20 |
| **MP-TF** | 0.18 | 0.16 | 0.55 | 0.69 |
| **PF-4** | **-0.37** | **-0.43** | **0.02** | 0.15 |

For APACHE II and SOFA scores, Spearman correlation coefficients are shown. Significant correlations ($p<0.05$ are highlighted in blue). Light blue denotes a correlation coefficient of <0.4 while dark blue denotes a coefficient of ≥0.4. For ventilator and vasopressor use, Mann-Whitney Test p value is shown for comparison of biomarker levels between patients receiving or not receiving ventilator or vasopressor support. Light blue denotes significance ($p<0.05$).

**Association of Biomarkers with DIC Score at Baseline**

As previously described, the ISTH DIC score was calculated in all sepsis patients, and patients were divided into three groups based on this score. Patients with a score of 0-2 were classified as “No DIC”, patients with a score of 3-4 were classified as “Non-Overt DIC”, and patients with a score of 5 or greater were classified as “Overt DIC”. Overt DIC describes a scenario of severe, decompensated coagulopathy with marked perturbations to multiple aspects of the hemostatic system. Non-overt DIC represents a heterogeneous phenotype, with a variable degree and manifestation of coagulopathy. Patients in the no DIC category were still severely ill with sepsis; however, these patients did not have significant coagulation dysfunction. Differences in biomarker levels between the three groups and from the healthy control cohort were assessed using the

Kruskal-Wallis ANOVA with Dunn's multiple comparison test and $p < 0.05$ as the cutoff for significance. Markers were measured in 50 healthy individuals as well as in samples from 20 patients with no DIC, 59 patients with non-overt DIC, and 24 patients with overt DIC.

**Hemostatic Biomarkers.** Platelet count, fibrinogen, INR and D-Dimer are included in the DIC scoring algorithm, and therefore are expected to vary significantly with DIC score. This variation was observed with D-Dimer, where significant differences were seen not only between the healthy controls and all patient groups but also between patients with sepsis without DIC and those with either non-overt or overt DIC. INR, platelet count, and fibrinogen were not measured in the healthy control population. However, both INR and platelet count, as expected, showed significant variation based on DIC score category. In contrast, fibrinogen showed no variation on the basis of DIC status. While in severe coagulopathy, fibrinogen levels may decrease as fibrinogen is converted into an insoluble fibrin clot, this phenomenon was not generally observed in this patient cohort. Fibrinogen is an acute phase reactant and is secreted by the liver under inflammatory conditions. In patients with sepsis, this increased fibrinogen release in response to inflammation appears to overcome the consumption due to coagulation, even in patients with severe coagulopathy. Accordingly, fibrinogen may not be an appropriate marker to use in the evaluation of coagulation status in this patient population.

The fibrinolysis regulator PAI-1 was significantly elevated in all categories of sepsis patients compared to healthy controls, but did not vary within the sepsis population based on DIC status. The thrombin generation marker F1.2 was only elevated in patients

with overt or non-overt DIC compared to healthy controls; the elevation in F1.2 in patients with sepsis without DIC was not statistically significant. Data is shown in Figure 17 and in Table 42 in Appendix B.

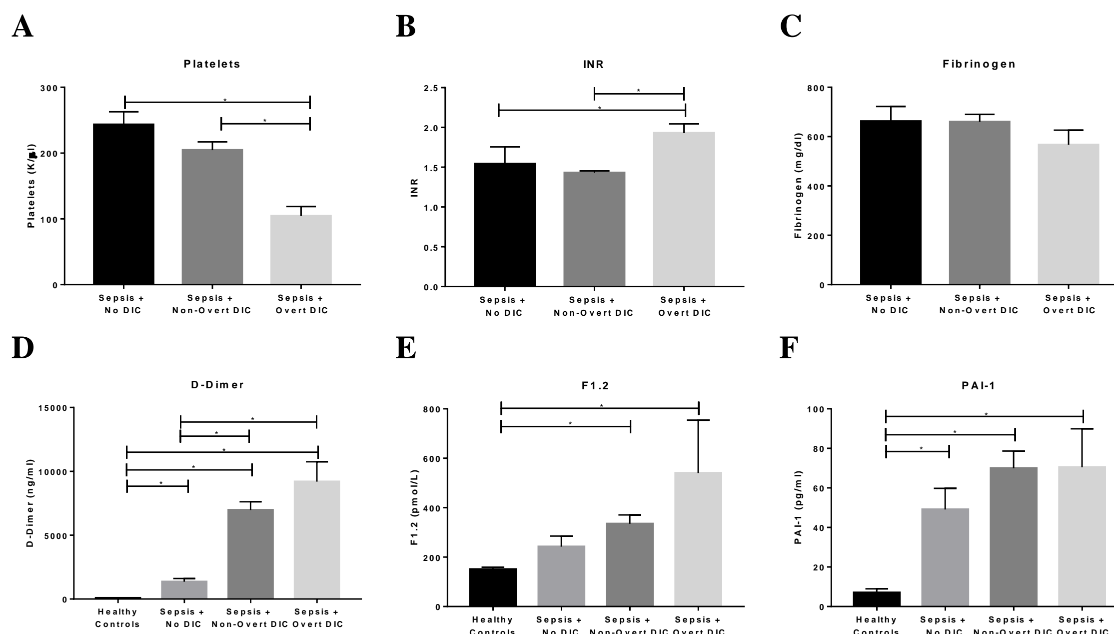


**Figure 17. Baseline Hemostatic Biomarker Levels Stratified by DIC Score**. Significance calculated between groups using the Kruskal-Wallis ANOVA with Dunn's multiple comparison test and p<0.05 as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Infection and Inflammation Biomarkers.** Numerous markers of infection and inflammation showed significant variation within the sepsis patient cohort based on DIC score category. Data is shown in Figure 18 and 19 and in Tables 43-44 in Appendix B.

Biomarkers of infection demonstrated a distinct association with coagulopathy. Nucleosomes showed significant elevation only in patients with overt DIC compared to healthy controls and to patients with sepsis without DIC. Septic patients without DIC showed no elevation in nucleosome levels. Although HMDB-1 trended towards

increasing levels with increasing severity of DIC, significant differences were only observed between healthy controls and all patient groups. This may be a function of limited statistical power due to the size of the patient cohort, as when analyzed separately from healthy controls (data not shown), differences in HMGB-1 levels were seen between patients with non-overt DIC and overt DIC. Procalcitonin demonstrated the greatest distinction between levels of coagulopathy. In addition to significant elevations compared to healthy controls in all sepsis patients regardless of DIC status, procalcitonin was also significantly elevated in both non-overt and overt DIC compared to sepsis alone. This supports the role of infection response, including infection-related nuclear material, in the molecular pathogenesis of DIC.

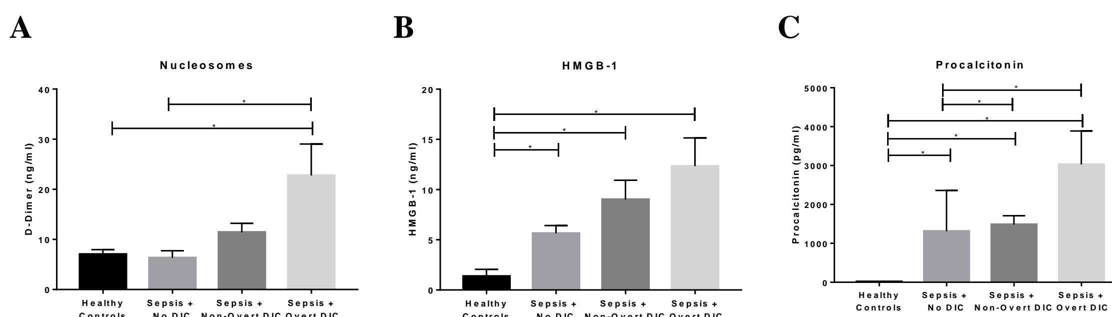


**Figure 18. Baseline Infection Biomarker Levels Stratified by DIC Score.** Significance calculated between groups using the Kruskal-Wallis ANOVA with Dunn's multiple comparison test and $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

Inflammatory cytokines, including the IL-6:IL-10 ratio which was calculated as a means to evaluate the balance between pro- and anti-inflammatory processes ongoing within a single patient, were significantly elevated in sepsis patients compared to healthy controls regardless of coagulation status. The exception to this elevation was IL-1β, which did not show elevation in the sepsis + no DIC group compared to controls.

Variation within sepsis patients based on DIC score was observed only for IL-8, which was significantly elevated in overt DIC compared to no DIC, and EGF, which was reduced in overt DIC compared to both no DIC and non-overt DIC. If analyzed without the inclusion of the healthy control population (data not shown), the increases in non-overt and overt DIC reached statistical significance in comparison to no DIC for IL-8, IL-8, and TNFα. Additional significant differences were noted for IL-10 in overt DIC compared to no DIC and in IL-1β and MCP-1 in non-overt DIC compared to overt DIC.

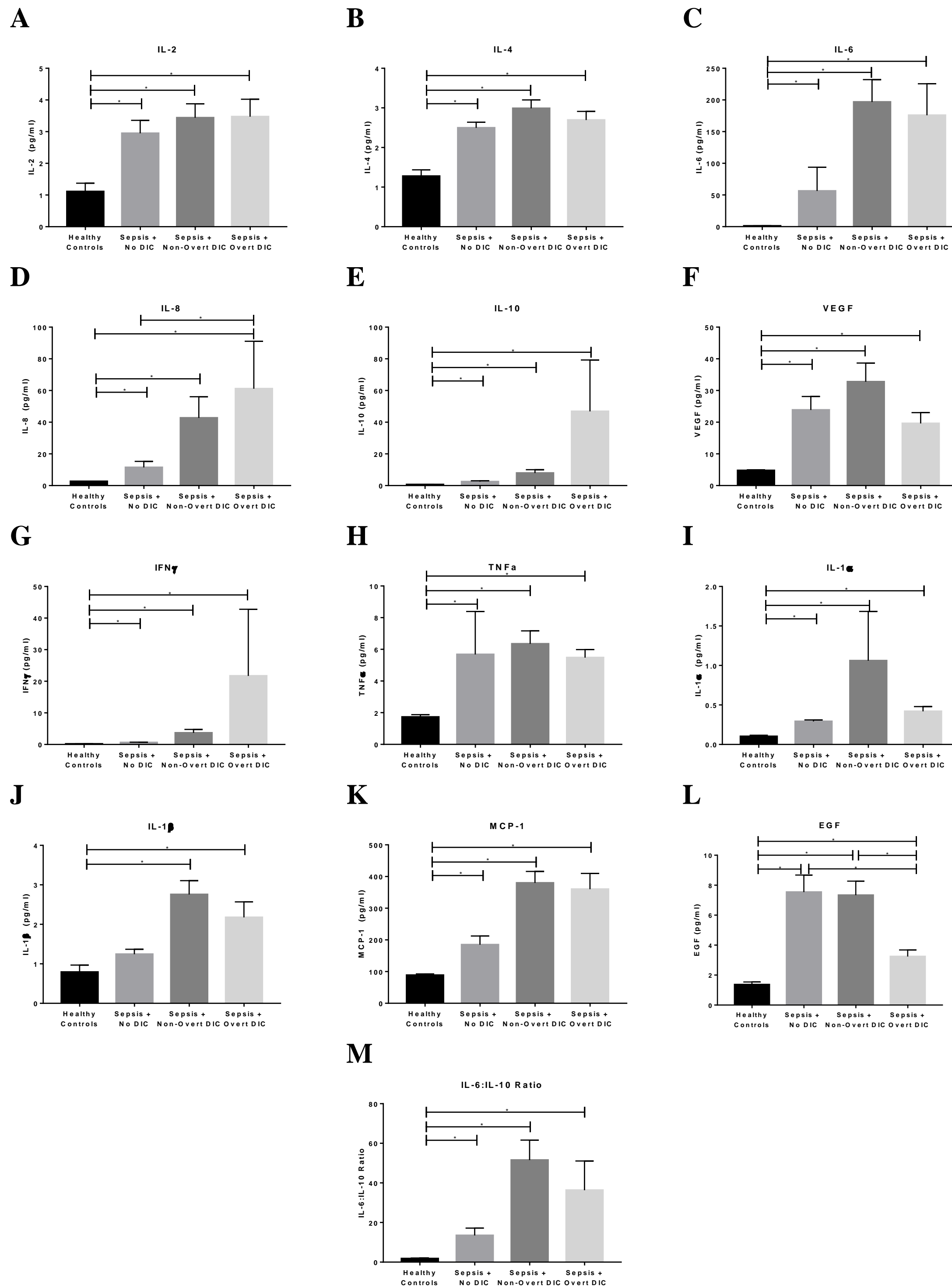


**Figure 19. Baseline Inflammatory Biomarker Levels Stratified by DIC Score.** Significance calculated between groups using the Kruskal-Wallis ANOVA with Dunn's multiple comparison test and $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Endothelial Biomarkers.** Significant variation of levels of endothelial biomarkers based on DIC score was observed, as shown in Figure 20 and in Table 45 in Appendix B.

Protein C is known to be implicated in the pathogenesis of sepsis-associated DIC, and decreased levels are generally associated with poor outcome. In this cohort, protein C was decreased in sepsis patients compared to healthy controls regardless of coagulation status. Additionally, protein C showed a significant decrease in patients with overt DIC compared to patients with sepsis and no DIC. This corroborates prior research regarding Protein C in sepsis-associated coagulopathy. Depletion of this endogenous anticoagulant contributes to the development of coagulopathy in sepsis patients, and this pathway is a major therapeutic target. In contrast, another endogenous anticoagulant, TFPI, showed no significant variation based on DIC status, although it was elevated in patients with sepsis compared to healthy controls regardless of DIC score. TFPI release is induced by heparin therapy. All patients enrolled in this study received prophylactic doses of UFH; no additional UFH or LMWH use was reported, therefore this treatment does not represent a confounding factor for TFPI levels in this cohort.

Ang-2 also varied significantly based on DIC status, with significant elevation in patients with overt DIC compared to those with sepsis and no DIC as well as significant elevations in all patient groups compared to healthy controls. Both endocan and vWF were elevated in sepsis patients compared to controls regardless of DIC status; however, no variation was seen within sepsis patients based on DIC status.

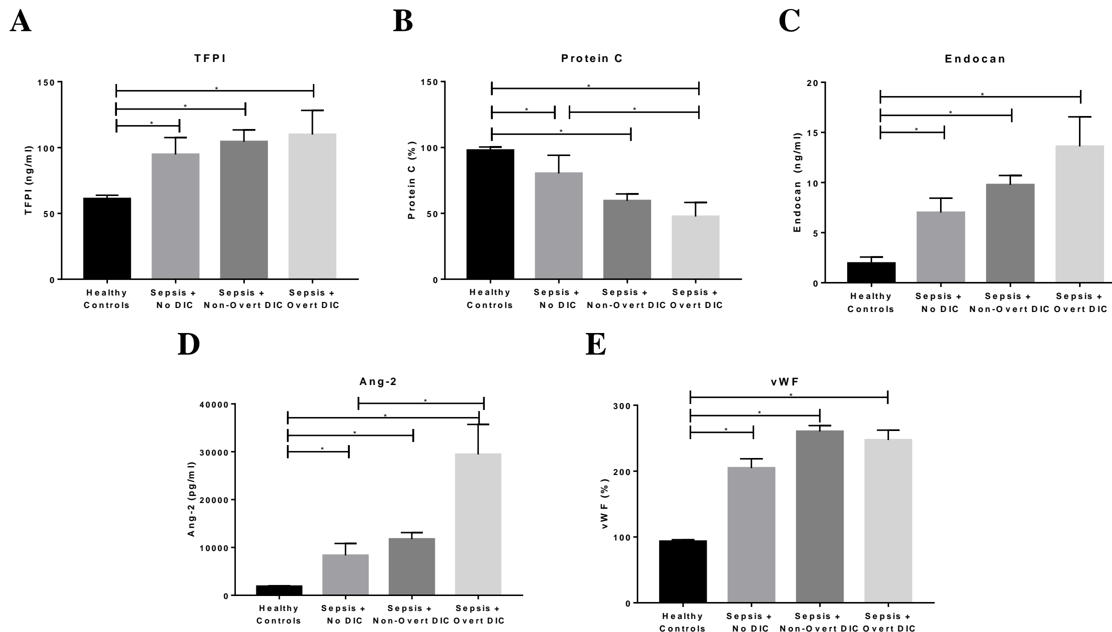


**Figure 20. Baseline Endothelial Biomarker Levels Stratified by DIC Score.** Significance calculated between groups using the Kruskal-Wallis ANOVA with Dunn's multiple comparison test and $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Platelet Biomarkers.** Although a trend towards changes in platelet markers was seen between patients in the different DIC score categories, no significant differences in CD40L, MP, MP-TF, or PF4 were observed based on DIC score status. Significant elevation in all platelet markers was observed in all groups compared to the healthy controls. Although platelets may be significantly activated in sepsis and therefore secrete high levels of platelet-derived biomarkers, this increase in biomarker level may be confounded by the depletion of platelets due to consumptive coagulopathy in severe DIC. The contrasting findings of elevated platelet function markers in sepsis despite reduced platelet count with inverse relationships between platelet function markers and SOFA scores are in line with these unclear observations about platelet function markers in sepsis-associated DIC. Data is shown in Figure 21 and in Table 46 in Appendix B.

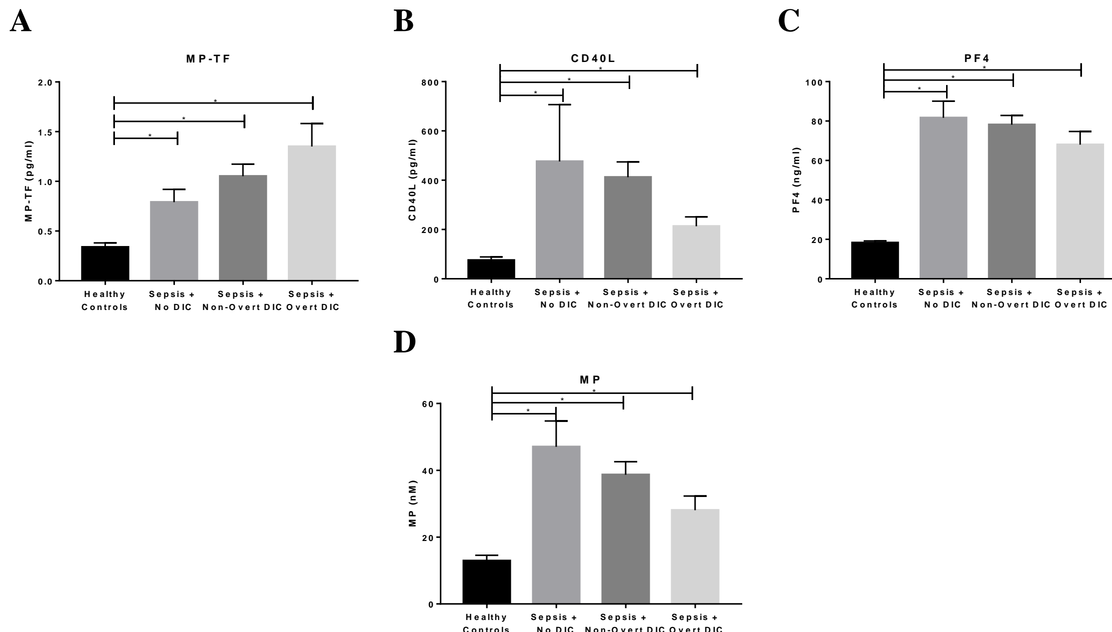


**Figure 21. Baseline Platelet Biomarker Levels Stratified by DIC Score.** Significance calculated between groups using the Kruskal-Wallis ANOVA with Dunn's multiple comparison test and $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM

## Association of Baseline Biomarkers with Mortality

The 28 day mortality in this patient cohort was 14.6% (88 survivors and 15 non-survivors). Information on time to mortality was not available. Differences in baseline biomarker levels between survivors and non-survivors were evaluated using the Mann-Whitney t-test with $p<0.05$ as the cutoff for significance. The predictive power of each biomarker for mortality was evaluated using receiver operating curve (ROC) analysis; the area under the curve (AUC) is reported as the quantification of this analysis. Using this analytical technique, an AUC value of 0.5 for a biomarker represents no ability to predict outcome while an AUC value of 1.0 represents ability to perfectly predict outcome with 100% sensitivity and 100% specificity.

**Hemostatic Biomarkers.** Baseline levels of hemostatic biomarkers were poor predictors of mortality in the sepsis patient population. Of the measured hemostatic markers, only PAI-1 showed a significant difference between survivors and non-survivors (p=0.015). Elevated PAI-1 has the potential to increase mortality in patients with DIC by preventing the fibrinolytic breakdown of clots in the microvasculature. This may increase vascular occlusion and lead to organ dysfunction out of proportion with the amount of thrombosis as quantified by D-Dimer, coagulation factor, or platelet levels. Interestingly, none of the markers typically used to describe coagulopathy in septic patients (INR, platelet count, D-Dimer, or fibrinogen) showed significant differences between survivors and non-survivors. Although the DIC score itself is not designed to predict mortality, the lack of association of these markers with patient outcome suggests that this scoring system may be missing important parameters. The AUC values for prediction of mortality with these markers were also poor, with values of 0.61 for platelets, 0.60 for INR, 0.51 for fibrinogen, 0.60 for D-Dimer, and 0.54 for F1.2. The predictive value for PAI-1 was slightly better, with an AUC of 0.70. Data is shown in Figure 22 and in Table 47 in Appendix B.

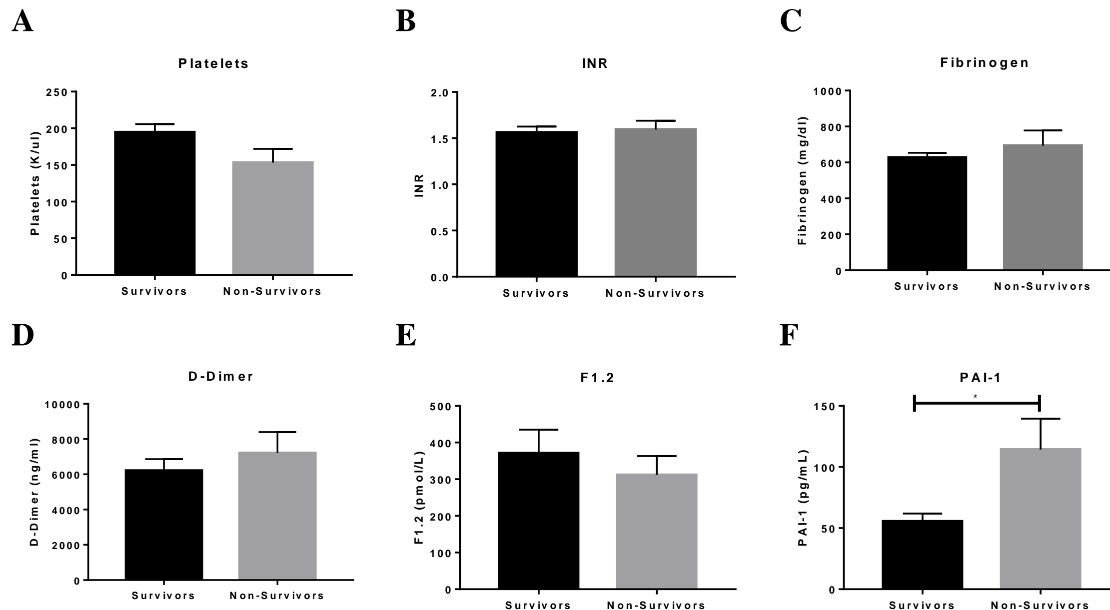


**Figure 22. Association of Baseline Hemostatic Biomarker Levels with Survival.** Significance calculated between groups using the Mann-Whitney test with p<0.05 as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Infection and Inflammation Biomarkers.** Significant associations were observed between markers of infection and mortality. Both HMGB-1 (p=0.031, AUC=0.67) and procalcitonin (p=0.0005, AUC=0.77) were significantly elevated in non-survivors compared to survivors, as shown in Figure 23 and in Table 48 in Appendix B. The elevation of HMGB-1 and procalcitonin in non-survivors demonstrates that infection and infection response are major determinants of patient outcome. Furthermore, procalcitonin had the highest AUC for the prediction of mortality of any biomarker measured in this study. While procalcitonin is not a therapeutic target, its predictive ability is important to recognize as procalcitonin is available as a clinical laboratory test and would therefore be relatively simple to incorporate into a new algorithm for evaluation of patients with sepsis or DIC. While a weaker predictor than procalcitonin,

HMGB-1 may be a direct mechanistic link between infection response and the physiological dysfunction that ultimately results in death. Despite the association of nucleosomes with severity of coagulopathy, the elevation of nucleosomes in non-survivors compared to survivors was not statistically significant and showed a poor predictive value (AUC = 0.58). This suggests that while infection is a critical determinant of both development of coagulopathy and patient outcome, different aspects of this response may play distinct physiological roles.

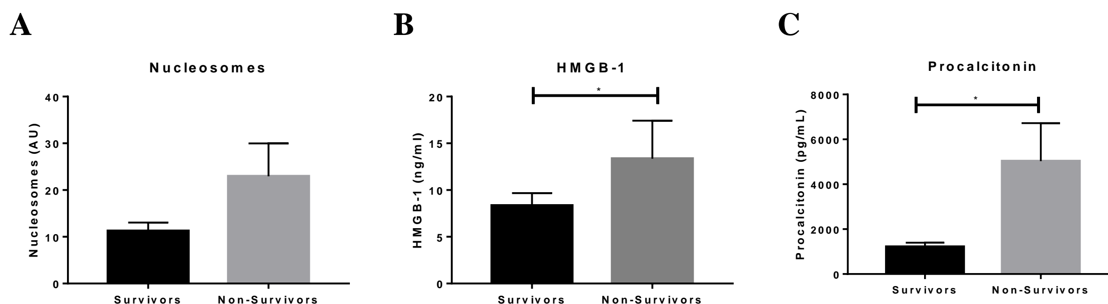


**Figure 23. Association of Baseline Infection Biomarker Levels with Survival.** Significance calculated between groups using the Mann-Whitney test with $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

Of the measured inflammatory markers, IL-6 (p = 0.02, AUC = 0.70) and IL-8 (p = 0.015, AUC = 0.70) were significantly elevated in non-survivors compared to survivors. Predictive values for mortality for all other inflammatory cytokines were relatively poor (IL-2, 0.52; IL-4, 0.55; IL-10, 0.58; VEGF, 0.57; IFNγ, 0.54; TNFα, 0.52; IL-1α, 0.60; IL-1β, 0.58; MCP-1, 0.56; EGF, 0.58, IL-6:IL-10 Ratio, 0.61). Data is shown in Figure 24 and in Table 49 in Appendix B.

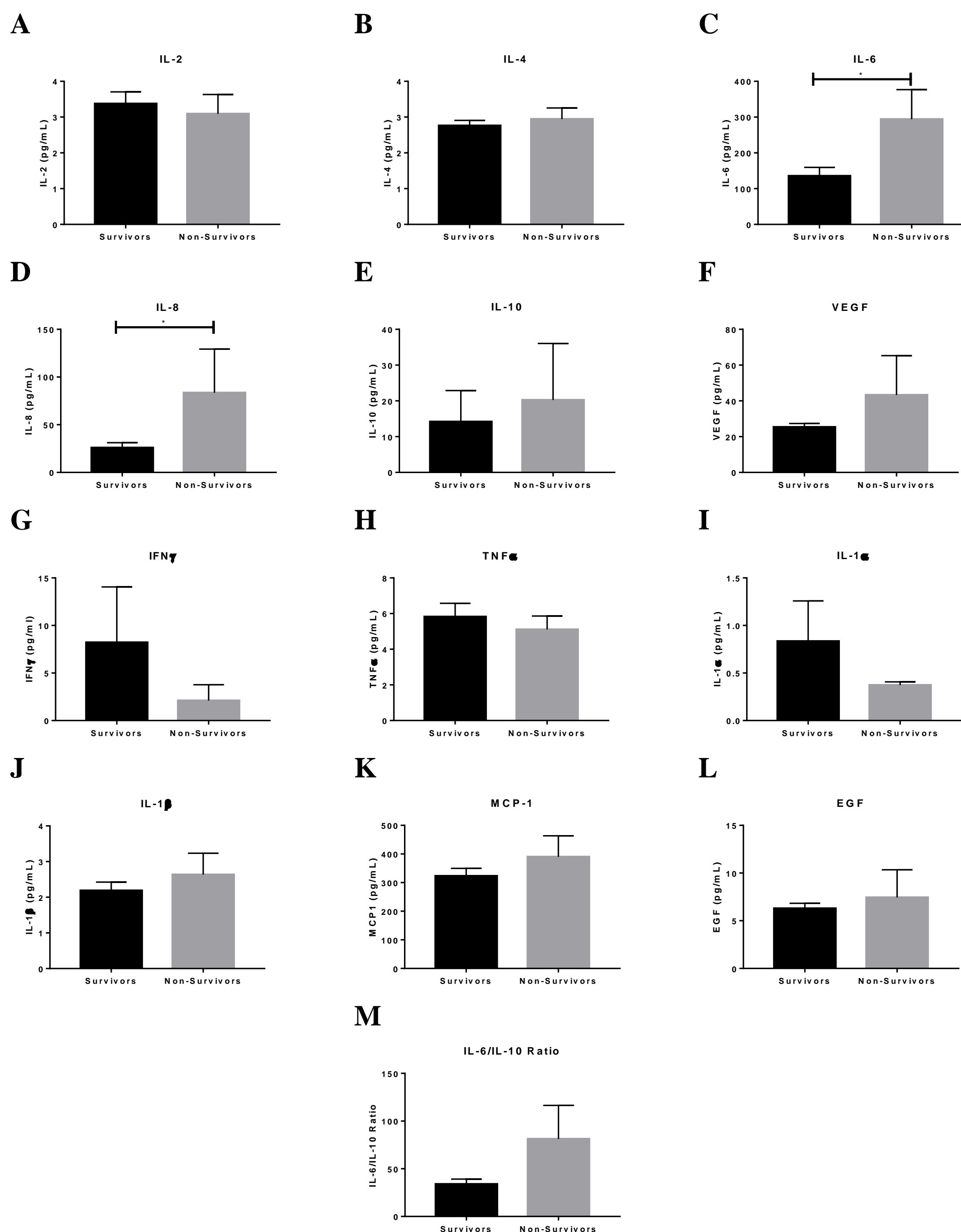


**Figure 24. Association of Baseline Inflammatory Biomarker Levels with Survival.** Significance calculated between groups using the Mann-Whitney test with $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Endothelial Biomarkers.** Significant association was seen between markers of endothelial function and mortality. TFPI (AUC=0.55) and vWF (AUC=0.58) showed no significant variation based on survival. However, significant variation was seen for the remainder of the endothelial markers. In contrast to almost all other evaluated markers, Protein C showed a significant reduction in non-survivors compared to survivors (p=0.0093, AUC=0.71). Both endocan (p=0.025, AUC=0.58) and Ang-2 (p=0.001, AUC=0.76) were significantly elevated in non-survivors compared to survivors. Data is shown in Figure 25 and in Table 50 in Appendix B.

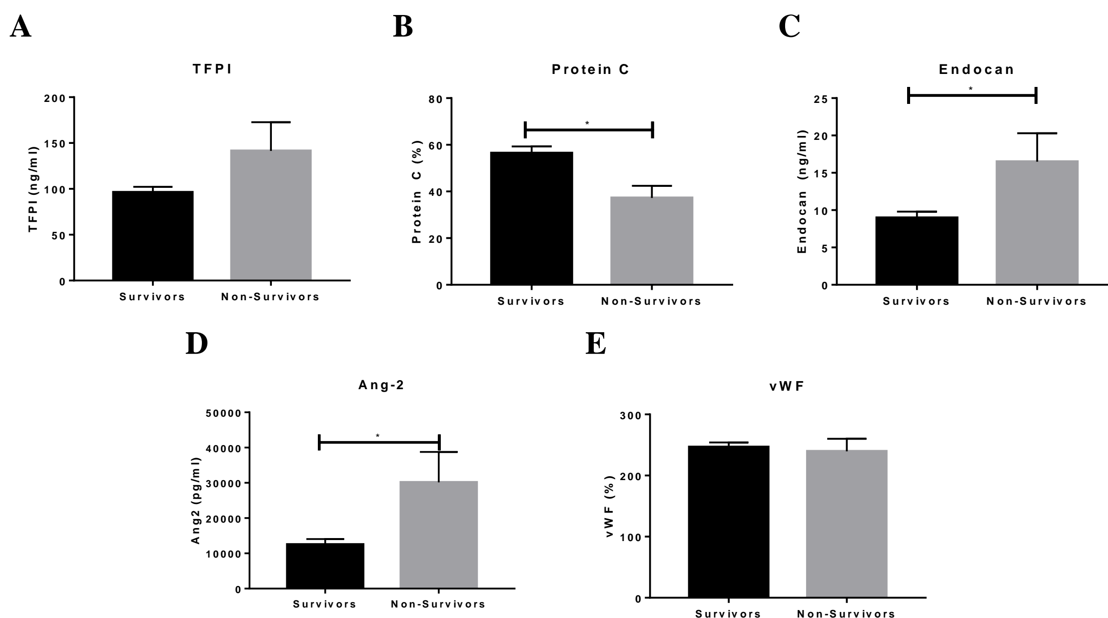


**Figure 25. Association of Baseline Endothelial Biomarker Levels with Survival.** Significance calculated between groups using the Mann-Whitney test with $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Platelet Biomarkers.** Overall, minimal associations were observed between markers of platelet function and patient outcome. Only PF4 showed a significant association with mortality (p=0.016, AUC=0.70), with significantly lower levels observed in non-survivors compared to survivors. All other platelet biomarkers had weak predictive values for mortality (CD40L, 0.55; MP, 0.53; MP/TF, 0.62). Data is shown in Figure 26 and in Table 51 in Appendix B.

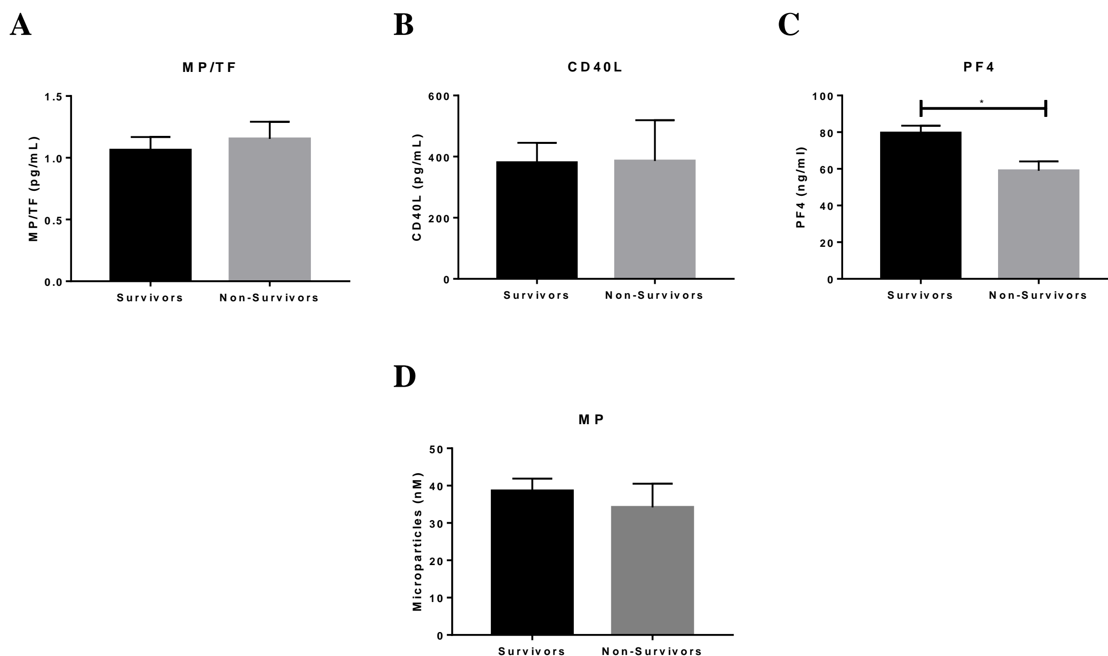


**Figure 26. Association of Baseline P1atelet Biomarker Levels with Survival.** Significance calculated between groups using the Mann-Whitney test with p<0.05 as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Association of Biomarkers with DIC Score and Mortality on ICU Days 4 and 8**

On Day 4, 57 patients remained in the ICU. Of the 46 patients who left the ICU before this time, 4 died and the remaining 42 were transferred to other units or discharged. Of the 57 patients remaining in the ICU on day 4, 11 had no DIC, 26 had non-overt DIC, and 10 had overt DIC. At this time, only D-Dimer and Protein C showed a significant association with DIC score category. 47 survivors and 10 non-survivors remained in the ICU on day 4. Significant elevations were observed in IL-6 ($p=0.028$), IL-8 ($p=0.005$) and endocan ($p=0.025$) at this time point. Data for day 4 biomarker levels is shown in Tables 52-56 and 62-66 in Appendix B.

On Day 8, 24 survivors and 6 non-survivors remained in the ICU. Of these, 8 had no DIC and 22 had non-overt DIC; no patients had overt DIC on day 8. Accordingly, comparisons were only made between patients with sepsis and patients with non-overt DIC. Significance between these two patient groups was calculated using the Mann-Whitney t test with $p<0.05$ as the cutoff for significance. Protein C and IL-2 were significantly reduced in patients with non-overt DIC compared to patients with no DIC. The reduction in protein C in patients with more severe coagulopathy is consistent with previous findings and with knowledge about the role of protein C in the pathophysiology of DIC. Significant elevations were observed in D-Dimer, procalcitonin, IL-6, IL-8, IL-10, MCP-1, MP/TF, endocan, and Ang-2 in patients with non-overt DIC compared to patients with sepsis and no DIC. The increase in the number of biomarkers demonstrating an association with DIC status on day 8 may be due to the decrease in the number of patient groups due to the absence of overt DIC patients at this time point. The elevations

in IL-6 (p=0.008) and IL-8 (p=0.009) that were observed on days 0 and 4 persisted on day 8. Additionally, on day 8, a significant elevation in D-Dimer (p=0.029) as well as a significant reduction in protein C (p=0.025) in non-survivors compared to survivors, suggesting a major role for coagulation dysfunction in determining outcome in patients remaining in the ICU at this time point. Data for Day 8 biomarker levels are shown in Tables 57-61 and 67-71 in Appendix B.

**Association of Biomarkers with Platelet Count**

Throughout this study, the association between markers of platelet function and outcome was relatively weak. However, numerous studies have demonstrated that the platelet response in sepsis and DIC is integral to the pathophysiology of this disease. The association of factors generated or released by platelets with clinical status and outcome may be confounded by the consumption of platelets during the coagulopathy characteristic of sepsis-associated DIC. Recently, significant associations were demonstrated between levels of platelets and hemostatic, inflammatory, and endothelial markers in patients with sepsis-associated coagulopathy (Claushuis 2016). In line with this analysis, patients were divided into groups based on platelet count on ICU Day 0 of <100 K/µl (n=21), 100-149 K/µl (n=20), or ≥150 K/µl (normal range; n = 61). The normal range for platelet count is 150-400 K/µl. Patients with a platelet count of 100-149 K/µl have thrombocytopenia; however, a platelet count within this range does not pose a significant bleeding risk. For patients with a typical platelet count of close to 150 K/µl, a platelet count within the 100-149 K/µl range may not represent a significant drop in platelet count. A platelet count of <100 K/µl indicates a marked drop in platelets from the

normal range and may lead to an increased risk of bleeding. A platelet count of <50 K/µl indicates a markedly increased bleeding risk; however, only 3 patients fell within this range in this cohort and thus these patients could not be analyzed separately. Differences in biomarker levels between these patient groups were quantified using the Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test and $p<0.05$ as the cutoff for significance.

**Hemostatic Biomarkers.** Of the hemostatic biomarkers, only INR and fibrinogen showed a significant association in platelet count, with an elevated INR observed in patients with a platelet count of 100-149 K/µl compared to those with a platelet count of ≥150 K/µl. Fibrinogen was significantly elevated in patients with a platelet count of ≥150 K/µl compared to those with a platelet count of >100 K/µl. No significant differences were observed in D-Dimer, F1.2, or PAI-1. Data is shown in Figure 27 and in Table 72 in Appendix B.

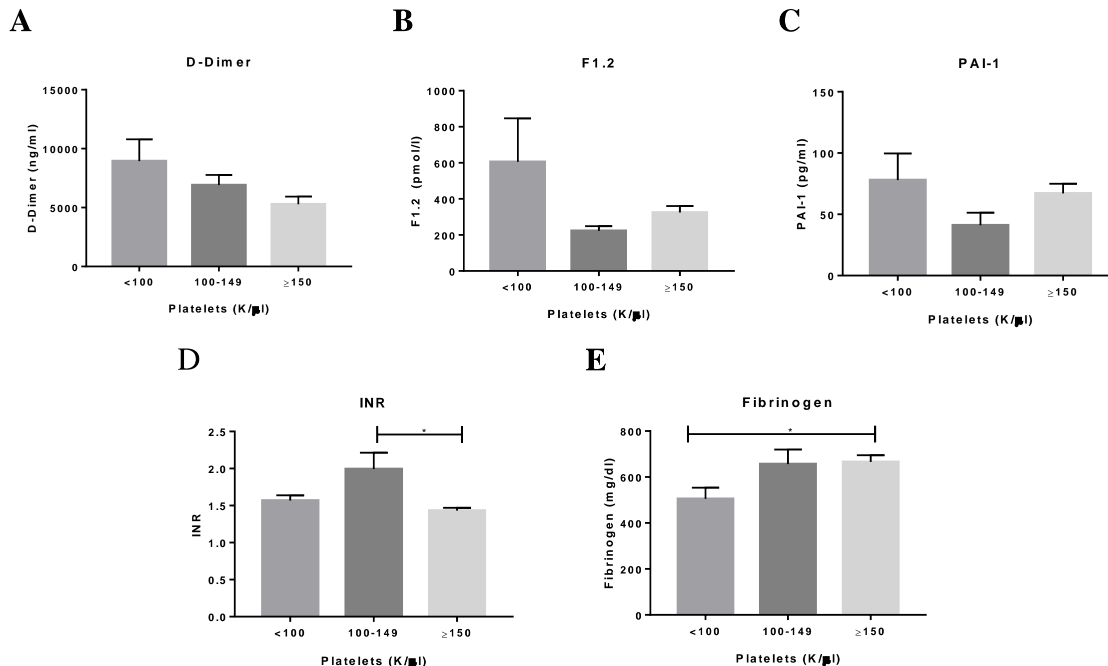


**Figure 27. Baseline hemostatic biomarker levels in patients stratified by platelet count**. Significance calculated between groups using the Kruskal-Wallis ANOVA with Dunn's multiple comparison test and p<0.05 as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Infection and Inflammation Biomarkers.** Of the infectious biomarkers, only procalcitonin showed significant variation based on platelet count. Patients with a platelet count of <100 K/µl or 100-149 K/µl had significantly elevated levels of procalcitonin compared to those with a platelet count within the normal range. This demonstrates an association between infection, as quantified by procalcitonin, and thrombocytopenia. Data is shown in Figure 28 and in Table 73 in Appendix B.

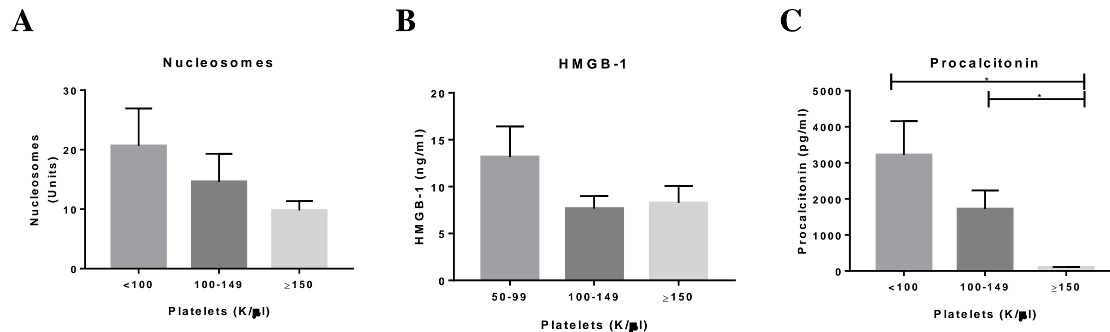


**Figure 28. Baseline Infection Biomarker Levels in Patients Stratified by Platelet Count.** Significance calculated between groups using the Kruskal-Wallis ANOVA with Dunn's multiple comparison test and p<0.05 as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

Of the inflammatory biomarkers, VEGF, TNFα, and EGF showed significant variation based on platelet count. The highest levels of VEGF and EGF were observed in patients with platelet counts of ≥150 K/µl, with significant differences between patients with platelet counts of ≥150 K/µl and <100 K/µl for both factors and for ≥150 K/µl and 100-149 K/µl for EGF. Conversely, the highest levels of TNFα were observed in patients with platelet counts of <100 K/µl. Data is shown in Figure 29 and in Table 74 in Appendix B

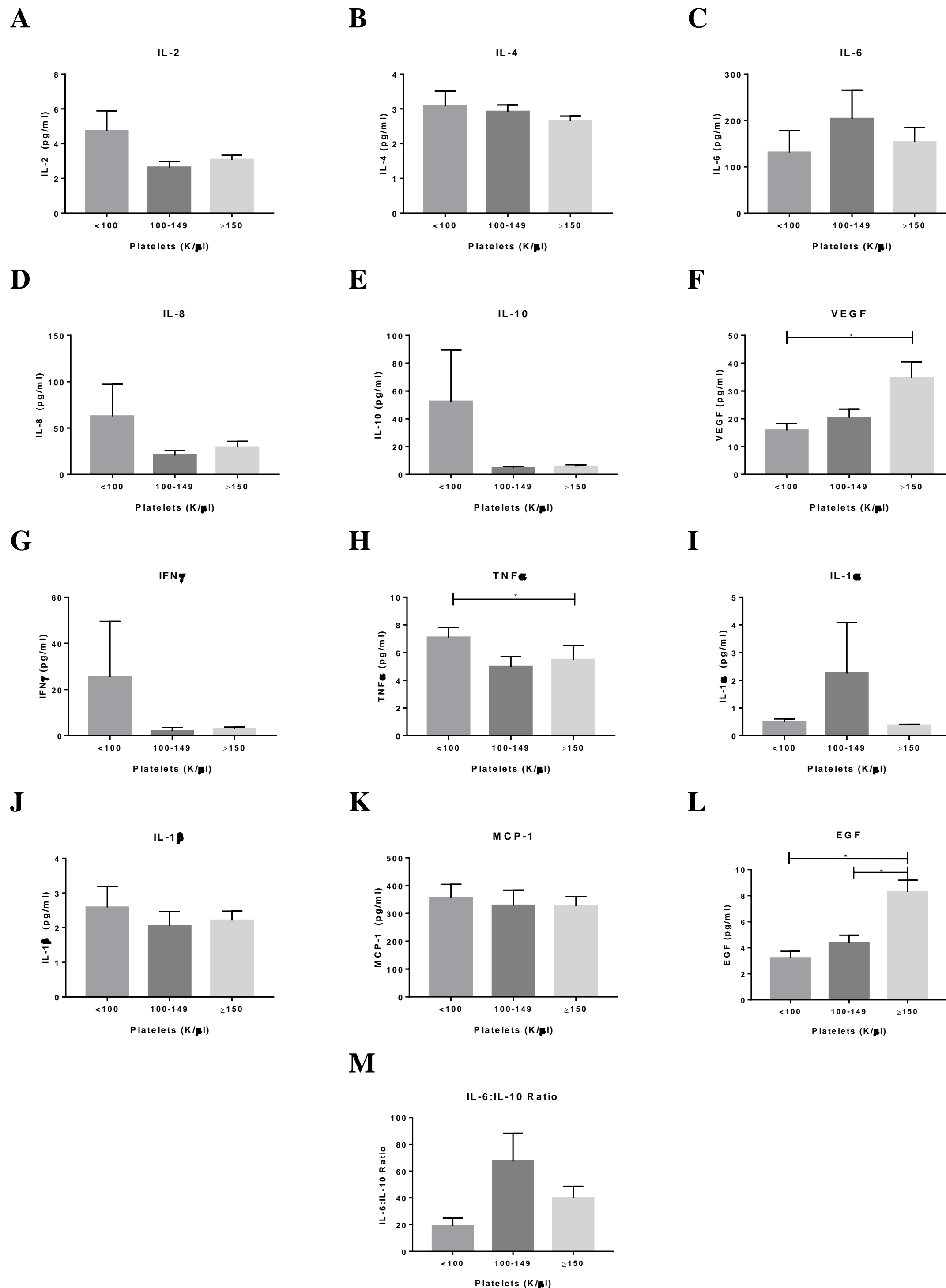


**Figure 29. Baseline Inflammatory Biomarker Levels in Patients Stratified by Platelet Count.** Significance calculated between groups using the Kruskal-Wallis ANOVA with Dunn's multiple comparison test and $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Endothelial Biomarkers.** Protein C and Ang-2 showed significant variation based on platelet count. Protein C was significantly reduced in patients with a platelet count of 100-149 K/µl compared to those with a platelet count of ≥150 K/µl. Ang-2 increased with decreasing platelet count, with significant differences observed between those with a normal platelet count and those with mild or severe thrombocytopenia. Data is shown in Figure 30 and in Table 75 in Appendix B.

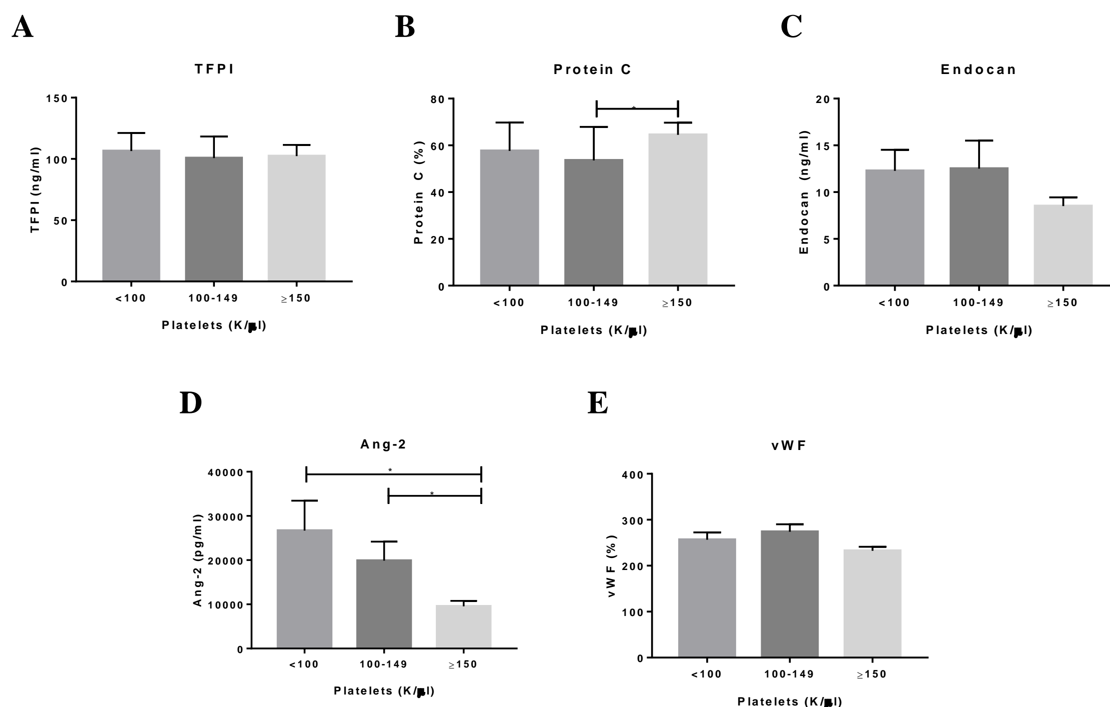


**Figure 30. Baseline Endothelial Biomarker Levels in Patients Stratified by Platelet Count**. Significance calculated between groups using the Kruskal-Wallis ANOVA with Dunn's multiple comparison test and $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Platelet Biomarkers.** Patients with sepsis-associated DIC have significantly elevated levels of several biomarkers associated with platelet activity. However, these same patients often have significantly reduced platelet counts due to consumption, which may confound the utility of these platelet biomarkers. Levels of some markers of platelet activity may be the most elevated in patients with platelet counts in the normal range of ≥150 as patients with severely depleted platelet levels may not be able to release these markers into circulation at high levels from their depleted pool of platelets. Alternatively, platelet markers may show the greatest elevation in patients with significantly reduced platelet counts (<100 K/µl if factors are released at high levels during platelet consumption. Furthermore, a combination of these two processes may contribute to the level of each biomarker in a given patient.

CD40L and MP increased with increasing platelet count, with statistically significant differences between patients with normal platelet counts of ≥150 K/µl compared to those of <100 K/µl for both parameters and for patients with a platelet count of ≥150 K/µl compared to those with a platelet count of 100-149 K/µl for MP. The trend observed for PF4 was more complex, with the highest level of PF4 measured in patients with platelets of 100-149 K/µl and significant differences between 100-149 K/µl and <100 K/µl and between ≤150 K/µl and <100 K/µl. Data is shown in Figure 31 and in Table 76 in Appendix B.

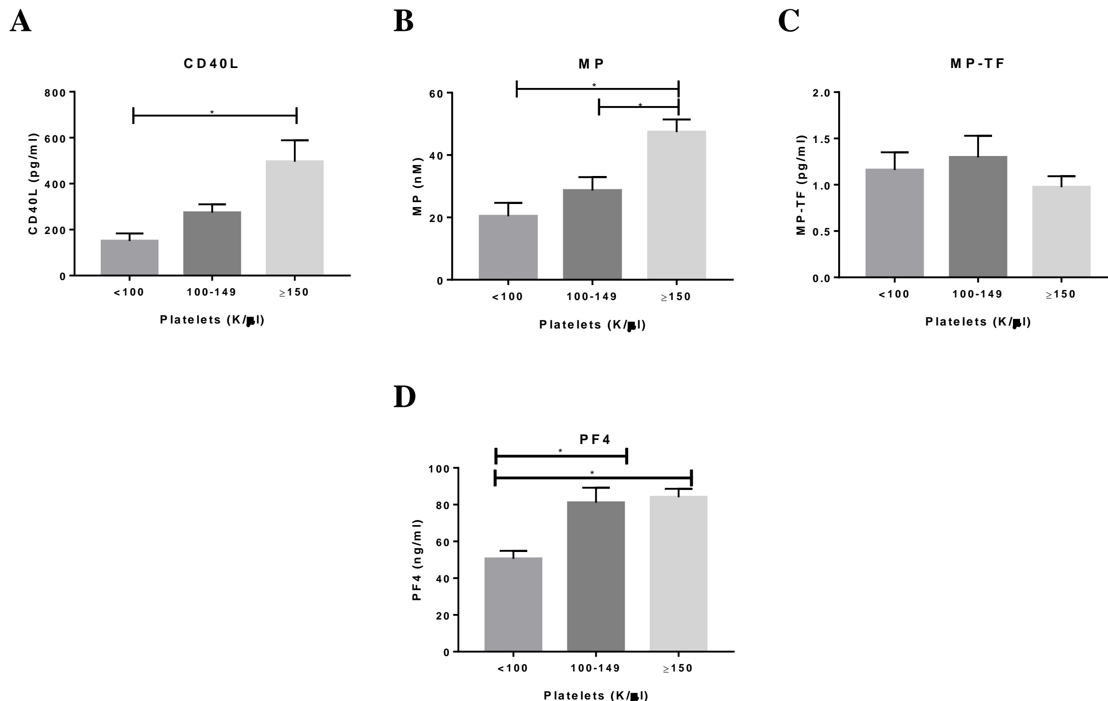


**Figure 31. Baseline Platelet Biomarker Levels in Patients Stratified by Platelet Count.** Significance calculated between groups using the Kruskal-Wallis ANOVA with Dunn's multiple comparison test and $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

## Changes in Biomarker Levels over Time

Several methods were used to analyze the change in biomarker levels over time within a given patient. For each marker in each patient, changes between baseline and day 4 levels were analyzed in terms of direct change (day 4 level minus baseline level), ratio of day 4 to baseline, and percent change from baseline to day 4. These parameters were calculated in the 37 survivors and 9 non-survivors with complete data at both baseline and day 4. Ratio and percent change could not be calculated in all patients due to undetectably low concentrations of factors in some samples, resulting in the presence of a zero in the denominator.

Using the direct comparison (subtraction method) approach, significant differences were observed between survivors and non-survivors for CD40L and protein C. When analyzed using the day 4 to baseline ratio and the percent change from baseline to day 4, significant differences were observed between survivors and non-survivors for VEGF and protein C. Contrary to previous studies, greater increases in protein C levels were seen in non-survivors than in survivors throughout the course of hospitalization. This is in direct contrast to previous reports that have demonstrated that increasing levels of protein C is a favorable prognostic indicator in sepsis-associated DIC. This may be due to the initial lower levels of protein C in non-survivors compared to survivors, allowing for a greater possible increase in these patients between baseline measurement and normal physiological levels.

**Potential Confounding Factors**

Age is one of the most significant confounding factors for biomarker analysis. Previous studies have demonstrated that older patients with sepsis have distinct biomarker profiles compared to younger patients, and that the associations between biomarkers and outcome may vary based on patient age (Opal, Girard, & Ely, 2005; Rondina 2015). Accordingly, the correlation between baseline biomarker level and age was calculated. Significant associations were seen between age and platelets ($p=0.018$, $r=-0.23$), INR ($p=0.009$, $r=0.26$), procalcitonin ($p=0.024$, $r=0.22$), VEGF ($p=0.033$, $r=-0.21$) IFNγ, ($p=0.031$, $r=-0.21$), endocan ($p<0.001$, $r=0.35$), and Ang-2 ($p=0.005$, $r=0.28$).

For further analysis, patients were divided into two categories based on age <65 vs. ≥65 years. Although aging is a continuous process with no universal cutoff point, the

use of 65 as a cutoff point was selected on the basis of similar analyses (Baldwin 2013; Martin 2006; Opal 2005; Rondina 2015). There were 66 patients younger than 65 and 37 patients older than 65. Data for markers with significant variation based on age or survival is shown in Figure 32. Significant differences were found based on age in INR (higher in the elderly, $p=0.017$), VEGF (lower in the elderly, $p=0.020$), Protein C (lower in the elderly, $p=0.0057$), and endocan (higher in the elderly, $p=0.0004$) using the Mann-Whitney t test with $p<0.05$ as the cutoff for significance.

In order to evaluate the potential interactions between age, survival, and biomarker levels, patients were subdivided into four groups; survivors younger than 65 (n=60), survivors aged 65 or older (n=27), non-survivors younger than 65 (n=6), and non-survivors aged 65 or older (n=9). Interaction between age and survival for each biomarker was assessed using an ordinary 2-way ANOVA with $p<0.05$ as the cutoff for significance.

For TFPI, significant variation was seen based on both age ($p<0.0001$) and survival category ($p=0.002$), with a statistically significant interaction between the two ($p<0.0001$). Nucleosomes varied significantly based on survival ($p=0.033$), but not on age. Procalcitonin varied significantly based on age ($p=0.0001$) and survival ($p<0.0001$), with a significant interaction between the two ($p=0.0006$). IL-6 varied significantly based on survival ($p=0.017$) but not age. IL-8 varied significantly based on age ($p=0.028$) and survival ($p=0.004$), with a significant interaction between the two ($p=0.012$). IL-6:IL-10 ratio varied significantly based on survival ($p=0.015$) but not age. PAI-1 varied significantly based on both age ($p=0.020$) and survival ($p=0.0004$), with no significant

interaction between the two (p=0.098). Endocan varied significantly based on survival (p=0.016) but not age. Ang-2 varied significantly based on both age (p=0.0124) and survival (p=0.0001), with a significant interaction between the two (p=0.002). PF-4 varied significantly based on survival (p=0.027) but not age. Other markers did not show any significant variation based on age or survival when analyzed in this manner.

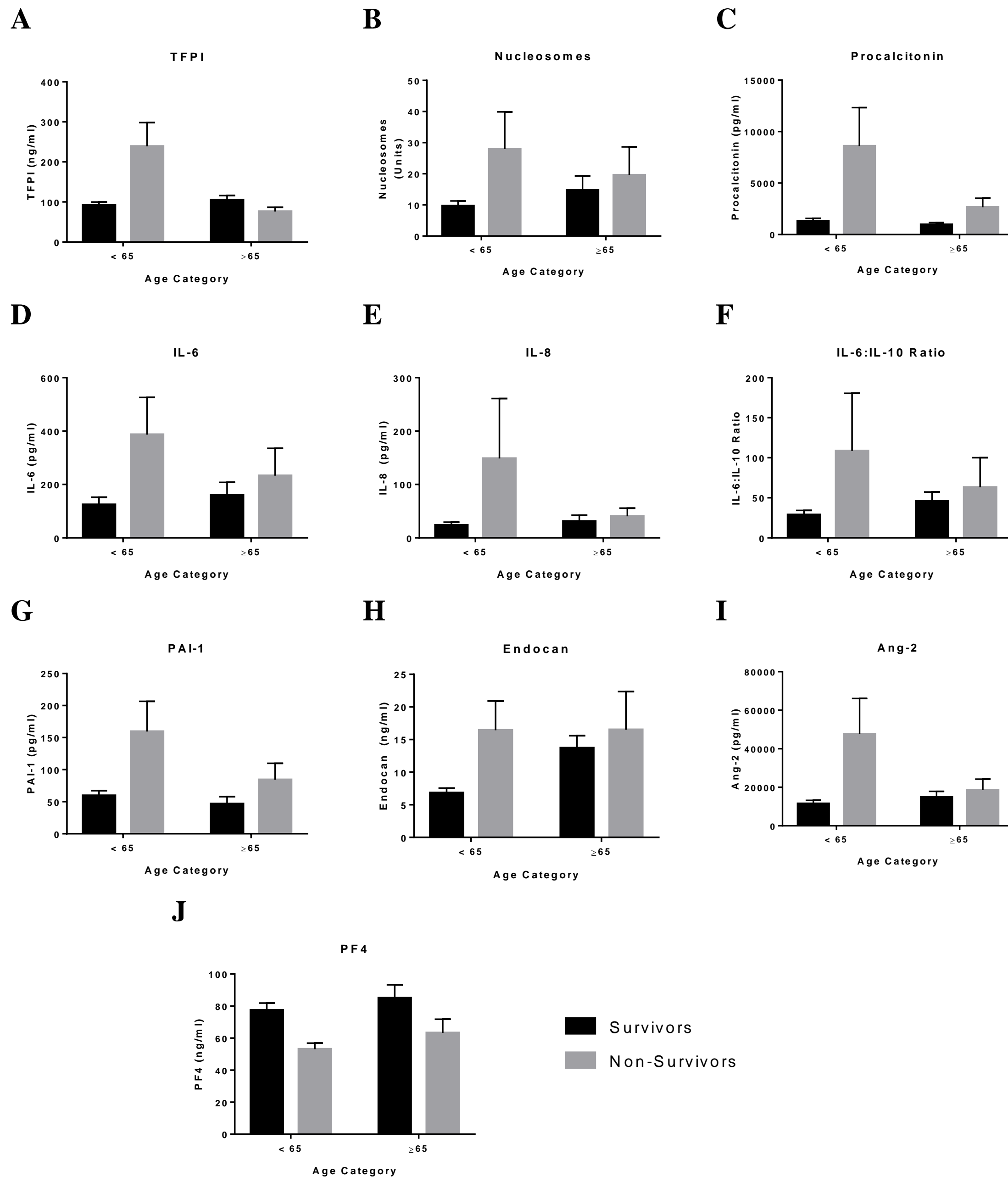


**Figure 32. Biomarker Levels Showing Significant Difference by Two-Way ANOVA when Subdivided by Age <65 vs. Age ≥65 and Survival or Non-Survival.** Data is shown as mean ± SEM.

Additional confounding factors include gender and BMI as well as comorbidities including history of hypertension, recent surgery, recent or active cancer, cirrhosis, pulmonary disease, congestive heart failure, diabetes, and cardiovascular disease were also analyzed. Differences between categorical variables were assessed using the Mann-Whitney t test, and correlations between biomarker levels and continuous variables (age and BMI) were assessed using Spearman correlation coefficients, summarized in Table 23. No significant differences were seen in the Day 0 levels of any biomarkers based on history of hypertension (present in 47 patients) or recent surgery (present in 23 patients). BMI showed no significant correlations with any analyzed variable. F1.2, IL-2, IL-4, IL-6, IL-8, IL-1β, MCP-1, EGF, HMGB-1, MP, MP/TF, and PF4 did not show any relationship with any analyzed confounding variable.

Of particular interest in any condition involving coagulation is liver function, as many coagulation factors are produced in the liver, and patients with liver dysfunction may not produce normal levels of some coagulation factors and related proteins. In this cohort, 6 patients had cirrhosis. These patients showed significant reductions in fibrinogen ($p=0.031$) and VEGF ($p=0.044$) compared to patients without cirrhosis.

The comorbidity with the greatest association with biomarker levels was history of pulmonary disease. D-Dimer ($p=0.006$), IFNγ ($p=0.018$), IL-10 ($p=0.012$), nucleosomes ($p=0.044$), and TNFα ($p=0.040$) were significantly elevated in patients without pulmonary disease compared to those with pulmonary disease (17 patients).

Common comorbidities included history of cardiovascular disease (22 patients) and diabetes (26 patients). Fibrinogen was significantly increased and (p=0.019) protein C significantly reduced (p=0.014) in patients with a history of cardiovascular disease compared to those without.IL-1α (p=0.013) and TFPI (p=0.021) were significantly elevated in patients with diabetes compared to non-diabetic patients (26 patients).

Several markers showed significant differences in patients with low-frequency comorbidities. vWF was significantly elevated (p=0.039) was seen in patients with recent or active cancer compared to those without (6 patients). IFNγ (p=0.035) was significantly elevated in patients with CHF compared to those without (9 patients). CD40L (p=0.0182) and fibrinogen (p=0.0380) were significantly reduced in patients with history of recent transfusion compared to those without (7 patients).

IL-1α (p=0.041) and TFPI (p=0.041) were higher in males than in females. PAI-1 (p=0.005) and platelets (p=0.025) were higher in females than in males.

**Table 23. Association of Biomarkers with Comorbid Conditions**

| Marker | Age | Sex | Cancer | CHF | Cirrhosis | CVD | Diabetes | Pulmonary | Transfusion |
|---|---|---|---|---|---|---|---|---|---|
| **D-Dimer** | 0.10 | 0.23 | 0.21 | 0.34 | 0.30 | 0.85 | 0.63 | 0.01 | 0.44 |
| **PAI-1** | -0.08 | 0.01 | 0.65 | 0.97 | 0.79 | 0.66 | 0.45 | 0.78 | 0.29 |
| **INR** | 0.26 | 0.98 | 0.89 | 0.15 | 0.48 | 0.31 | 0.02 | 0.33 | 0.28 |
| **Platelets** | -0.23 | 0.03 | 0.17 | 0.65 | 0.09 | 0.84 | 0.61 | 0.21 | 0.23 |
| **Fibrinogen** | -0.01 | 0.23 | 0.63 | 0.47 | 0.03 | 0.02 | 0.93 | 0.89 | 0.04 |
| **Nucleosomes** | 0.05 | 0.58 | 0.09 | 0.24 | 0.46 | 0.32 | 0.75 | 0.04 | 0.51 |
| **Procalcitonin** | 0.22 | 1.00 | 0.11 | 0.65 | 0.29 | 0.66 | 0.83 | 0.45 | 0.59 |
| **IL-10** | -0.11 | 0.77 | 0.76 | 0.34 | 0.55 | 0.26 | 0.69 | 0.01 | 0.54 |
| **VEGF** | -0.21 | 0.92 | 0.38 | 0.35 | 0.04 | 0.32 | 0.94 | 0.85 | 0.79 |
| **IFNγ** | -0.21 | 0.62 | 0.58 | 0.03 | 0.66 | 0.60 | 0.40 | 0.02 | 0.69 |
| **TNFα** | 0.05 | 0.46 | 0.18 | 0.36 | 0.59 | 0.96 | 0.45 | 0.04 | 0.78 |
| **IL-1α** | -0.08 | 0.04 | 0.34 | 0.58 | 0.98 | 0.32 | 0.01 | 0.50 | 0.20 |
| **TFPI** | 0.16 | 0.04 | 0.57 | 0.56 | 0.64 | 0.87 | 0.02 | 0.91 | 0.37 |
| **Protein C** | -0.23 | 0.99 | 0.56 | 0.16 | 0.57 | 0.01 | 0.12 | 0.60 | 0.51 |
| **Endocan** | 0.35 | 0.15 | 0.40 | 0.12 | 0.92 | 0.68 | 0.44 | 0.91 | 0.49 |
| **Ang-2** | 0.28 | 0.89 | 0.44 | 0.69 | 0.44 | 0.96 | 0.37 | 0.09 | 0.34 |
| **vWF** | 0.19 | 0.08 | 0.04 | 0.78 | 0.30 | 0.65 | 0.49 | 0.36 | 0.61 |
| **CD40L** | -0.07 | 0.80 | 0.34 | 0.28 | 0.39 | 0.35 | 0.75 | 0.32 | 0.02 |

Spearman r value (age) and Mann-Whitney test p values (all other markers) for the relationship of biomarker levels to comorbidities. Significant relationships ($p<0.05$) are highlighted in blue. BMI, history of hypertension or recent surgery showed no associations with biomarker level and were not included in this table.

**Correlations**

Correlations between baseline biomarker levels were assessed using Spearman correlation coefficients with $p<0.05$ as the cutoff for significance. A correlation matrix relating each measured biomarker to each other biomarker was generated and is shown split into separate tables for hemostatic markers (D-Dimer, F1.2, PAI-1, INR, platelets, and fibrinogen), infection markers (nucleosomes, HMGB-1, and procalcitonin), inflammatory markers (IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFNγ, TNFα, IL-1α, IL-1β, MCP-1, and EGF), endothelial markers (TFPI, Protein C, endocan, Ang-2, and vWF), and platelet markers (CD40L, MP, MP-TF, and PF4). Correlation coefficients are shown in Tables 24-28 with significant correlations highlighted in blue. Significant correlations with an $r<0.4$ are highlighted in light blue whereas correlations with an $r\geq0.4$ are highlighted in dark blue.

Hemostatic biomarkers, shown in Table 24, correlated moderately with each other as well as with biomarkers of infection, inflammation, and endothelial function. PAI-1 did not correlate with any other measured hemostatic marker, suggesting that PAI-1 is involved in an independently regulated process relevant to patient outcome in sepsis-associated DIC. As anticipated based on prior analysis of the relationship of platelet counts with platelet-associated biomarkers, platelets correlated strongly with CD40L, MP, and PF4.

The infection markers nucleosomes, HMGB-1, and procalcitonin, shown in Table 25, correlated moderately with each other. Surprisingly, nucleosomes and HMGB-1 had minimal relationships to the classic inflammatory markers, with the exception of weak

but significant correlations between nucleosomes and IL-8 and HMGB-1 and IL-1β. In contrast, procalcitonin, an indicator but not a physiological mediator of infection, correlated strongly with numerous inflammatory markers including IL-6, IL-8, IL-10, TNFα, and MCP-1 as well as D-Dimer and Ang-2. Procalcitonin exhibited the greatest degree of correlation of any single biomarker, also correlating with hemostatic markers, including a strong correlation with D-Dimer, and endothelial markers, including a strong correlation with Ang-2.

Inflammatory markers, shown in Table 26, correlated strongly with each other; the majority of strong correlations between biomarkers were observed among inflammatory biomarkers. In addition to the previously mentioned strong correlations with procalcitonin, the inflammatory markers demonstrated significant but weak correlations with hemostatic markers, endothelial markers, and platelet markers. The majority of these correlations were focused in a few inflammatory cytokines, particularly IL-6, IL-8, IL-10, TNFα and MCP-1. This suggests that analysis of a small number of biomarkers with association with coagulopathy or mortality (i.e. IL-6, IL-8, or IL-10) may be sufficient to describe the inflammatory status of a patient with sepsis and DIC.

Endothelial biomarkers correlated significantly but weakly with each other and showed relatively few strong correlations with anything else, as shown in Table 27. The exceptions to this were a strong relationship between Protein C and INR, strengthening the association between low Protein C levels and coagulopathy, strong associations between Ang-2 and HMGB-1 and procalcitonin, and a strong negative association

between endocan and VEGF. Virtually no associations were seen between endothelial and platelet markers.

Platelet count showed limited associations with other factors, as shown in Table 28. With the exception of the previously mentioned strong correlation of platelet count with CD40L, MP, and PF4, the only other strong correlations observed were between VEGF and CD40L and between MP and EGF. Minimal associations were seen between platelet markers and endothelial markers, infection markers, and hemostatic parameters.

**Table 24. Spearman Correlation Coefficients for Hemostatic Markers**

| | D-Dimer | F1.2 | PAI-1 | INR | Platelets | Fibrinogen |
|---|---|---|---|---|---|---|
| **D-Dimer** | | 0.46 | 0.17 | 0.11 | -0.27 | 0.03 |
| **F1.2** | 0.46 | | 0.17 | -0.15 | -0.01 | -0.12 |
| **PAI-1** | 0.17 | 0.17 | | 0.08 | 0.19 | -0.06 |
| **INR** | 0.11 | -0.15 | 0.08 | | -0.30 | -0.11 |
| **Platelets** | -0.27 | -0.01 | 0.19 | -0.30 | | 0.26 |
| **Fibrinogen** | 0.03 | -0.12 | -0.06 | -0.11 | 0.26 | |
| **Nucleosomes** | 0.34 | 0.19 | 0.29 | 0.17 | -0.14 | 0.04 |
| **HMGB-1** | 0.19 | 0.18 | 0.09 | 0.16 | -0.18 | 0.10 |
| **Procalcitonin** | 0.47 | 0.29 | 0.18 | 0.24 | -0.35 | 0.02 |
| **IL-2** | 0.13 | 0.11 | -0.14 | -0.04 | -0.03 | 0.01 |
| **IL-4** | -0.04 | -0.02 | -0.11 | 0.05 | -0.07 | 0.01 |
| **IL-6** | 0.34 | 0.12 | 0.28 | 0.33 | -0.03 | 0.21 |
| **IL-8** | 0.34 | 0.13 | 0.39 | 0.32 | -0.16 | 0.06 |
| **IL-10** | 0.36 | 0.32 | 0.31 | 0.17 | -0.21 | -0.09 |
| **VEGF** | 0.12 | 0.17 | 0.14 | -0.15 | 0.45 | 0.41 |
| **IFNγ** | 0.05 | 0.21 | 0.10 | 0.01 | -0.09 | -0.06 |
| **TNFα** | 0.31 | 0.33 | 0.12 | 0.16 | -0.28 | -0.02 |
| **IL-1α** | 0.15 | 0.08 | -0.23 | -0.05 | -0.15 | 0.01 |
| **IL-1β** | 0.25 | 0.20 | 0.03 | 0.13 | -0.05 | 0.11 |
| **MCP-1** | 0.23 | 0.22 | 0.25 | 0.30 | -0.14 | 0.09 |
| **EGF** | -0.01 | 0.02 | 0.13 | -0.22 | 0.58 | 0.25 |
| **IL-6:IL-10** | 0.11 | -0.12 | 0.05 | 0.20 | 0.12 | 0.36 |
| **TFPI** | 0.21 | 0.15 | 0.26 | 0.06 | -0.04 | -0.11 |
| **Protein C** | -0.22 | -0.13 | -0.11 | -0.49 | 0.26 | -0.10 |
| **Endocan** | 0.16 | -0.02 | 0.19 | 0.27 | -0.22 | -0.30 |
| **Ang-2** | 0.32 | 0.01 | 0.16 | 0.38 | -0.39 | -0.01 |
| **vWF** | 0.31 | -0.01 | -0.06 | 0.15 | -0.22 | 0.18 |
| **CD40L** | -0.05 | -0.01 | 0.13 | 0.02 | 0.40 | 0.15 |
| **MP** | -0.10 | -0.06 | 0.26 | 0.01 | 0.58 | 0.10 |
| **MP-TF** | 0.24 | -0.04 | 0.11 | 0.19 | -0.13 | 0.03 |
| **PF4** | -0.09 | 0.03 | 0.05 | 0.00 | 0.42 | 0.12 |

Spearman correlations are shown. Significant correlations ($p<0.05$ are highlighted in blue). Light blue denotes a correlation coefficient of <0.4 while dark blue denotes a coefficient of ≥0.4

**Table 25 Spearman Correlation Coefficients for Infection Markers**

| | Nucleosomes | HMGB-1 | Procalcitonin |
|---|---|---|---|
| **D-Dimer** | 0.34 | 0.19 | 0.47 |
| **F1.2** | 0.19 | 0.18 | 0.29 |
| **PAI-1** | 0.29 | 0.09 | 0.18 |
| **INR** | 0.17 | 0.16 | 0.24 |
| **Platelets** | -0.14 | -0.18 | -0.35 |
| **Fibrinogen** | 0.04 | 0.10 | 0.02 |
| **Nucleosomes** | | 0.25 | 0.31 |
| **HMGB-1** | 0.25 | | 0.28 |
| **Procalcitonin** | 0.31 | 0.28 | |
| **IL-2** | -0.07 | -0.03 | 0.11 |
| **IL-4** | -0.19 | -0.03 | 0.19 |
| **IL-6** | 0.19 | 0.14 | 0.41 |
| **IL-8** | 0.24 | 0.17 | 0.41 |
| **IL-10** | 0.18 | 0.07 | 0.45 |
| **VEGF** | -0.02 | 0.04 | -0.11 |
| **IFNγ** | -0.08 | -0.12 | 0.16 |
| **TNFα** | 0.03 | 0.14 | 0.52 |
| **IL-1α** | 0.00 | -0.01 | 0.12 |
| **IL-1β** | 0.07 | 0.19 | 0.37 |
| **MCP-1** | 0.16 | 0.14 | 0.46 |
| **EGF** | -0.16 | -0.09 | -0.26 |
| **IL-6:IL-10** | 0.02 | 0.12 | 0.09 |
| **TFPI** | 0.21 | 0.20 | 0.18 |
| **Protein C** | -0.17 | -0.17 | -0.29 |
| **Endocan** | 0.37 | 0.02 | 0.21 |
| **Ang-2** | 0.17 | 0.43 | 0.62 |
| **vWF** | 0.26 | 0.19 | 0.30 |
| **CD40L** | 0.04 | 0.10 | -0.08 |
| **MP** | 0.05 | 0.12 | -0.14 |
| **MP-TF** | 0.25 | 0.03 | 0.17 |
| **PF4** | 0.05 | 0.06 | -0.16 |

Spearman correlations are shown. Significant correlations ($p<0.05$ are highlighted in blue). Light blue denotes a correlation coefficient of <0.4 while dark blue denotes a coefficient of ≥0.4

**Table 26. Spearman Correlation Coefficients for Inflammatory Markers**

| | IL-2 | IL-4 | IL-6 | IL-8 | IL-10 | VEGF | IFNγ | TNFα | IL-1α | IL-1β | MCP-1 | EGF | IL-6:10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **D-Dimer** | 0.13 | -0.04 | 0.34 | 0.34 | 0.36 | 0.12 | 0.05 | 0.31 | 0.15 | 0.25 | 0.23 | -0.01 | 0.11 |
| **F1.2** | 0.11 | -0.02 | 0.12 | 0.13 | 0.32 | 0.17 | 0.21 | 0.33 | 0.08 | 0.20 | 0.22 | 0.02 | -0.12 |
| **PAI-1** | -0.14 | -0.11 | 0.28 | 0.39 | 0.31 | 0.14 | 0.10 | 0.12 | -0.23 | 0.03 | 0.25 | 0.13 | 0.05 |
| **INR** | -0.04 | 0.05 | 0.33 | 0.32 | 0.17 | -0.15 | 0.01 | 0.16 | -0.05 | 0.13 | 0.30 | -0.22 | 0.20 |
| **Platelets** | -0.03 | -0.07 | -0.03 | -0.16 | -0.21 | 0.45 | -0.09 | -0.28 | -0.15 | -0.05 | -0.14 | 0.58 | 0.12 |
| **Fibrinogen** | 0.01 | 0.01 | 0.21 | 0.06 | -0.09 | 0.41 | -0.06 | -0.02 | 0.01 | 0.11 | 0.09 | 0.25 | 0.36 |
| **Nucleosomes** | -0.07 | -0.19 | 0.19 | 0.24 | 0.18 | -0.02 | -0.08 | 0.03 | 0.00 | 0.07 | 0.16 | -0.16 | 0.02 |
| **HMGB-1** | -0.03 | -0.03 | 0.14 | 0.17 | 0.07 | 0.04 | -0.12 | 0.14 | -0.01 | 0.19 | 0.14 | -0.09 | 0.09 |
| **PCT** | 0.11 | 0.19 | 0.41 | 0.41 | 0.45 | -0.11 | 0.16 | 0.52 | 0.12 | 0.37 | 0.46 | -0.26 | 0.12 |
| **IL-2** | | 0.28 | -0.03 | 0.05 | 0.08 | 0.10 | 0.51 | 0.26 | 0.28 | 0.46 | 0.06 | 0.09 | -0.04 |
| **IL-4** | 0.28 | | 0.22 | 0.20 | 0.13 | 0.02 | 0.23 | 0.33 | 0.29 | 0.47 | 0.27 | 0.04 | 0.15 |
| **IL-6** | -0.03 | 0.22 | | 0.61 | 0.50 | 0.30 | 0.07 | 0.38 | 0.04 | 0.42 | 0.68 | -0.06 | 0.77 |
| **IL-8** | 0.05 | 0.20 | 0.61 | | 0.57 | 0.19 | 0.09 | 0.49 | 0.06 | 0.31 | 0.66 | -0.07 | 0.31 |
| **IL-10** | 0.08 | 0.13 | 0.50 | 0.57 | | 0.08 | 0.28 | 0.57 | 0.11 | 0.36 | 0.61 | -0.16 | -0.07 |
| **VEGF** | 0.10 | 0.02 | 0.30 | 0.19 | 0.08 | | -0.01 | 0.04 | 0.14 | 0.09 | 0.14 | 0.55 | 0.36 |
| **IFNγ** | 0.51 | 0.23 | 0.07 | 0.09 | 0.28 | -0.01 | | 0.46 | 0.12 | 0.41 | 0.30 | 0.06 | -0.09 |
| **TNFα** | 0.26 | 0.33 | 0.38 | 0.49 | 0.57 | 0.04 | 0.46 | | 0.06 | 0.49 | 0.64 | -0.09 | 0.08 |
| **IL-1α** | 0.28 | 0.29 | 0.04 | 0.06 | 0.11 | 0.14 | 0.12 | 0.06 | | 0.18 | 0.03 | 0.04 | -0.01 |
| **IL-1β** | 0.46 | 0.47 | 0.42 | 0.31 | 0.36 | 0.09 | 0.41 | 0.49 | 0.18 | | 0.53 | 0.01 | 0.24 |
| **MCP-1** | 0.06 | 0.27 | 0.68 | 0.66 | 0.61 | 0.14 | 0.30 | 0.64 | 0.03 | 0.53 | | -0.06 | 0.35 |
| **EGF** | 0.09 | 0.04 | -0.06 | -0.07 | -0.16 | 0.55 | 0.06 | -0.09 | 0.04 | 0.01 | -0.06 | | 0.14 |
| **IL-6:IL-10** | -0.04 | 0.15 | 0.77 | 0.31 | -0.07 | 0.36 | -0.09 | 0.08 | -0.01 | 0.24 | 0.35 | 0.14 | |

| | IL-2 | IL-4 | IL-6 | IL-8 | IL-10 | VEGF | IFNγ | TNFα | IL-1α | IL-1β | MCP-1 | EGF | IL-6:10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **TFPI** | -0.08 | 0.06 | 0.11 | 0.13 | 0.11 | -0.18 | -0.03 | 0.09 | -0.12 | 0.13 | 0.18 | -0.09 | -0.06 |
| **Protein C** | -0.06 | 0.05 | -0.36 | -0.30 | -0.18 | -0.07 | -0.11 | -0.18 | 0.02 | -0.13 | -0.36 | 0.23 | -0.28 |
| **Endocan** | -0.06 | 0.09 | 0.13 | 0.13 | 0.00 | -0.44 | -0.01 | 0.06 | -0.15 | 0.18 | 0.15 | -0.30 | 0.01 |
| **Ang-2** | -0.12 | 0.08 | 0.38 | 0.37 | 0.36 | -0.18 | -0.05 | 0.39 | -0.05 | 0.19 | 0.37 | -0.34 | 0.16 |
| **vWF** | 0.01 | 0.09 | 0.19 | 0.26 | 0.12 | 0.07 | -0.03 | 0.32 | -0.15 | 0.12 | 0.20 | -0.13 | 0.17 |
| **CD40L** | -0.02 | -0.14 | 0.14 | 0.17 | -0.06 | 0.41 | -0.12 | -0.10 | -0.17 | -0.05 | 0.06 | 0.37 | 0.23 |
| **MP** | -0.19 | -0.05 | 0.03 | -0.15 | -0.21 | 0.28 | -0.21 | -0.27 | -0.18 | -0.08 | -0.09 | 0.62 | 0.14 |
| **MP-TF** | 0.02 | -0.01 | 0.17 | 0.25 | 0.03 | 0.04 | 0.00 | 0.05 | -0.17 | 0.00 | 0.08 | -0.11 | 0.12 |
| **PF4** | -0.23 | -0.02 | -0.06 | -0.09 | -0.23 | 0.21 | -0.10 | -0.15 | -0.24 | -0.18 | -0.11 | 0.30 | 0.04 |

Spearman correlations are shown. Significant correlations ($p<0.05$ are highlighted in blue). Light blue denotes a correlation coefficient of $<0.4$ while dark blue denotes a coefficient of $\geq 0.4$

**Table 27. Spearman Correlation Coefficients for Endothelial Markers**

| | TFPI | Protein C | Endocan | Ang-2 | vWF |
|---|---|---|---|---|---|
| **D-Dimer** | 0.21 | -0.22 | 0.16 | 0.32 | 0.31 |
| **F1.2** | 0.15 | -0.13 | -0.02 | 0.01 | -0.01 |
| **PAI-1** | 0.26 | -0.11 | 0.19 | 0.16 | -0.06 |
| **INR** | 0.06 | -0.49 | 0.27 | 0.38 | 0.15 |
| **Platelets** | -0.04 | 0.26 | -0.22 | -0.39 | -0.22 |
| **Fibrinogen** | -0.11 | -0.10 | -0.30 | -0.01 | 0.18 |
| **Nucleosomes** | 0.21 | -0.17 | 0.37 | 0.17 | 0.26 |
| **HMGB-1** | 0.20 | -0.17 | 0.02 | 0.43 | 0.19 |
| **Procalcitonin** | 0.18 | -0.29 | 0.21 | 0.62 | 0.30 |
| **IL-2** | -0.08 | -0.06 | -0.06 | -0.12 | 0.01 |
| **IL-4** | 0.06 | 0.05 | 0.09 | 0.08 | 0.09 |
| **IL-6** | 0.11 | -0.36 | 0.13 | 0.38 | 0.19 |
| **IL-8** | 0.13 | -0.30 | 0.13 | 0.37 | 0.26 |
| **IL-10** | 0.11 | -0.18 | 0.00 | 0.36 | 0.12 |
| **VEGF** | -0.18 | -0.07 | -0.44 | -0.18 | 0.07 |
| **IFNγ** | -0.03 | -0.11 | -0.01 | -0.05 | -0.03 |
| **TNFα** | 0.09 | -0.18 | 0.06 | 0.39 | 0.32 |
| **IL-1α** | -0.12 | 0.02 | -0.15 | -0.05 | -0.15 |
| **IL-1β** | 0.13 | -0.13 | 0.18 | 0.19 | 0.12 |
| **MCP-1** | 0.18 | -0.36 | 0.15 | 0.37 | 0.20 |
| **EGF** | -0.09 | 0.23 | -0.30 | -0.34 | -0.13 |
| **IL-6:IL-10** | -0.06 | -0.28 | 0.01 | 0.16 | 0.17 |
| **TFPI** | | 0.24 | 0.34 | 0.28 | 0.08 |
| **Protein C** | 0.24 | | -0.18 | -0.29 | -0.26 |
| **Endocan** | 0.34 | -0.18 | | 0.28 | 0.10 |
| **Ang-2** | 0.28 | -0.29 | 0.28 | | 0.33 |
| **vWF** | 0.08 | -0.26 | 0.10 | 0.33 | |
| **CD40L** | -0.02 | 0.07 | -0.19 | -0.12 | 0.03 |
| **MP** | 0.08 | 0.15 | -0.01 | -0.14 | -0.16 |
| **MP-TF** | 0.03 | -0.14 | 0.21 | 0.11 | 0.32 |
| **PF4** | 0.08 | 0.17 | -0.08 | -0.16 | 0.13 |

Spearman correlations are shown. Significant correlations (p<0.05 are highlighted in blue). Light blue denotes a correlation coefficient of <0.4 while dark blue denotes a coefficient of ≥0.4

**Table 28. Spearman Correlation Coefficients for Platelet Markers**

| | CD40L | MP | MP-TF | PF4 |
|---|---|---|---|---|
| **D-Dimer** | -0.05 | -0.10 | 0.24 | -0.09 |
| **F1.2** | -0.01 | -0.06 | -0.04 | 0.03 |
| **PAI-1** | 0.13 | 0.26 | 0.11 | 0.05 |
| **INR** | 0.02 | 0.01 | 0.19 | 0.00 |
| **Platelets** | 0.40 | 0.58 | -0.13 | 0.42 |
| **Fibrinogen** | 0.15 | 0.10 | 0.03 | 0.12 |
| **Nucleosomes** | 0.04 | 0.05 | 0.25 | 0.05 |
| **HMGB-1** | 0.10 | 0.12 | 0.03 | 0.06 |
| **Procalcitonin** | -0.08 | -0.14 | 0.17 | -0.16 |
| **IL-2** | -0.02 | -0.19 | 0.02 | -0.23 |
| **IL-4** | -0.14 | -0.05 | -0.01 | -0.02 |
| **IL-6** | 0.14 | 0.03 | 0.17 | -0.06 |
| **IL-8** | 0.17 | -0.15 | 0.25 | -0.09 |
| **IL-10** | -0.06 | -0.21 | 0.03 | -0.23 |
| **VEGF** | 0.41 | 0.28 | 0.04 | 0.21 |
| **IFNγ** | -0.12 | -0.21 | 0.00 | -0.10 |
| **TNFα** | -0.10 | -0.27 | 0.05 | -0.15 |
| **IL-1α** | -0.17 | -0.18 | -0.17 | -0.24 |
| **IL-1β** | -0.05 | -0.08 | 0.00 | -0.18 |
| **MCP-1** | 0.06 | -0.09 | 0.08 | -0.11 |
| **EGF** | 0.37 | 0.62 | -0.11 | 0.30 |
| **IL-6:IL-10** | 0.23 | 0.14 | 0.12 | 0.04 |
| **TFPI** | -0.02 | 0.08 | 0.03 | 0.08 |
| **Protein C** | 0.07 | 0.15 | -0.14 | 0.17 |
| **Endocan** | -0.19 | -0.01 | 0.21 | -0.08 |
| **Ang-2** | -0.12 | -0.14 | 0.11 | -0.16 |
| **vWF** | 0.03 | -0.16 | 0.32 | 0.13 |
| **CD40L** | | 0.32 | 0.05 | 0.30 |
| **MP** | 0.32 | | 0.00 | 0.45 |
| **MP-TF** | 0.05 | 0.00 | | 0.13 |
| **PF4** | 0.30 | 0.45 | 0.13 | |

Spearman correlations are shown. Significant correlations ($p<0.05$ are highlighted in blue). Light blue denotes a correlation coefficient of $<0.4$ while dark blue denotes a coefficient of $\geq 0.4$

The most notable markers to emerge from this study are the biomarkers of infection. The relationship between these markers, particularly between nucleosomes and HMGB-1, is of interest as it may provide information regarding the source of these markers in the circulation. To assess this association, patients were divided into tertiles based on HMGB-1 levels. Data is shown in Figure 33. The highest tertile included patients with HMGB-1 in the range of 7.11-86.77 ng/ml. The middle tertile included patients in the range of 4.16-7.10 ng/ml. The lowest tertile included patients in the range of from 0.18-4.15 ng/ml. For each group, n=34. Differences in nucleosome levels based on HMGB-1 tertile were assessed using the Kruskal-Wallis test with Dunn's multiple comparison test and $p<0.05$ as the cutoff for significance. Significant variance was seen in nucleosome levels based on HMGB-1 levels ($p=0.019$), with significance reached between the 1$^{st}$ and 2$^{nd}$ tertiles ($p=0.020$). The significant but relatively weak relationship between nucleosomes and HMGB-1 supports the related physiological role of these molecules; however, it also suggests that there is some independent regulation of these factors.

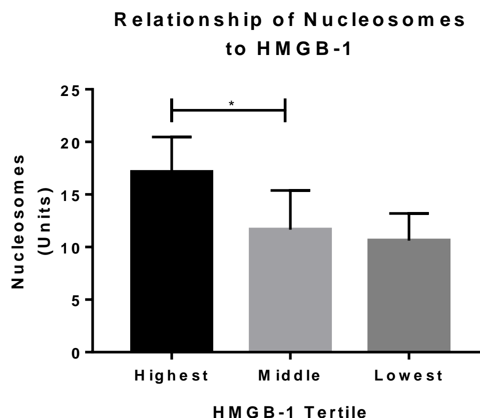


**Figure 33. Relationship of Nucleosomes to HMGB-1.** Patients were divided into tertiles based on HMGB-1 levels (n=34 per tertile). Differences in nucleosome levels by tertile were assessed using the Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison test and $p<0.05$ as the cutoff for significance. Significant differences in nucleosome levels were seen between the highest tertile (HMGB-1 between 7.11 and 86.88 ng/ml) and the middle tertile (HMGB-1 between 4.16-7.10 ng/ml).

### Stepwise Linear Regression Modeling

Stepwise linear regression modeling using MATLAB software was performed in order to create an algorithm for the prediction of mortality in patients with sepsis and sepsis-associated DIC. Stepwise linear regression is a computational technique in which an iterative algorithm is employed to construct an equation to predict the value of a "response variable" based on a subset of "predictor variable" selected by the algorithm from among all input "predictor values". Two starting assumptions are possible for this model; a "constant" starting assumption in which all predictor variables are assumed to be included in the model or a "linear" starting assumption in which no predictor variables are assumed to be included in the model. With a constant starting assumption, variables are added to the model if inclusion yields a statistically significant improvement to model

fit. This process continues until no variables remain which improve the fit of the model when added. Using a linear starting assumption, variables are removed from the model if elimination does not significantly change model fit, and this process is repeated until no variables remain that can be removed without altering model fit.

The output of this process is an equation composed of a constant term and coefficients for each included predictor variable. This equation is used to predict the value of the response variable for a given patient. Model performance was assessed using receiver operating curve (ROC) analysis.

MATLAB code is shown in Appendix D. Data tables defining mortality as the response variable and baseline biomarker levels as the predictor values were imported into MATLAB from Microsoft Excel. Models were developed using the “stepwiselm” function. Both linear and constant model starting assumptions were used as specified in the data analysis. Predictive equations generated using the linear starting assumption included more terms than those generated using the constant assumption. Model coefficients were recorded and model output value for each patient was calculated from the appropriate biomarker levels

Two different approaches were used for the prediction of mortality in the Utah cohort. In the first approach, only measured biomarkers (levels of D-Dimer, F1.2, PAI-1, INR, platelets, fibrinogen, nucleosomes, HMGB-1, procalcitonin, IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFNγ, TNFα, IL-1α, IL-1β, MCP-1, EGF, IL-6:IL-10 Ratio, TFPI, Protein C, endocan, Ang-2, vWF, CD40L, MP, MP-TF, and PF4) were included. In the second approach, the additional clinical parameters of DIC score, hemoglobin, white blood cell

count, BMI, age, MODS score, and SOFA score, and APACHE-II score were included in the analysis. Accordingly, four different models were generated: linear starting assumption and biomarkers alone, linear starting assumption and biomarkers plus clinical data, constant starting assumption and biomarkers alone, and constant starting assumption and biomarkers plus clinical data.

As shown in Table 29, models were successfully generated for the prediction of mortality in Utah cohort patients using this approach. All four models generated using this approach had greater predictive value (AUC of 0.84-0.95) than any individual biomarker in this patient cohort (maximum individual AUC=0.77 for procalcitonin). Furthermore, the models generated using this approach incorporated biomarkers representative of multiple physiological systems and processes.

The best model was generated using a constant starting assumption and biomarkers alone. This model included 5 variables: procalcitonin, representative of infection; VEGF and the IL-6:IL-10 ratio, representative of inflammation; endocan, representative of endothelial function; and PF4, representative of platelet activation. The overall AUC for prediction of mortality using this model was 0.87. The inclusion of clinical variables did not improve AUC; the model generated using the constant starting assumption and biomarkers plus clinical data had an AUC value of 0.84. This model supported previous results on the importance of infection response to the disease progression and outcome of sepsis and DIC; both procalcitonin and white blood cell count, markers of infection response, were included in this model. The models generated using the linear starting assumption showed some improvement in predictive value over

the constant starting assumption model, with the biomarker alone model producing an AUC of 0.95 and the biomarker plus clinical model producing an AUC of 0.89. Despite the high predictive value of these model, the practical utility of these models is limited by the inclusion of in excess of 10 biomarkers.

The results of this modeling analysis support the hypothesis that while a single biomarker cannot accurately predict outcome in this complex patient population, a combination of biomarkers representative of multiple physiological systems will have improved predictive value. The model generated using the constant starting assumption and biomarker data alone provides a superior predictive value for outcome than any single measured biomarker and accounts for the activity of multiple physiological systems. This model should be further validated in additional patient cohorts.

**Table 29. Stepwise Linear Regression Modeling for Prediction of Mortality in All Utah Sepsis Cohort Patients**

| Components | Assumption | Components | Coefficient | AUC |
|---|---|---|---|---|
| Biomarkers | Constant | Intercept | -1.9E-3 | 0.87 |
| | | Procalcitonin | 4.1E-5 | |
| | | VEGF | 2.6E-3 | |
| | | IL-6:IL-10 Ratio | 8.5E-4 | |
| | | Endocan | 0.010 | |
| | | PF4 | -1.6E-3 | |
| Biomarkers + Clinical | Constant | Intercept | -0.27 | 0.84 |
| | | APACHE II | 9.8E-3 | |
| | | WBC | 0.013 | |
| | | Procalcitonin | 4.47E-5 | |
| Biomarkers | Linear | Intercept | 0.13 | 0.95 |
| | | D-Dimer | -8.6E-6 | |
| | | PCT | 4.3E-5 | |
| | | MCP1 | -2.5E-4 | |
| | | EGF | 0.016 | |
| | | IL-6:IL-10 Ratio | 1.2E-3 | |
| | | PAI-1 | 1.3E-3 | |
| | | CD40L | 1.2E-4 | |
| | | Protein C | -1.5E-3 | |
| | | Endocan | 9.7E-3 | |
| | | PF4 | -9.8E-4 | |
| | | HMGB-1 | 3.8E-3 | |
| | | MP | -4.6E-3 | |
| Biomarkers + Clinical | Linear | Intercept | -0.11 | 0.89 |
| | | WBC | 0.015 | |
| | | D-Dimer | -1.0E-5 | |
| | | PCT | 4.3E-5 | |
| | | EGF | 0.011 | |
| | | IL-6:IL-10 Ratio | 8.8E-4 | |
| | | PAI-1 | 1.1E-3 | |
| | | CD40L | 1.2E-4 | |
| | | Endocan | 9.5E-3 | |
| | | PF4 | -1.1E-3 | |
| | | MP | -4.6E-3 | |

A difficulty in designing tools to predict outcome in patients with sepsis is the heterogeneity of the patient population. For example, different factors may contribute to mortality in patients with sepsis who develop coagulopathy compared to patients who do not. Accordingly, patients were subdivided based on DIC score and separate models for the prediction of mortality were generated for patients with sepsis + no DIC, sepsis + non-overt DIC, and sepsis + overt DIC. These models are described in Table 30, Table 31, and Table 32.

This approach is limited by the low number of patients in each category, particularly the low number of non-survivors. The models generated using this approach have extraordinarily high predictive value for this patient cohort, but would not be expected to be generalizable to a larger population. However, this analysis does serve as proof of concept of the potential utility of developing different predictive models for sepsis patients on the basis of coagulation status.

The largest group of patients when subdivided on the basis of coagulopathy is sepsis + non-overt DIC, encompassing a total of 59 patients including 7 non-survivors and 52 survivors. The model generated for this patient group using biomarkers only and a constant starting assumption, shown in Table 31, is very similar to that generated using the total patient population, also including procalcitonin, VEGF, IL-6:IL-10 ratio, and PF4 but excluding endocan.

The groups of patients with no DIC and with overt DIC were significantly smaller than the non-overt DIC group. 20 patients had no DIC, including 18 survivors and 2 non-survivors. 24 patients had overt DIC, including 6 non-survivors and 18 survivors. Models

generated on the basis of these patients' data incorporated different markers than those generated from the total cohort or non-overt DIC patients.

**Table 30. Stepwise Linear Regression Modeling for Prediction of Mortality in Patients with Sepsis Alone**

| Components | Assumption | Components | Coefficient | AUC |
|---|---|---|---|---|
| Biomarkers<br><br>Biomarkers + Clinical | Constant | Intercept | 0.19 | 1 |
| | | INR | -0.14 | |
| | | IL-8 | 0.013 | |
| | | VEGF | -2.4E-3 | |
| | | TNFα | -2.4E-3 | |
| | | Protein C | -1.9E-3 | |
| | | HMGB-1 | 0.035 | |
| Biomarkers<br><br>Biomarkers + Clinical | Linear | Intercept | -0.40 | 1 |
| | | F1.2 | 5.8E-5 | |
| | | IL-10 | 0.026 | |
| | | VEGF | -5.0E-3 | |
| | | IFNγ | -0.21 | |
| | | IL-1α | -0.82 | |
| | | IL-1β | 0.30 | |
| | | MCP-1 | -4.8E-4 | |
| | | EGF | 0.036 | |
| | | MP-TF | -0.37 | |
| | | IL-6:IL-10 Ratio | -3.5E-3 | |
| | | PAI-1 | -1.8E-3 | |
| | | CD40L | 3.2E-4 | |
| | | Endocan | 0.033 | |
| | | Ang2 | 7.8E-6 | |
| | | PF4 | 4.2E-4 | |
| | | HMGB-1 | 0.047 | |
| | | vWF | 1.5E-3 | |
| | | MP | -5.7E-3 | |

Incorporation of clinical data in addition to biomarkers did not result in the generation of a distinct model for patients with sepsis alone using either the linear or constant starting assumption.

**Table 31. Stepwise Linear Regression Modeling for Prediction of Mortality in Patients with Non-Overt DIC**

| Components | Assumption | Components | Coefficient | AUC |
|---|---|---|---|---|
| Biomarkers | Constant | Intercept | -0.010 | 0.94 |
| | | Procalcitonin | 8.5E-5 | |
| | | VEGF | 3.8E-3 | |
| | | IL-6:IL-10 Ratio | 1.4E-3 | |
| | | PF4 | -1.9E-3 | |
| Biomarkers | Linear | Intercept | -1.7 | 1 |
| | | INR | 0.95 | |
| | | F1.2 | -3.9E-4 | |
| | | Procalcitonin | 1.3E-4 | |
| | | IL-2 | -0.037 | |
| | | IL-10 | -6.5E-3 | |
| | | IL-1α | -0.015 | |
| | | IL-1β | 0.034 | |
| | | EGF | 0.023 | |
| | | MP-TF | 0.34 | |
| | | CD40L | 2.1E-4 | |
| | | Endocan | 0.012 | |
| | | PF4 | 1.5E-3 | |
| | | MP | -8.7E-3 | |
| Clinical | Constant | Intercept | -0.87 | 0.99 |
| | | SOFA | 0.043 | |
| | | WBC | 0.019 | |
| | | Nucleosomes | 0.01 | |
| | | PCT | 6.2E-5 | |
| | | IL-6:IL-10 Ratio | 1.7E-3 | |
| | | Protein C | 3.2E-3 | |
| Clinical | Linear | Intercept | -1.6 | 1 |
| | | WBC | 0.020 | |
| | | Platelets | 1.5E-3 | |
| | | INR | 0.81 | |
| | | Procalcitonin | 1.2E-4 | |
| | | IL-2 | -0.045 | |
| | | IL-10 | -0.011 | |
| | | IL-1β | 0.045 | |
| | | EGF | 0.027 | |
| | | Endocan | 0.016 | |
| | | PF4 | -3.6E-4 | |
| | | MP | -0.012 | |

**Table 32. Stepwise Linear Regression Modeling for Prediction of Mortality in Patients with Overt DIC**

| Components | Assumption | Components | Coefficient | AUC |
|---|---|---|---|---|
| Biomarkers | Constant | Intercept | 0.20 | 1 |
| | | TFPI | -5.8E-3 | |
| | | Procalcitonin | 3.4E-5 | |
| | | IL-4 | 0.17 | |
| | | TNFα | -0.051 | |
| | | MCP1 | -7.2E-4 | |
| | | PAI-1 | 4.6E-3 | |
| | | HMGB-1 | 0.017 | |
| | | MP | 2.9E-3 | |
| Clinical | Constant | Intercept | -0.68 | 1 |
| | | Age | 7.7E-3 | |
| | | Nucleosomes | -3.9E-3 | |
| | | IL-6:IL-10 Ratio | 2.0E-3 | |
| | | PAI-1 | 2.6E-3 | |
| | | Protein C | -2.6E-3 | |
| | | Endocan | 8.4E-3 | |
| | | HMGB-1 | 0.025 | |
| Biomarkers<br><br>Biomarkers + Clinical Data | Linear | Intercept | 0.94 | 1 |
| | | PCT | -5.9E-5 | |
| | | IL-8 | 2.1E-3 | |
| | | IL-10 | 4.0E-3 | |
| | | VEGF | -0.026 | |
| | | IFNγ | -8.1E-3 | |
| | | TNFα | 0.22 | |
| | | IL-1α | 1.6 | |
| | | IL-1β | -0.26 | |
| | | MCP1 | -5.2E-4 | |
| | | EGF | -0.14 | |
| | | MP-TF | 0.45 | |
| | | PAI-1 | -5.4E-3 | |
| | | CD40L | 1.1E-3 | |
| | | Protein C | -0.027 | |
| | | Endocan | 6.1E-3 | |
| | | Ang2 | -9.96E-6 | |
| | | PF4 | 7.0E-3 | |
| | | HMGB-1 | 0.034 | |
| | | vWF | -6.9E-3 | |
| | | MP | 0.028 | |

## *In vitro* Coagulation Profiles of rTM, AT, and UFH

Endogenous anticoagulants including AT and rTM have been pursued as treatments for DIC in part due to the reduced potential for bleeding with these drugs when compared to traditional anticoagulants, such as heparin. These drugs may only prevent pathological coagulation without interfering with the normal hemostatic process to the same degree as heparin.

Prior to testing the effects of rTM, AT, and heparin in an animal model of sepsis, the relative anticoagulant effects of rTM, AT, and heparin were compared *in vitro* in human whole blood and plasma and in rat plasma. rTM was supplemented into plasma at concentrations from 0.625-10 µg/ml and in whole blood at concentrations from 1.25-5 µg/ml. This is representative of the circulating level of rTM in the management of patients with sepsis-associated DIC, which is typically within the range of 0.5-1.5 µg/ml (Moll 2004; Vincent 2013). Heparin was used at concentrations of 0.0625-1 U/ml in plasma and 0.125-0.5 U/ml in whole blood. For similar indications, therapeutic levels of heparin range from 1.5-5.0 µg/ml (0.15-0.5 U/ml) in blood. Antithrombin was used at concentrations of 0.0625-1 U/ml in plasma and 1.25-5 U/ml in whole blood. In DIC, therapeutic blood levels of AT range from 1-2.5 U/ml (Choi 2014; Kienast 2006).

### Clotting

The anticoagulant effects of rTM, AT, and heparin on plasma based coagulation tests were assessed *in vitro* in both human and rat plasma using physiologically relevant drug concentrations. Endogenous anticoagulants such as AT and rTM have been pursued as treatments for DIC in part due to the reduced potential for bleeding with these drugs

when compared to traditional anticoagulants, such as heparin. PT was used to assess the effect of the drugs on the extrinsic pathway and aPTT was used to assess the effect of the drugs on the intrinsic pathway.

The results of *in vitro* clotting tests in human samples are shown in Table 33. All tests were performed in citrated plasma from healthy normal volunteers. Drugs were supplemented into blood at the specified concentrations. Data is shown as the mean of two independent experiments. Both rTM and AT had minimal anticoagulant activity in *in vitro* clot formation as measured by PT and aPTT at physiologically relevant blood concentrations. At supertherapeutic concentrations, prolongation of clotting times was minimal. Heparin showed strong anticoagulant activity, producing maximum results in the aPTT and lesser prolongations in PT. This was expected, as aPTT is used clinically to monitor heparin therapy and PT is not sensitive to heparin treatment.

As shown in Table 34, the modulation of coagulation by rTM, AT, and UFH in rat plasma is comparable to that in human plasma. Drugs were supplemented in pooled plasma from healthy rats at the specified concentrations. No elevation in PT was present as a result of supplementation with rTM, AT, or UFH. UFH supplementation caused a dose-dependent increase in aPTT, while neither rTM nor AT supplementation increased aPTT.

**Table 33. Comparison of rTM, AT, and UFH in Clotting Tests in Human Whole Blood**

| Recombinant Thrombomodulin | | | Antithrombin | | | Heparin | | |
|---|---|---|---|---|---|---|---|---|
| **Concentration** | **PT (s)** | **aPTT (s)** | **Concentration** | **PT (s)** | **aPTT (s)** | **Concentration** | **PT (s)** | **aPTT (s)** |
| **10 µg/ml** | | | 1 U/ml | 18.6 | 49.3 | 1 U/ml | 25.1 | 300 |
| **5 µg/ml** | 16.5 | 80.4 | 0.5 U/ml | 16.4 | 42.8 | 0.5 U/ml | 20.8 | 270.9 |
| **2.5 µg/ml** | 15.9 | 61.4 | 0.25 U/ml | 16.2 | 42.6 | 0.25 U/ml | 16.6 | 120.8 |
| **1.25 µglml** | 15.9 | 53 | 0.125 U/ml | 15.9 | 42.3 | 0.125 U/ml | 16.4 | 73.3 |
| **0.625 µg/ml** | 15.8 | 47 | 0.0625 U/ml | 15.0 | 41.3 | 0.0625 U/ml | 15.9 | 49.1 |
| **0 µg/ml** | 15.6 | 37.3 | 0 U/ml | 15.9 | 37.3 | 0 U/ml | 15.6 | 37.3 |

Drugs were supplemented into whole blood acquired from healthy volunteers at the specified concentrations. Data is shown as the mean of two independent experiments. The maximum reported time for aPTT is 300 seconds.

**Table 34. Comparison of rTM, AT, and UFH in Clotting Tests in Rat Plasma**

| Recombinant Thrombomodulin | | | Antithrombin | | | Heparin | | |
|---|---|---|---|---|---|---|---|---|
| **Concentration** | **PT (s)** | **aPTT (s)** | **Concentration** | **PT (s)** | **aPTT (s)** | **Concentration** | **PT (s)** | **aPTT (s)** |
| 10 µg/ml | 9.7 | 27.8 | 1 U/ml | 9.4 | 20.0 | 1 U/ml | 9.6 | 91.4 |
| 5 µg/ml | 10.0 | 23.9 | 0.5 U/ml | 9.3 | 21.3 | 0.5 U/ml | 9.5 | 35.2 |
| 2.5 µg/ml | 9.4 | 21.7 | 0.25 U/ml | 9.4 | 20.9 | 0.25 U/ml | 9.7 | 24.0 |
| 1.25 µglml | 9.5 | 21.1 | 0.125 U/ml | 9.2 | 20.5 | 0.125 U/ml | 9.2 | 21.2 |
| 0.625 µg/ml | 8.9 | 21.2 | 0.0625 U/ml | 9.0 | 20.5 | 0.0625 U/ml | 9.5 | 20.2 |
| 0 µg/ml | 9.5 | 20.2 | 0 U/ml | 9.5 | 20.2 | 0 U/ml | 9.5 | 20.2 |

Drugs were supplemented into pooled plasma from healthy rats at the specified concentrations.

**Thromboelastography**

Thromboelastography (TEG) was used to assess the anticoagulant activity of rTM, AT, and UFH in human whole blood. R time, K time, angle, and maximum amplitude were recorded as methods to describe clot formation. R is reflective of the time required for clot formation to start; a longer R time indicates greater anticoagulant activity. K time is the interval between clot initiation and the time that amplitude reaches 20 mm in size and is a descriptor of the speed of clot formation. The angle provides similar information to the K time and is the angle of the tangent to the thromboelastograph curve at the K time; a wider angle describes faster clot formation. Maximum amplitude (MA) is the maximum size reached by the clot and describes clot strength.

Thromboelastography was used to compare the anticoagulant properties of rTM, AT, and UFH at concentrations ranging from 0-5μg/ml for rTM, 0-5 μg/ml for UFH, and 0-5 U/ml for AT. Four concentrations of each drug were run simultaneously in the blood of a single donor. The same donor was used for all three drugs. Thromboelastography plots and tabulated data is shown in Figure 34.

Heparin demonstrated strong anticoagulant properties at all doses, with an R time greater than 60 minutes and no K time, angle, or maximum amplitude computed within the time limit. AT also demonstrated anticoagulant properties at higher drug concentrations. In contrast, the anticoagulant effects of rTM were minimal, even at the highest utilized concentration of 5 μg/ml. This suggests that rTM may have an improved

safety profile in the treatment of sepsis-associated DIC due to a reduced risk of bleeding compared to AT and heparin.

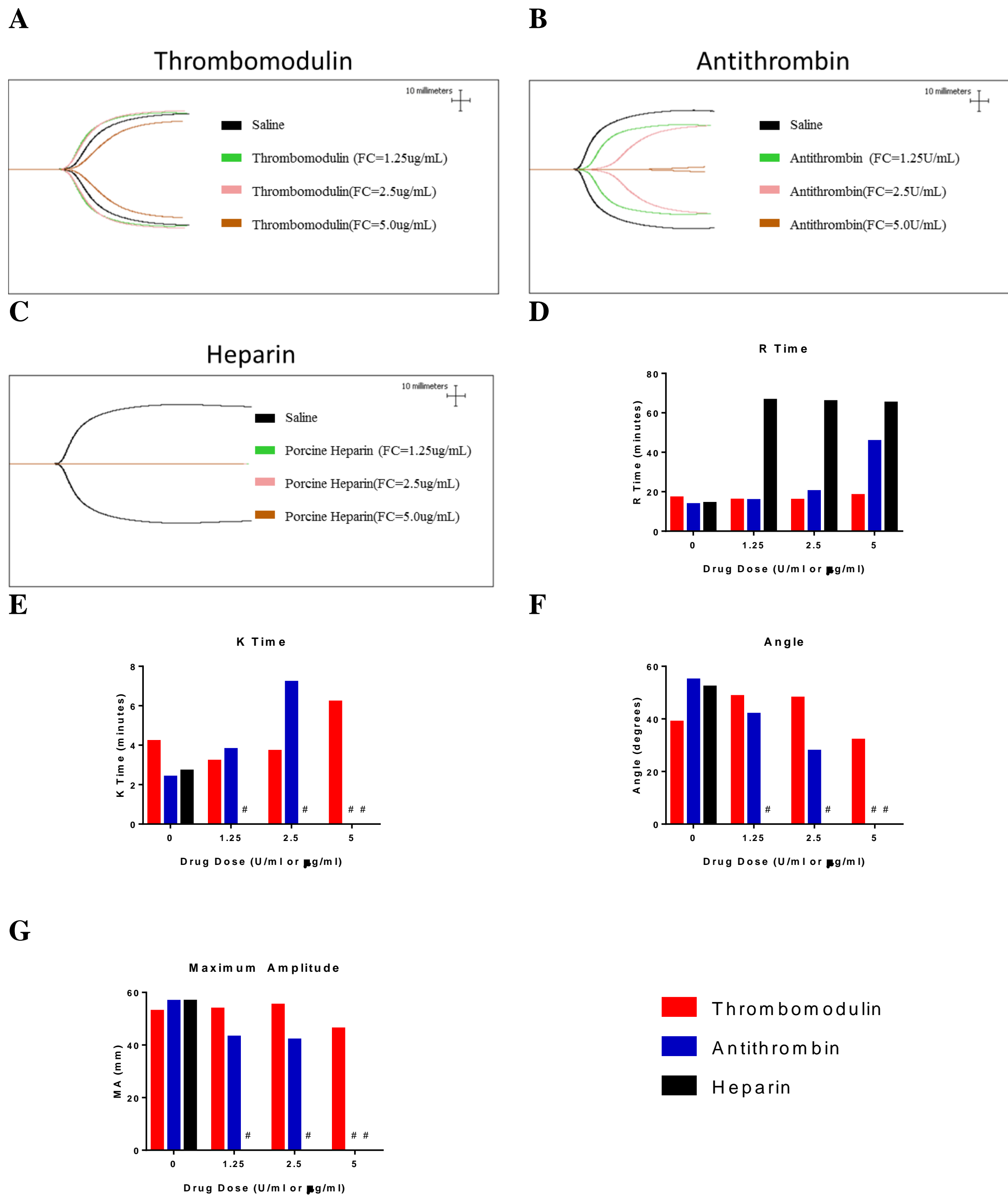


**Figure 34. Comparison of Anticoagulant Properties of rTM, AT, and UFH via Thromboelastography**. (A-C) Plots showing the anticoagulant properties of AT, UFH, and rTM in whole blood as measured via thromboelastography in the blood of a single donor. (D-G) Graphs of thromboelastograph parameters of UFH, AT, and rTM. # indicates that parameter could not be measured at a given concentration as clot formation within the allocated time frame was insufficient.

In order to compare the anticoagulant effects of rTM, AT, and heparin in a single donor, one concentration of each drug and a saline control was run simultaneously in a single donor. Three combinations of drug doses were used, and each set of dosages was repeated with three individual donors. The time allotted for clot formation in each test was limited to one hour. Data is show in Figures 35-38

Direct comparisons were performed between AT at 5 U/ml, rTM at 2.5µg/ml, heparin at 2.5µg/ml, and saline control in 3 donors. Direct comparisons were also performed in 3 donors between AT at 2.5 U/ml, rTM at 2.5 µg/ml, and heparin at 1.25 µg/ml saline. A third set of comparisons was performed in 3 donors between AT at 1.25 µ/ml, rTM at 1.25 µg/ml, heparin at 1.25 µg/ml, and saline.

As shown in Figure 35, heparin demonstrated the greatest prolongation in R time, with clot formation only occurring in under one hour with a heparin concentration of 1.25 µg/ml. AT treatment led to R time prolongation, with no clot formation detected within the time limit at an AT concentration of 5 U/ml. The R time prolongation observed with rTM was minimal.

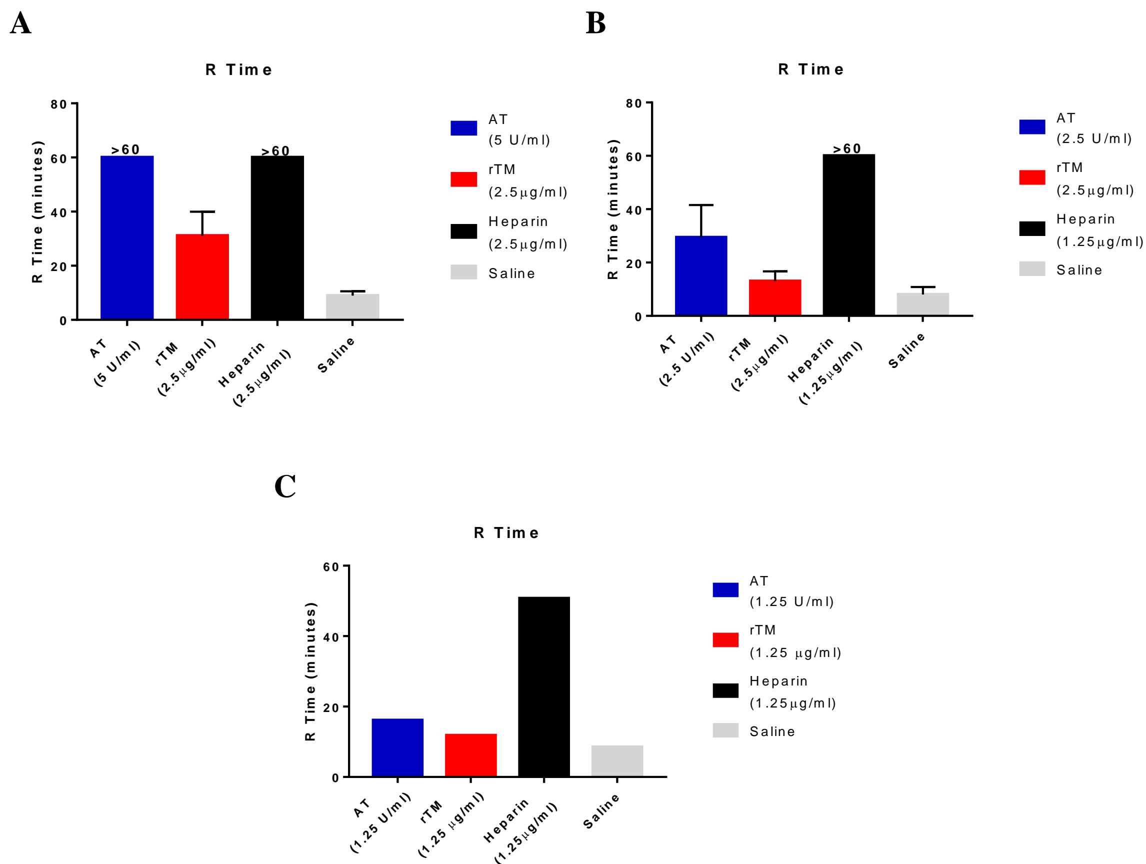


**Figure 35. R Times of rTM, AT, and UFH Treated Whole Blood**. Data is shown as mean ± SD of three independent experiments with blood collected from individual donors. For conditions in which no clot formed within the time limit, R time is shown as 60 minutes.

As shown in Figures 36-38, clot formation was slow in all heparin treated samples, resulting in no measureable K times, angles, or maximum amplitudes. Clot formation in AT treated samples was also accelerated, with no measurable K time, angle, or maximum amplitude at the highest dose of 5 U/ml. rTM showed minimal, if any, slowing of clot formation at all evaluated doses.

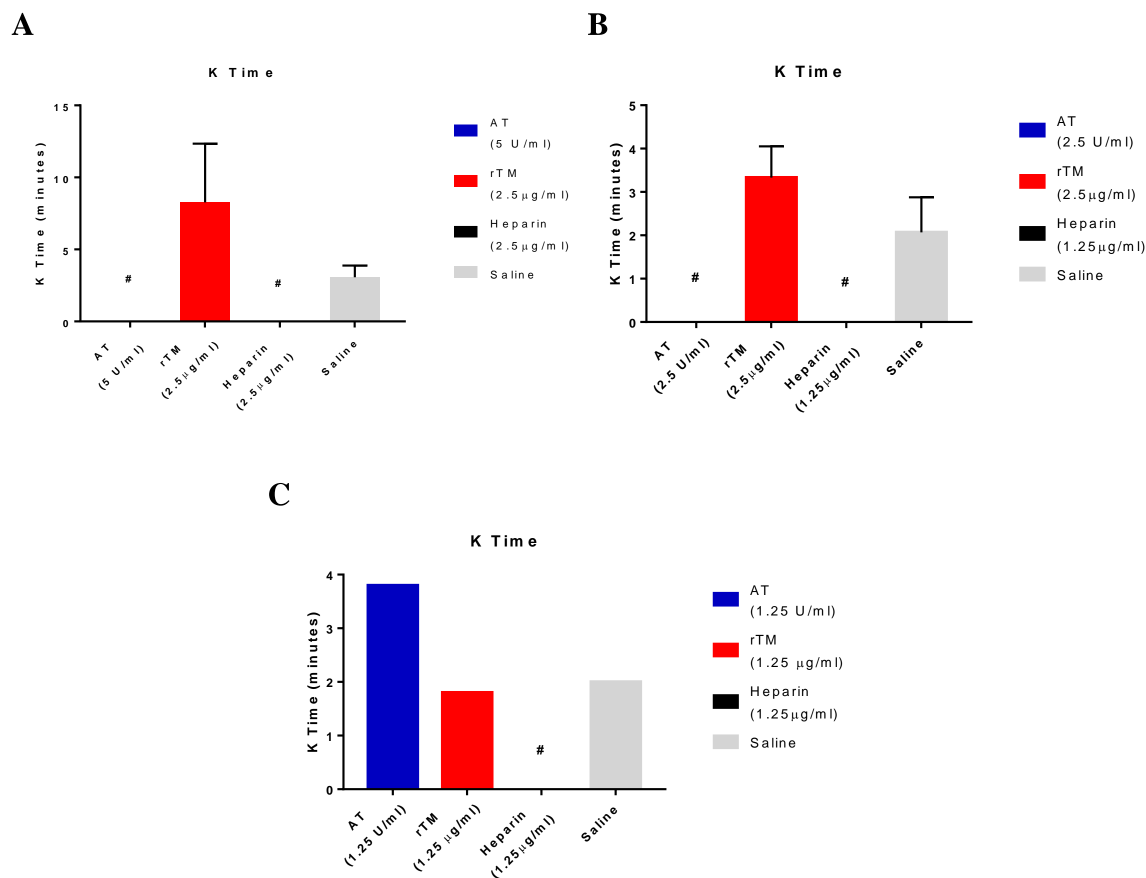


**Figure 36. K times of rTM, AT, and UFH Treated Whole Blood**. Data is shown as mean ± SD of three independent experiments with blood collected from individual donors. # indicates that no clot was formed within the time limit of one hour for a given condition.

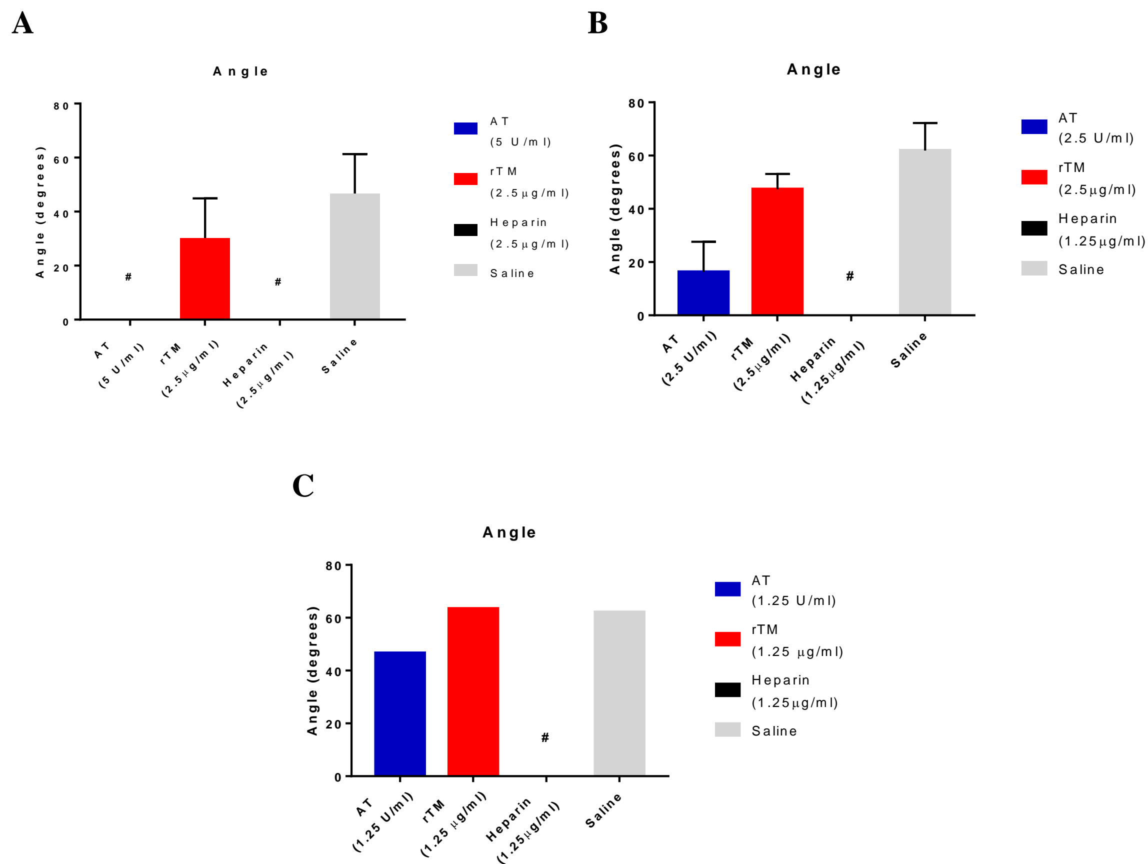


**Figure 37. Angles of rTM, AT, and UFH Treated Whole Blood**. Data is shown as mean ± SD of three independent experiments with blood collected from individual donors. # indicates that no clot was formed within the time limit of one hour for a given condition.

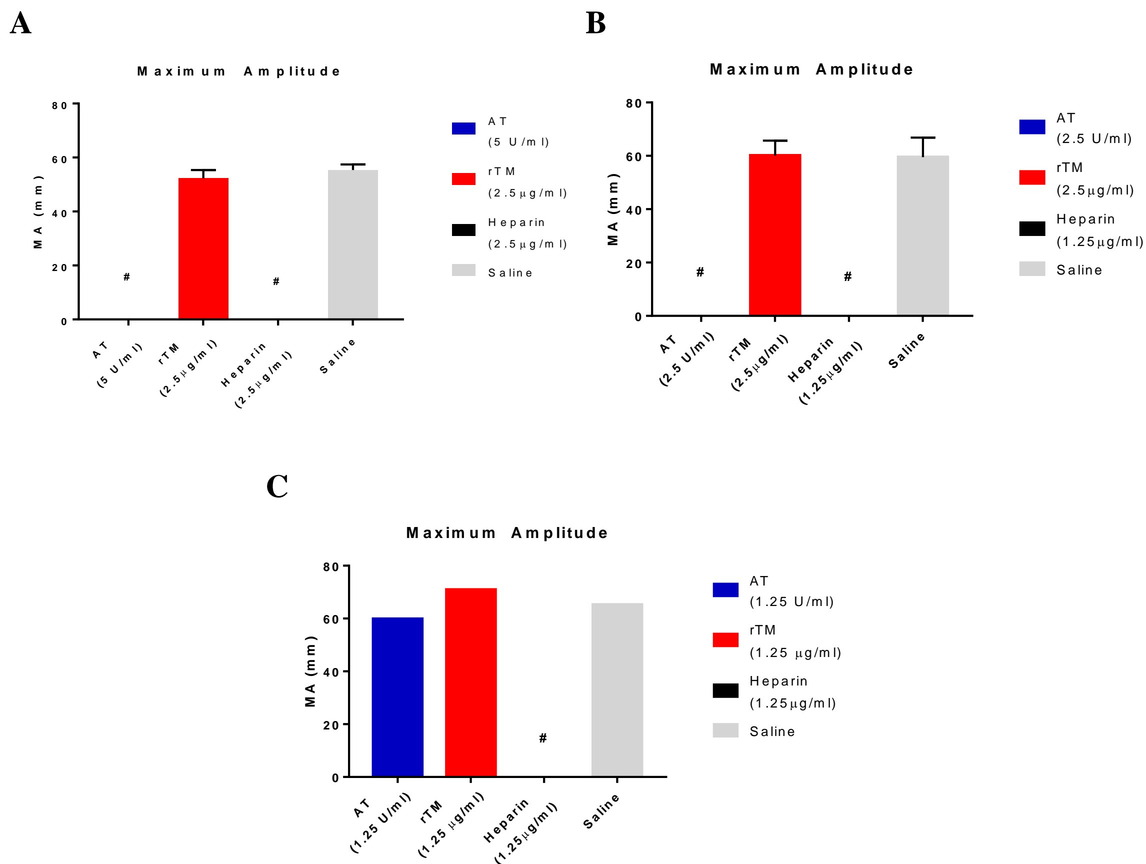


**Figure 38. Maximum Amplitudes of rTM, AT, and UFH Treated Whole Blood**. Data is shown as mean ± SD of three independent experiments with blood collected from individual donors. # indicates that no clot was formed within the time limit of one hour for a given condition.

## Animal Models

The cecal ligation and puncture (CLP) model, described previously, was performed in rats to induce sepsis and concomitant coagulopathy. The purpose of this study was to evaluate the effects of treatment with rTM, AT, and UFH on the molecular pathophysiology of DIC. Accordingly, it was important to develop a model system in which rats developed sepsis and coagulopathy but survived for at least 72 hours to allow for drug administration and blood collection.

Biomarkers were selected for analysis in rat plasma on the basis of findings in human plasma. Platelet count was selected as the primary marker of hemostatic status in the rats, with a reduction in platelet count indicative of consumptive coagulopathy. PT was tested in all samples in order to ensure that samples collected were plasma rather than serum. PAI-1 was also measured. Due to the traumatic nature of the cardiac puncture blood draw and the associated activation of coagulation, other coagulation parameters could not be reliably measured. Procalcitonin was the strongest individual predictor of outcome in the Utah patients, demonstrated a significant association with severity of coagulopathy, and is an established biomarker of infection; accordingly, procalcitonin was measured in rats to confirm and monitor infection. IL-10 was included as a measure of the anti-inflammatory processes ongoing to oppose pro-inflammatory activation. The drugs studied are hypothesized to have non-anticoagulant activity mediated partly through interaction with nuclear material. Nucleosomes were measured as a tool to analyze these properties. Weight loss was also monitored as an indicator of general health status.

**Validation of the CLP Model for Sepsis and DIC**

In order to validate this model system, CLP was performed in 22 rats according to the protocol described previously. Of these rats, 2 (9.1%) died within 72 hours. All rats showed signs of illness including subdued behavior 24 hours following surgery. All rats received appropriate analgesia throughout the experimental procedure. Sham surgery, in which an incision was made through the skin and muscle layers but no ligation or puncture of the cecum occurred, was performed in 6 rats, all of which survived.

Blood was collected via cardiac puncture 72 hours following surgery from CLP and sham rats. As shown in Figure 39, changes in biomarker levels consistent with sepsis-associated coagulopathy occurred. The significant elevation in procalcitonin in the CLP group compared to the sham group ($p=0.038$) is indicative of infection in CLP rats. The reduction in platelets in the CLP group ($p=0.013$) is indicative of the development of consumptive coagulopathy in addition to infection in these rats. CLP rats also demonstrated significant elevation in IL-10 ($p=0.002$) and nucleosomes ($p=0.011$) compared to sham rats, consistent with results seen in human sepsis and DIC patients. No significant differences in PAI-1 were noted between sham and CLP rats ($p=0.74$). CLP rats also exhibited significant weight loss over the 72 hour period, with a higher percent loss of body weight than sham operated animals ($p=0.012$). No rats lost more than 12% of pre-surgical body weight through the course of this study.

These results validate this model in terms of the development of sepsis and DIC with characteristics similar to those observed in human patients. Furthermore, the mortality in this model was sufficiently low to facilitate study of drug mechanisms.

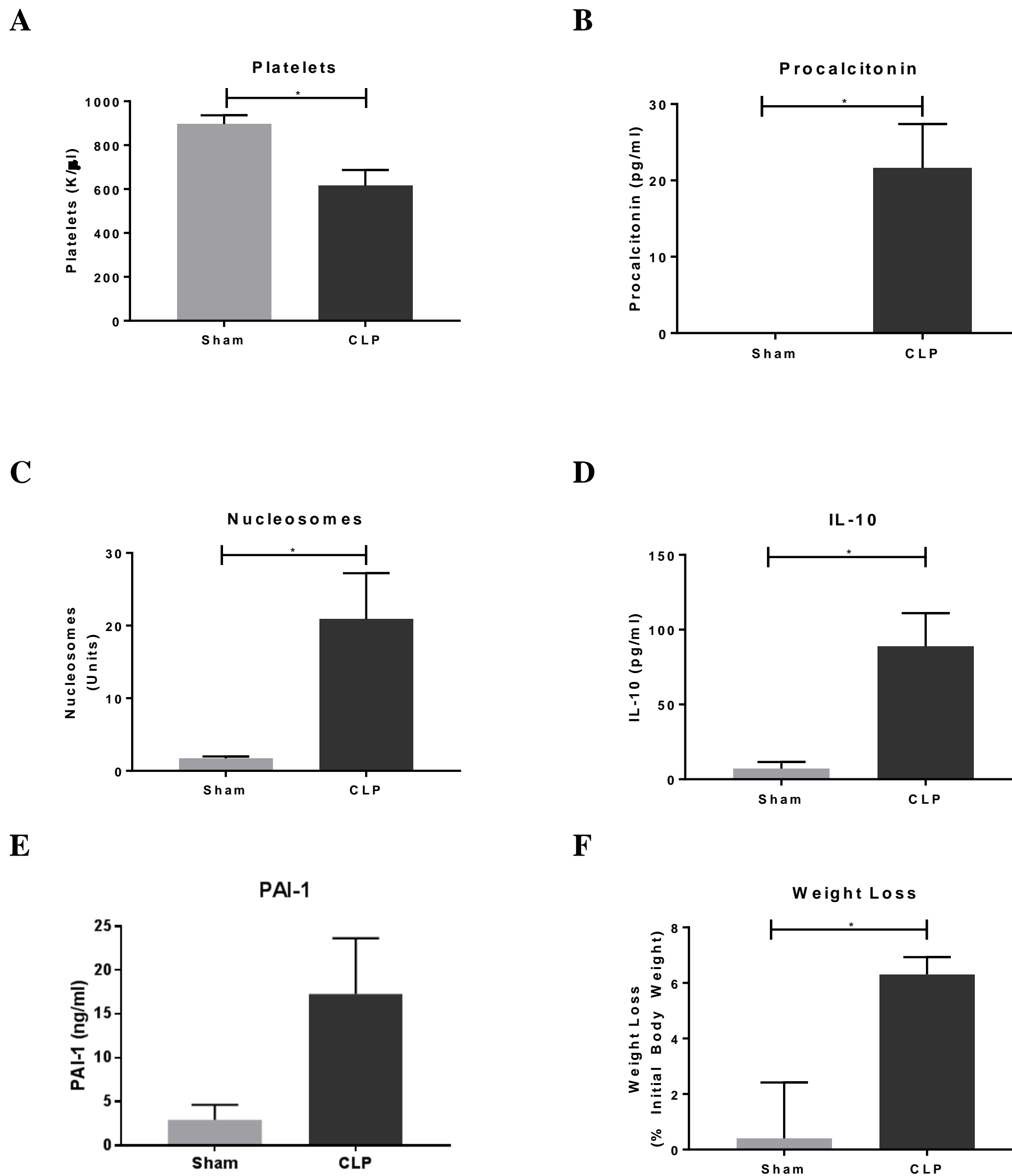


**Figure 39. Validation of rat CLP Model of Sepsis and DIC**. CLP was performed in 22 rats and sham surgery in 6 rats. Biomarker analysis was performed in 16 CLP rats and 5 sham rats for which significant activation of coagulation did not occur during blood draw. Groups were compared using the Mann-Whitney t test with $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

**Comparison of rTM, AT, and UFH in Rats with CLP-Induced Sepsis and DIC**

The mechanisms of action of rTM, AT, and UFH were studied in rats with CLP-induced sepsis and DIC. All drugs were administered intravenously via bolus injection into the tail vein at 24 and 48 hours following surgery. This replicated a realistic clinical scenario in which drugs are not administered until after a patient presents with sepsis and coagulopathy. Blood was collected for analysis 72 hours following surgery (24 hours after the second drug dose).

Two doses of UFH were originally selected for analysis. Heparin is administered in studies of sepsis and DIC at an extremely wide range of doses (Li 2011). The goal in selecting a dose of UFH for use in this study was to maximize the potential non-anticoagulant effect while minimizing bleeding risk. UFH was administered to 5 rats at a high dose of 70 IU/kg. One rat died between 30 and 48 hours following surgery (6 to 24 hours following the administration of the first dose of heparin). Blood was visible in the nares, and necropsy revealed significant bleeding into the gastrointestinal tract. Heparin dose was reduced to 25 IU/kg for the subsequent 10 rats, all of which survived for 72 hours. As shown in Figure 40, no significant differences in biomarker levels were detected between rats treated with 70 or 25 IU/kg UFH. Due to the minimized risk of bleeding complications, 25 IU/kg UFH was selected as the dose for further analysis. Heparin treatment resulted in significant reduction in nucleosomes in rats treated with 25 IU/kg UFH compared to CLP alone ($p=0.004$) and in IL-10 in rats treated with either 25 IU/kg ($p=0.0098$) or 70 IU/kg ($p=0.028$) compared to CLP alone. The reduction in PCT

in heparin treated rats was not statistically significant (Kruskal-Wallis ANOVA p=0.060). No changes in platelet count, weight loss, or PAI-1 were observed in heparin treated rats.

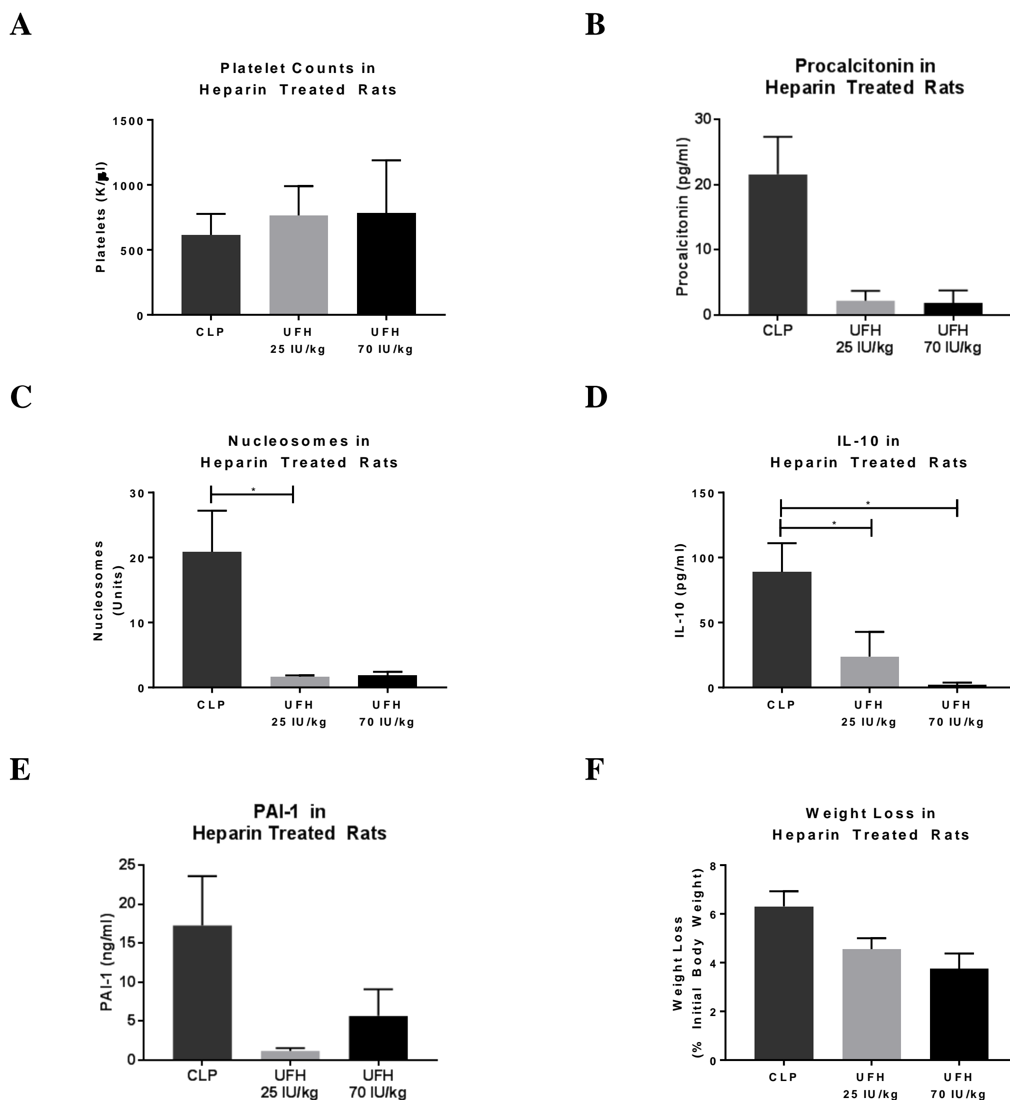


**Figure 40. Comparison of High and Low Dose UFH in the Treatment of Sepsis and DIC.** Data represents rats from which plasma was successfully collected treated with CLP (n=16), CLP + 25 IU/kg UFH (n=9), and CLP + 70 IU/kg UFH (n=4) Groups were compared using the Kruskal-Wallis One-Way ANOVA with Dunn's Multiple Comparison Test and p<0.05 as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

Two doses of AT were also tested in the treatment of rats with sepsis-associated DIC. A low dose of 50 IU/kg was used to achieve clinically relevant blood concentrations. A clinically validated dose of 3000 IU/day (Allingstrup 2016; Iba 2016; Iba 2012) in a 70kg “standard man” corresponds to a dose of 43 IU/kg. However, it is hypothesized that higher doses of AT may be necessary to achieve anti-inflammatory effects (Uchiba 1998); AT is commonly administered to rats at doses of 125 IU/kg or higher (Uchiba 1998; Yamashiro 2001; Yang 1994). 5 rats were treated with 50 IU/kg AT and 9 with 125 IU/kg; all survived for 72 hours.

As shown in Figure 41, no significant differences were noted between rats treated with 50 IU/kg or 125 IU/kg AT. Although bleeding complications with AT have been reported, none were observed. 125 IU/kg was used in further analysis in order to maximize the ability to detect non-anticoagulant effects. Treatment with both high and low dose AT resulted in a reduction in nucleosomes and reduced weight loss.

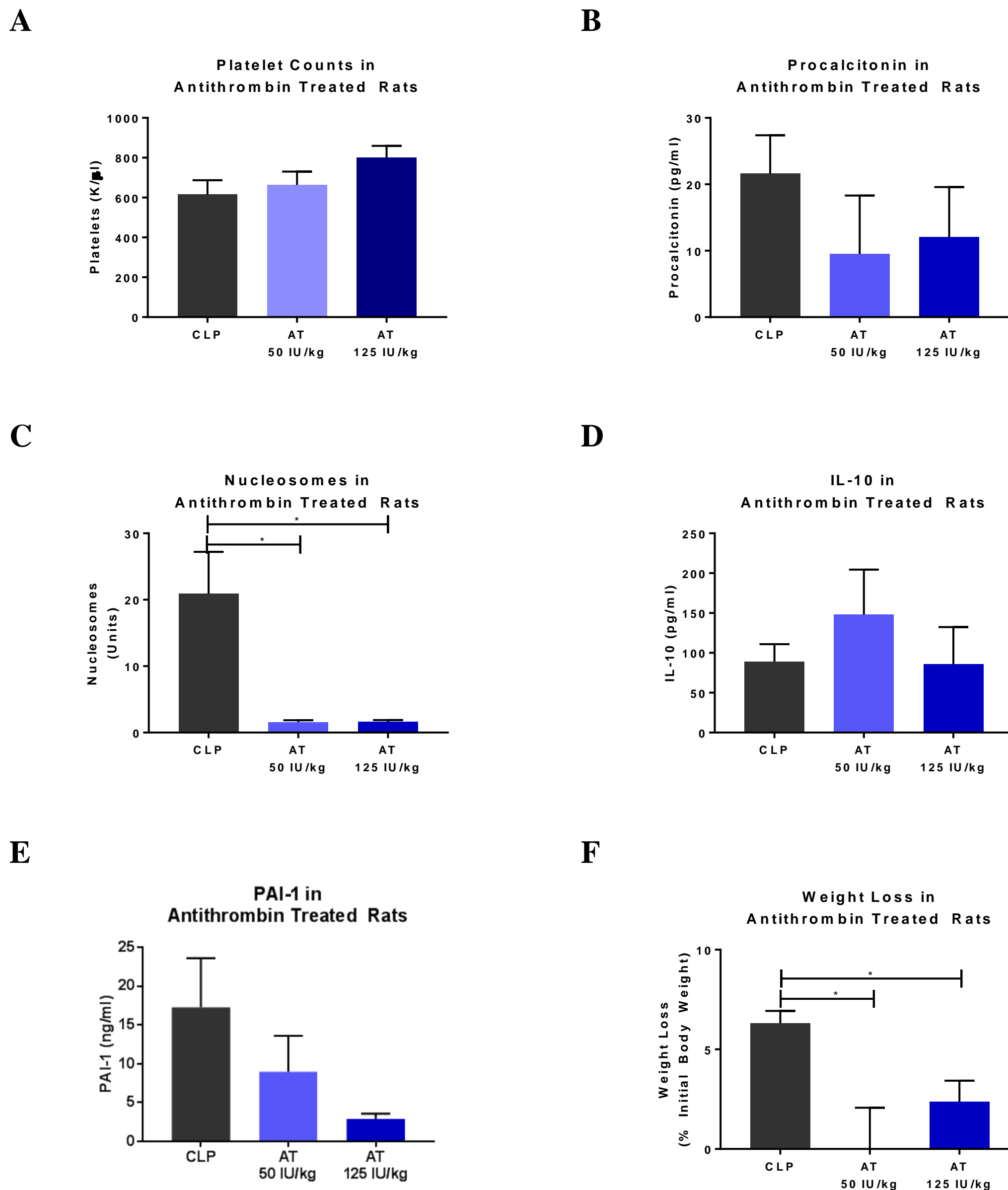


**Figure 41. Comparison of High and Low Dose AT in the Treatment of Sepsis and DIC**. Data represents rats from with plasma was successfully collected treated with CLP (n=16), CLP + 50 IU/kg AT (n=5), and CLP + 125 IU/kg AT (n=8) Groups were compared using the Kruskal-Wallis One-Way ANOVA with Dunn's Multiple Comparison Test and $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

rTM was used at the single dose of 1 mg/kg throughout this study. This is higher than the 60 µg/kg dose at which rTM is typically administered in clinical trials in humans. However, previous studies have demonstrated that higher doses of rTM are required to achieve comparable effects in rats, and 1 mg/kg is a commonly accepted dose in both the original pre-clinical development of rTM and in more recent work (Gonda 1993; Hagiwara 2010; Iba 2013; Mohri 1994; Nagato 2009). rTM was administered to 11 rats at a dose of 1 mg/kg; 3 rats (27%) died within 72 hours.

In order to compare the mechanisms of rTM, AT, and UFH, the levels of the selected biomarkers were compared between rats treated with one concentration of each drug as well as CLP rats without drug and sham operated rats. As shown in Figure 42, no drugs led to a significant change in platelet count (Kruskal-Wallis ANOVA p=0.090), suggesting that drug treatment led to at best incomplete resolution of coagulopathy. Significant variation in procalcitonin based on treatment condition was observed (Kruskal-Wallis ANOVA p=0.040); however, significance was not reached between any individual treatment groups. All drugs showed drastic reductions in nucleosomes (rTM, p=0.031; AT, p=0.016, UFH, p=0.028) compared to untreated rats. UFH exhibited the greatest effects, also leading to significant reduction in both IL-10 (p=0.046) and PAI-1 (p=0.035). In contrast, AT treatment led to a reduction in weight loss (p=0.034). No changes in PT or aPTT were present between any groups.

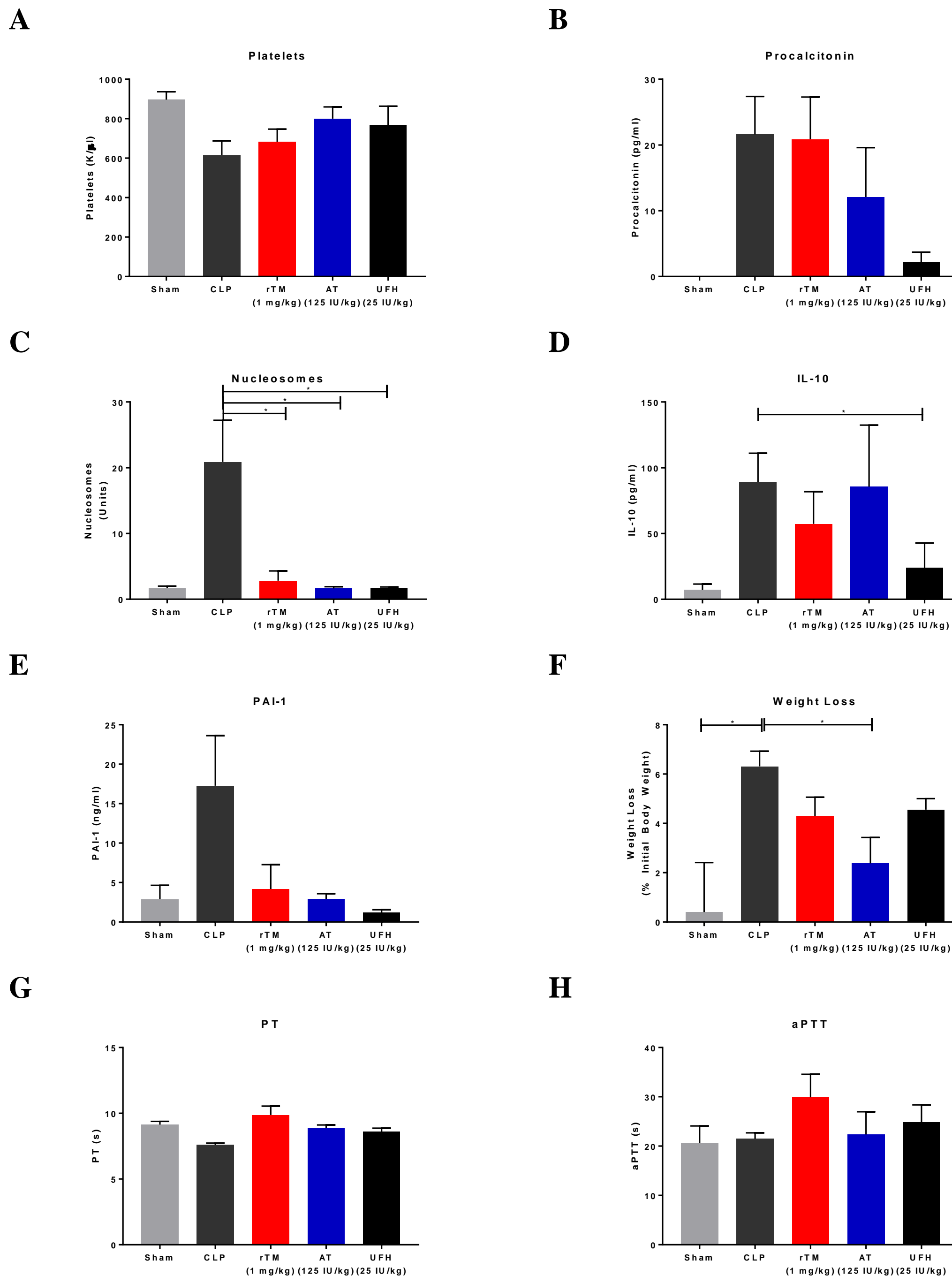


**Figure 42. Comparison of rTM, AT, and UFH in the Treatment of CLP-Induced Sepsis and DIC.** Data represents rats from which plasma was successfully collected from sham (n=5), CLP (n=16), 1 mg/kg rTM (n=7), 125 IU/kg AT (n=8), and 25 IU/kg UFH (n=9) groups. Groups were compared using the Kruskal-Wallis One-Way ANOVA with Dunn’s Multiple Comparison Test and $p<0.05$ as the cutoff for significance (indicated by *). Data is shown as mean ± SEM.

# CHAPTER FIVE

## DISCUSSION

Sepsis is a severe systemic response to infection, defined by a pathological and overwhelming immune and inflammatory response. Although a robust immune response is necessary to overcome infection, the response seen in sepsis can have detrimental effects on the host. The inappropriate immune response in sepsis has both quantitative aspects, typically characterized by elevated plasma cytokine levels, and qualitative aspects, such as changes in the function of neutrophils and other immune cells. Sepsis may progress to septic shock, in which hypotension and hypoperfusion contribute to the failure of multiple organ systems. Furthermore, sepsis can lead to coagulation dysfunction, which contributes significantly to morbidity and mortality.

Sepsis-associated disseminated intravascular coagulation (DIC) is a serious and often fatal medical condition occurring as a complication of sepsis which causes significant morbidity and mortality worldwide. DIC is the severe manifestation of a spectrum of coagulation disorders which occur secondary to sepsis. This disorder is characterized by both thrombotic and hemorrhagic complications. At present, DIC is identified in sepsis patients on the basis of the consumptive coagulopathy characteristic of this disease. Inappropriate and widespread coagulation consumes platelets and coagulation factors, resulting in measurable decreases in platelet count and fibrinogen

levels and elevation of the INR due to the consumption of coagulation proteins. Fibrin split products or D-Dimer are also measured and reflect the presence and ongoing breakdown of thrombi in the circulation. Given the current lack of specific therapeutic agents for DIC, this diagnostic scheme may be sufficient. However, it does not account for the underlying pathophysiology of DIC or the ways in which this may influence standard laboratory test results. When drugs for the treatment of DIC do become available, an improved diagnostic approach will be necessary to identify patients most likely to benefit from therapies and to monitor the effectiveness of treatment. An improved understanding of the underlying molecular pathophysiology of DIC may contribute to both the development of drugs for this disease and the identification of patients who will benefit from treatment.

In order to better understand the pathophysiology of sepsis-associated coagulopathy, a systematic approach taking into account all of the processes contributing to the development of this disease—not only the resulting coagulation dysfunction—is required. Both sepsis and DIC are complex clinical scenarios with pathophysiology encompassing all aspects of the blood and vasculature. This includes not only coagulation and inflammatory processes but also the response to infection as well as endothelial and platelet activation. While this complicates the modeling of this disease process, it also means that the blood and the factors it contains provide a window through which to understand this disease. Many biomarkers have been evaluated for their utility in understanding and diagnosing sepsis, and numerous reviews have been written on this

topic (Biron, Ayala, & Lomas-Neira, 2015; Paulus, Jennewein, & Zacharowski, 2011; Pierrakos & Vincent, 2010; Sims, Nguyen, & Mayeux, 2016). However, analysis of the association of these factors with well-defined coagulopathy is often lacking. Furthermore, these biomarkers may also be useful in the validation of the physiological relevance of animal models for sepsis-associated DIC.

An improved understanding of the molecular pathophysiology of sepsis-associated DIC will also provide information for the development and implementation of therapeutics for this disease. One of the guiding principles of medicine is “first, do no harm”. The opposing risks for thrombosis and bleeding in this patient population make the development of a drug to treat coagulopathy without causing harm difficult. Any anticoagulant drug may carry significant bleeding risk in this already vulnerable patient population, while any treatment designed to promote coagulation and prevent bleeding may fuel ongoing coagulation.

In addition to exogenous anticoagulants such as heparin, potential treatments for DIC include the replacement of endogenous anticoagulants such as thrombomodulin, antithrombin, and protein C. Under normal physiological circumstances, these proteins serve to prevent excessive or inappropriate coagulation. However, these factors become depleted or dysregulated in DIC. These factors are also pleiotropic, with cytoprotective and anti-inflammatory effects in addition to their major hemostatic functions. Two endogenous anticoagulants, antithrombin (AT) and recombinant thrombomodulin (rTM) are currently approved for use in the treatment of DIC in Japan, where post-approval studies have demonstrated efficacy in sepsis-associated DIC, particularly in more severe

cases (Kienast 2006; Umemura 2016; Yamakawa 2015). However, AT in particular may be associated with an elevated risk of bleeding. A previously investigated endogenous anticoagulant, activated protein C (APC) was approved for use in patients with sepsis-associated coagulopathy and subsequently withdrawn from the market due to serious bleeding events. *Post hoc* analyses of the clinical trials for APC have demonstrated that patients with the most severe disease may have benefitted while patients with less severe disease may have experienced adverse events (Dhainaut 2004; Kienast 2006). Accordingly, an understanding of the additional mechanisms of action of these drugs in conjunction with an improved understanding of the pathophysiology of DIC may contribute to optimal targeting of treatments to patients who will benefit the most from intervention.

Thus, the purpose of this dissertation was twofold. First, a wide array of biomarkers were assessed in the plasma of a cohort of patients with sepsis and strictly defined coagulopathy. This allowed for the evaluation of the association of these markers with both mortality and the degree of coagulopathy and the identification of markers representative of multiple aspects of disease that were relevant to outcome. Secondly, three potential therapeutics for DIC, recombinant thrombomodulin, antithrombin, and heparin, were compared *in vitro* for their anticoagulant effects and *in vivo* in a rat model of sepsis-associated DIC in order to provide insight into the actions of these agents in the setting of this disease.

**Coagulation Profiling in DIC vs. Warfarin Treated Patients**

The initial identification of patients with sepsis-associated DIC is commonly based on the presence of an elevated INR. As a component of the DIC scoring algorithm, INR is by definition associated with the severity of DIC, as was observed in the Utah patient cohort. Elevated INR is often associated with poor outcome or increased mortality in sepsis patients (Dhainaut 2005; Kinasewitz 2004) as well as in other critically ill patient populations (MacLeod 2003; Walsh 2010), although no association between INR and mortality was seen in the Utah patient cohort. As INR is one of the key parameters used clinically to identify patients with sepsis-associated DIC, it is important to understand the appropriate interpretation of this measure in this patient population. Prolonged PT or elevated INR is generally indicative of a hypocoagulable state. In patients receiving warfarin anticoagulant therapy, the degree of INR elevation is indicative of the degree of anticoagulation resulting from treatment and is used to guide therapy and maintain an appropriate balance between thrombotic and bleeding risks. However, DIC patients with an elevated INR are at risk of complications due to both thrombosis and bleeding. This leads to the hypothesis that the same INR result does not indicate the same status of the coagulation cascade in DIC patients compared to warfarin-treated patients. In order to better understand the appropriate interpretation of INR in DIC patients, the relationship between INR, other global coagulation tests, and coagulation factor levels was compared in patients with sepsis-associated DIC to patients receiving warfarin anticoagulation. In addition to PT, aPTT, and fibrinogen, functional and protein levels of Factors VII, IX, and X were measured. Although warfarin also effects Factor II,

it is the slowest of the coagulation factors to respond to warfarin therapy. Therefore, Factor II would be expected to remain unaltered in patients with warfarin levels within the therapeutic range and was not included in this study.

Markedly different relationships between INR and other laboratory coagulation tests and coagulation factor levels were observed in DIC patients compared to patients receiving warfarin anticoagulation. In DIC patients, increased INR was associated with increased aPTT. This supports the hypothesis that the coagulation dysfunction indicated by a given INR level is different in warfarin treated patients, where changes in INR are the result of a targeted disruption to the coagulation cascade, than in DIC patients, where an elevated INR is indicative of a more diffuse insult to the coagulation system.

Functional and antigenic levels of coagulation factors VII, IX, X were decreased in both warfarin treated and DIC patients compared to healthy controls. However, the pattern of decrease in factor levels was markedly different between the two patient groups. In warfarin treated patients, the decrease in factor levels corresponded strongly with an increase in INR. In contrast, the factor levels in DIC patients were uniformly low across all INR levels; additional increases in INR did not correspond with an additional drop in coagulation factor levels.

When correlations between all coagulation tests and factor levels were assessed, strikingly different patterns were observed in DIC and warfarin treated patients. In warfarin treated patients, functional and antigenic levels of Factors VII, IX, and X showed strong correlations with each other. The highly correlated levels of coagulation

factors in these patients are a reflection of the unified mechanism by which warfarin inhibits coagulation. These factors also correlate strongly with INR, the test designed to monitor the effects of warfarin on the coagulation cascade. In contrast, minimal significant correlations were observed between coagulation factors in DIC patients. While coagulation factor levels were overall decreased compared to healthy controls in this patient population, the patient to patient variation in the nature of this decrease was high, indicated by the lack of correlation between factor levels. Furthermore, the levels of individual coagulation factors were not predictable based on INR in the DIC patient cohort.

These results support the hypothesis that the meaning of an elevated INR is substantially different in warfarin treated patients than in DIC patients. In warfarin treated patients, an elevated INR suggests a uniform and predictable reduction in the detectable and functional levels of multiple coagulation factors without an accompanying alteration in the other global coagulation parameter of aPTT. In this patient population, elevated INR is solely a measure of a specific type of hypocoagulability induced by warfarin in order to prevent thrombotic complications. In contrast, an elevated INR in DIC patients provides different information and is accompanied by global coagulation dysfunction, suggested by the strong correlation between elevated INR and elevated aPTT in this patient population. This demonstrates that unlike warfarin anticoagulation, DIC has strong effects on both the extrinsic and intrinsic pathways of the coagulation cascade. However, this alteration in coagulation is not accompanied by predictable or consistent changes in levels of individual coagulation factors in this patient population. .

Despite the elevation of both INR and aPTT in DIC patients, INR remains the superior of the two tests for diagnosis of sepsis-associated coagulopathy due to its standardization between clinical sites. In DIC patients, elevated INR is accompanied not only by bleeding risk but also by thrombosis in this patient population, a risk that is not demonstrable by analysis of traditional hemostatic parameters.

This study underscores the need for an improved understanding of the relationship of hemostatic laboratory parameters to the ongoing coagulation processes and the associated risks of both bleeding and thrombosis specific to the DIC patient population. Furthermore, this demonstrates that the coagulation tests available in the clinical laboratory may not provide an accurate description of the nature or severity of the coagulopathy in patients with sepsis-associated DIC. Additional hemostatic parameters as well as biomarkers representing the processes that lead to the development of coagulopathy such as infection response and endothelial dysfunction must be analyzed for their association with the severity of coagulopathy. This understanding has implications for both the diagnosis of patients with sepsis-associated DIC and the development and administration of safe and effective treatments for these patients.

**Biomarker Profiling of Utah Cohort Patient Plasma Samples**

In order to understand the molecular pathogenesis of sepsis-associated DIC, biomarkers of hemostatic dysregulation, inflammation, infection, endothelial function, and platelet function were measured in the plasma of patients with sepsis and associated DIC. The results of these studies, summarized in Table 35, supported the hypothesis that

a combination of biomarkers representative of multiple physiological systems would provide greater insight into the pathophysiology and outcome of sepsis-associated DIC than markers of a single system. Biomarkers representative of multiple physiological systems were relevant to all studied aspects of disease, including organ failure, severity of coagulation dysfunction, and association with mortality. However, inflammation and coagulation were particularly relevant to organ failure whereas infection response and coagulation function were particularly relevant to severity of coagulopathy and mortality. Furthermore, the use of biomarkers representative of multiple physiological systems permitted the development of predictive algorithm for patient outcome. This algorithm incorporated procalcitonin (a biomarker of infection), VEGF and the IL-6:IL-10 ratio (biomarkers of inflammation), endocan (a biomarker of endothelial function), and PF-4 (a biomarker of platelet function). The predictive ability of this algorithm was superior to that of any individual biomarker.

Procalcitonin, a biomarker of infection, was associated with organ failure, severity of coagulopathy, and mortality outcome. While associations were present between all systems and organ failure, severity of DIC, and mortality, organ failure was predominantly associated with biomarkers of hemostasis (D-Dimer, INR, and platelet count) and inflammation (IL-6, IL-8, IL-10, TNFα, and MCP-1) whereas severity of coagulopathy and mortality were predominately associated with biomarkers of infection (nucleosomes, HMGB-1, and procalcitonin) and endothelial function (protein C, endocan, and Ang-2).

Disease severity and patient outcome were predominately associated with changes in levels of biomarkers representing infection and endothelial function. These findings support the hypothesized roles of infection response (particularly as characterized by the presence of extracellular nuclear material) and endothelial function in the molecular pathogenesis of sepsis-associated DIC and promote further study of these systems, particularly with respect to their involvement in the response to treatment.

Throughout this study, procalcitonin demonstrated the most significant association with patient clinical status and outcome. Procalcitonin was the only marker to demonstrate associations with organ failure, severity of coagulopathy, and mortality as well as to be included in the predictive algorithm for outcome. While procalcitonin is well established as an indicator of ongoing infectious processes, this study also identified a significant association between procalcitonin and the severity of coagulopathy. Procalcitonin is clinically available as a laboratory test and is used as an indicator of whether a systemic inflammatory process is infectious or sterile in origin. This would facilitate the inclusion of procalcitonin in any testing panel for use in patients with sepsis or DIC. Nucleosomes and HMGB-1 levels were individually weaker markers than procalcitonin in terms of ability to distinguish survivors from non-survivors. However, in addition to diagnostic relevance, both HMGB-1 and nucleosomes have the mechanistic relevance to the development of DIC through their pro-coagulant, pro-inflammatory, and endothelial damaging properties and may be involved in the response to treatment with heparin, rTM (Iba 2014; Nakahara 2013; Osada 2017), or AT (Iba 2017). Therefore,

these nuclear factors should be included in further studies of both the pathophysiology and treatment of DIC.

Endothelial markers also demonstrated particularly strong associations with severity of coagulopathy and with outcome. Protein C is well documented to play a role in the pathophysiology of sepsis-associated DIC, and the protein C pathway is a major therapeutic target in this disease. Ang-2 also emerged as strongly associated with both severity of coagulopathy and mortality, representing a new potential avenue for study in this disease process.

**Table 35. Summary of Utah Cohort Biomarker Profiling Results**

| Marker | Significant Association | | | Included in Predictive Model | |
|---|---|---|---|---|---|
| | SOFA | DIC | Mortality (AUC) | Constant AUC=0.87 | Linear AUC=0.95 |
| **D-Dimer** | **0.21** | **Yes** | No (0.60) | No | **Yes** |
| **F1.2** | 0.12 | Yes | No (0.54) | No | No |
| **PAI-1** | 0.04 | No | **Yes (0.70)** | No | **Yes** |
| **INR** | **0.20** | **Yes** | No (0.60) | No | No |
| **Platelets** | **-0.36** | **Yes** | No (0.61) | No | No |
| **Fibrinogen** | -0.02 | No | No (0.51) | No | No |
| **Nucleosomes** | -0.07 | **Yes** | No (0.58) | No | No |
| **HMGB-1** | -0.07 | No | **Yes (0.67)** | No | **Yes** |
| **Procalcitonin** | **0.28** | **Yes** | **Yes (0.77)** | **Yes** | **Yes** |
| **IL-2** | 0.06 | No | No (0.52) | No | No |
| **IL-4** | 0.01 | No | No (0.55) | No | No |
| **IL-6** | **0.26** | No | **Yes (0.70)** | No | No |
| **IL-8** | **0.32** | **Yes** | **Yes (0.70)** | No | No |
| **IL-10** | **0.23** | No | No (0.58) | No | No |
| **VEGF** | 0.00 | No | No (0.57) | **Yes** | No |
| **IFNγ** | 0.01 | No | No (0.54) | No | No |
| **TNFα** | **0.22** | No | No (0.52) | No | No |
| **IL-1α** | 0.14 | No | No (0.60) | No | No |
| **IL-1β** | 0.11 | No | No (0.58) | No | No |
| **MCP-1** | **0.33** | No | No (0.53) | No | **Yes** |
| **EGF** | -0.17 | **Yes** | No (0.58) | No | **Yes** |
| **IL-6:IL-10** | 0.17 | No | No (0.61) | **Yes** | **Yes** |
| **TFPI** | 0.00 | No | No (0.55) | No | No |
| **Protein C** | **-0.22** | **Yes** | **Yes (0.71)** | No | **Yes** |
| **Endocan** | -0.02 | No | **Yes (0.58)** | **Yes** | **Yes** |
| **Ang-2** | 0.14 | **Yes** | **Yes (0.76)** | No | No |
| **vWF** | -0.12 | No | No (0.58) | No | No |
| **CD40L** | -0.18 | No | No (0.55) | No | **Yes** |
| **MP** | **-0.26** | No | No (0.53) | No | **Yes** |
| **MP-TF** | 0.16 | No | No (0.62) | No | No |
| **PF-4** | **-0.43** | No | **Yes (0.70)** | **Yes** | **Yes** |

Spearman correlation coefficients shown for relationship with SOFA score. Significant correlations are highlighted in blue with bold text. Significant association with DIC score or mortality determined on the basis of significant difference between survivors and non-survivors or significant difference between patient groups based on DIC score.

**Patient Cohort Baseline Characteristics**

The Utah patient cohort samples used throughout this study were collected from consenting adult patients following IRB approved protocols. The enrollment criteria used to define sepsis for the purpose of this cohort are well defined (Levy 2003) and are the criteria most commonly used in the literature to define sepsis (Alhamadi 2015; Bozza 2007; Claushuis 2016; Collins 2006; Davis 2010; Hovinga 2007; Ioakeimidou 2017; Jones 2009; Kaplan 2015; Livaditi 2006; Mihajlovic 2014; Ogura 2014; Ricciuto 2011; Rondina 2015; Scherpereel 2006; Siner 2009; Soriano 2005; Sunden-Cullberg 2005; van der Heijden 2009; Wildhagen 2015). The most recent guidelines for the diagnosis of sepsis (Singer 2016) were published after the collection of all patient samples and the initialization of this study. Based on available information, it was not possible to determine which patients in this cohort met the SEPSIS-3 guidelines for the diagnosis of sepsis. As the focus of the studies in this dissertation is not sepsis epidemiology but rather the pathogenesis of the associated DIC, this cohort was appropriate for this analysis.

The demographics of this cohort are within the range typical for sepsis patients in the literature. This includes the age distribution (57 ± 18.5 years, mean ± SD) and the gender balance (46.6% male) (Abraham 2003; Chen 2012; Claushuis 2016; Fisher 2016; Jones 2009; Yaroustovsky 2013). The racial and ethnic composition of this cohort is reasonable for the region in which the samples were collected. The highest prevalence comorbidities in this cohort included hypertension (45.6%), diabetes mellitus (25.2%), and cardiovascular disease (21.4%), all of which are common medical conditions. History

of recent surgery was also highly prevalent in this cohort (22.3%). This is reasonable, as sepsis often develops as a complication of surgery.

**Association of Biomarkers with Organ Failure**

The severity of organ failure, quantified by SOFA score, was associated with changes in markers of hemostasis (D-Dimer, INR, and platelets), infection (procalcitonin), inflammation (IL-6, IL-8, IL-10, TNFα, and MCP-1), endothelial function (protein C) and platelet function (PF-4). While organ failure was predominately associated with inflammation and coagulation dysfunction, this demonstrates that infection, endothelial function, and platelet function also play a role in this process.

The severity of disease, quantified by mortality as well as through clinical scoring systems such as SOFA and APACHE II are highly variable based on factors such as study inclusion criterial, standard of care, and variability between institutions and services. The overall 28-day mortality of patients included in this cohort, 14.6%, is relatively low, as mortality in sepsis is often estimated at greater than 20%. However, many studies reporting high mortality are designed to enroll only patients with severe sepsis or septic shock, both of which are associated with increased mortality. Numerous studies enrolling patients with sepsis have reported mortality of under 20% (Abraham 2005; Davis 2010; Gogos 2000; Rondina 2015; Scherpereel 2006; Sunden-Cullberg 2005). Similarly, the SOFA and APACHE II scores were at the low end of the range typically reported for cohorts of sepsis patients. Many studies enrolling sepsis patients report mean SOFA scores between 6 and 9 and mean APACHE II scores between 18 and 25 (Alhamadi 2015; Bozza 2007; Davis 2010; Jones 2009; Ogura 2014; Park 2016; Siner

2009; Sunden-Cullberg 2005; Wildhagen 2015). In this cohort, the SOFA score was 5.9 ± 3.7 (mean ± SD) and the APACHE II score was 17.4 ± 7.3 (mean ± SD). Insufficient information about the causative pathogen was available to allow for analyses based on pathogen type.

The severity of coagulopathy was significantly associated with organ failure, as quantified by SOFA score. DIC is well established to lead to increased organ failure in sepsis patients (Okabayashi 2004). The formation of microthrombi in the vasculature can lead directly to organ failure. Additionally, the status of the hemostatic system as measured by platelet count is included in the SOFA score.

A strong association was also observed between organ failure and inflammation. SOFA score correlated significantly with IL-6, IL-8, IL-10, MCP-1, and TNFα. Notably, this association was not present between SOFA score and the infection markers, nucleosomes and HMGB-1.

Of the inflammatory cytokines, MCP-1 exhibited the most notable association with the severity of illness. In addition to correlating significantly with SOFA score, MCP-1 was significantly elevated in patients who required vasopressor or ventilator support compared to those who did not. Although MCP-1 is relatively less studied than other inflammatory cytokines in the context of sepsis, it has been suggested that MCP-1 may contribute to the development of septic shock and subsequent organ failure through increased vascular leakage, mediated by effects on endothelial tight junctions. Treatment of mice with either LPS or CLP-induced sepsis with a blocker of MCP-1 synthesis was

shown to reduce liver and lung injury, as quantified by myeloperoxidase (MPO) levels (Ramnath 2008).

The associations of hemostatic and endothelial biomarkers with organ failure were relatively weak, and association with ventilator or vasopressor use was minimal. Somewhat surprising was the degree of association between platelet biomarkers and organ failure. It is interesting to note that these relationships were inverse, despite the overall elevation of these biomarkers. This suggests that the involvement of platelets and platelet-associated factors in sepsis and associated DIC may be highly variable through the time course of disease, as platelets progress from activation to consumption.

The main limitation of the relatively mild sepsis and low mortality rate in this patient cohort was the limited statistical power this provides for the comparison of survivors to non-survivors, particularly when the cohort was further subdivided based on other factors such as DIC score. This may explain the lack of association between DIC score and mortality in this patient cohort. Numerous studies have demonstrated an association between DIC score and mortality; however, no such association was observed in this patient population. Other studies have also seen no significant increase in mortality among DIC patients compared to non-DIC sepsis patients (Okabayashi 2004).

Surviving patients in this cohort were assessed for the presence of thrombosis at the time of hospital discharge using ultrasound of the venous system. Major thrombotic events including PE, MI, or thromboembolic stroke were also reported. No associations were noted between baseline levels of any measured biomarker and thrombosis at or prior

to discharge. The thrombosis identified through this protocol, most commonly asymptomatic DVT, is distinct from the sepsis-associated coagulopathy that is the focus of this study, which typically manifests as microthrombi and bleeding due to the consumption of platelets and coagulation factors. Thrombosis is common in hospitalized patients due to a combination of immobilization-induced stasis with other predisposing factors including inflammation and the presence of interventional devices in the vasculature. Without further information regarding the complicated hospital courses of the patients involved in this study, no further analysis of factors associated with this outcome can be performed.

## Association of Biomarker Levels with DIC Score

This study examined the relationship of biomarkers representing hemostasis, infection, inflammation, endothelial function, and platelet function with the severity of DIC in a cohort of patients with sepsis and well defined coagulopathy. The severity of coagulation dysfunction, quantified by DIC score, was associated with markers of hemostasis (D-Dimer, INR, and platelet count), infection (nucleosomes and procalcitonin), inflammation (IL-8 and EGF), and endothelial function (protein C and Ang-2). While multiple systems are involved in the development of coagulation dysfunction, this demonstrates that the processes underlying the development of DIC may be distinct from those classically associated with sepsis. In particular, extracellular nuclear material released into the circulation as a component of the response to infection as well as the function of the endothelium contribute to DIC and should therefore be addressed in the evaluation and treatment of this disease.

The Utah cohort is composed of patients with sepsis and DIC defined according to well-established criteria. While the overall severity of illness, as defined by 28-day mortality, SOFA score, and APACHE-II score was relatively mild, patients were well distributed in terms of severity of DIC. Using the ISTH criteria, 19.4% of patients were diagnosed with no DIC, 57.3% with non-overt DIC, and 23.3% with overt DIC. This distribution of DIC scores enables analysis of the association of biomarker levels with the severity of coagulopathy.

DIC was strictly defined and patients were subdivided into three groups based on DIC score. Many studies categorize patients as either overt DIC (ISTH score ≥5) or no DIC (ISTH score <5) (Bakhtiari 2004; Cauchie 2006; Dhainaut 2004; Jesmin 2012; Joo 2010; Kim 2015; Park 2016; Seo 2009). In these studies, non-overt DIC (ISTH score 3-4) is not treated as an independent category. This results in a highly heterogeneous patient population in the no DIC category, resulting in reduced ability to identify factors associated with the development of severe coagulation dysfunction. Separation of patients with non-overt DIC from those who do not demonstrate coagulation dysfunction (ISTH score ≤2), as was performed in this study, is required for improved understanding of factors involved in the development of coagulation dysfunction. Furthermore, *post hoc* analysis of clinical trials has demonstrated that patients with overt DIC may respond differently to treatments than patients with less severe manifestations of coagulopathy (Dhainaut 2004; Shakoory 2016). In this study, 57.3% of patients had non-overt DIC at baseline.

The majority of measured biomarkers were significantly elevated in all patient groups compared to the healthy control group, regardless of coagulation status. Sepsis represents a severe dysfunction of all major physiological systems; this was reflected in changes in markers of hemostasis, infection, inflammation, platelet activity, and endothelial function in all patients. Notable differences were apparent in markers of infection and endothelial function not only on the basis of sepsis but also on the basis of severity of DIC.

Among the markers of infection, both nucleosomes and procalcitonin were significantly elevated in patients with overt DIC compared to those with less severe coagulopathy. Although this trend was also apparent for HMGB-1, statistical significance was not achieved.

Procalcitonin was significantly elevated in both overt and non-overt DIC patients compared to patients with sepsis alone. Unlike many of the other markers measured in this study, there is no hypothesized direct role for procalcitonin in the development of coagulopathy. Under normal physiological conditions, procalcitonin is produced by the parafollicular cells of the thyroid as the precursor to the hormone calcitonin, which is involved in calcium homeostasis. The mechanism increase in circulating procalcitonin under infectious circumstances is not fully understood, although expression of procalcitonin in numerous tissues has been demonstrated in response to LPS and IL-1β (Riedel, 2012). In animal models, circulating levels of procalcitonin have been shown to correlate well with the quantity of bacteria administered (Becker 2010; Nylen 1998; Steinwald 1999). However, procalcitonin is widely studied in sepsis and accepted as a

means to distinguish infectious conditions from non-infectious inflammatory states such as SIRS (Annane 2005; Biron 2015; Harbarth 2001; Livaditi 2006; Pierrakos 2010; Riedel 2012; Riedel 2011; Wacker 2013; Wunder 2004; Zakariah 2008). The association of procalcitonin with the severity of coagulopathy in addition to the presence of infection supports the importance of infection in the development of coagulopathy. As procalcitonin is readily available as a laboratory test in the hospital setting, further study of the relationship of procalcitonin to DIC may have the potential to improve DIC diagnosis with minimal logistical challenges.

Nucleosomes represent a more novel biomarker in the field of sepsis and DIC than procalcitonin. In this study, nucleosomes showed a significant association with coagulopathy but not with sepsis alone. No difference in nucleosome levels was apparent between patients with sepsis and no or non-overt DIC and the healthy control group. However, nucleosomes were significantly elevated in patients with sepsis and overt DIC compared to both healthy controls and patients with sepsis and no DIC. Both histones and DNA, the constituent parts of nucleosomes, have been shown to have direct procoagulant and prothrombotic properties in addition to effects on platelets, the endothelium, and inflammation. The elevation of nucleosomes only in patients with overt DIC supports the involvement of nucleosomes or processes that they represent in the development of coagulopathy.

The association of procalcitonin and nucleosomes with DIC is largely independent of general inflammatory processes. Of the inflammatory cytokines, only IL-8 and EGF showed significant variation based on coagulation status in addition to overall

elevation in sepsis. This points as infection rather than inflammation as the major driver of the development of DIC in sepsis patients. Although the relationship between inflammation and coagulation is bi-directional, evidence in the literature points towards infection and infection-related neutrophil activity as a mediator of coagulation (Massberg 2010; McDonald 2017) as opposed to the converse. Platelets activated with LPS but not those activated with traditional platelet activators have been shown to stimulate NET formation (Clark 2007). This is evolutionary advantageous as it can trap bacteria and reduce bacterial dissemination throughout the body. However, as evidenced in DIC, it can also be detrimental to the host if coagulation occurs to excess.

Endothelial dysfunction also demonstrated significant association with the development of DIC. This is logical from a pathophysiological perspective, as endothelial damage is cited in Virchow's Triad as one of the main requirements for thrombosis. The variation of protein C based on DIC score is expected as the role of this endogenous anticoagulant in the pathophysiology of DIC is well accepted. Protein C was notable in this study as the only biomarker to maintain an association with DIC status throughout the course of hospitalization.

Ang-2 also demonstrated a significant association with DIC, with elevation in overt DIC patients compared to sepsis and no DIC. Ang-2 has not previously been strongly associated with coagulopathy in sepsis patients, although an association of Ang-2 with coagulopathy in trauma patients has been noted (Ganter 2008). Ang-2 has been more strongly tied to regulation of endothelial barrier function (Gallagher 2008; Parikh 2006) and the development of respiratory dysfunction in critically ill patients (Gallagher

2008; Kumpers 2008; Lin 2015; Parikh 2006). Ang-2 acts as an antagonist to Ang-1 at the Tie2 receptor on the endothelial cell surface. While Ang-1 promotes vascular stability and preserves cell-cell contacts, Ang-2 acts in opposition to these effects. In addition to Ang-2, Ang-1 and the Ang-Tie system may represent a new avenue of study in sepsis-associated DIC.

**Association of Biomarkers with Mortality**

Mortality was associated with changes in the levels of markers of hemostasis (PAI-1), infection (HMGB-1 and procalcitonin), inflammation (IL-6 and IL-8), endothelial function (protein C, endocan, and Ang-2), and platelet function (PF-4). Sepsis is commonly described as a disease of excessive inflammation with potential associated coagulation dysfunction. However, biomarkers of infection and endothelial function demonstrated a greater degree of association with mortality than inflammatory or hemostatic markers. These endothelial and inflammatory markers were also strongly associated with the severity of coagulopathy. This shows that infection and endothelial function in addition to inflammation and hemostatic function are relevant to disease progression and outcome in patients with sepsis-associated DIC and therefore must be considered in the diagnosis, evaluation, and treatment of these patients

Procalcitonin was significantly elevated in non-survivors compared to survivors and demonstrated the best predictive value for outcome of any measured biomarker (AUC = 0.77). This supports previous research demonstrating elevated procalcitonin in non-survivors compared to survivors of sepsis (Harbarth 2001; Wunder 2004) and further

validates procalcitonin as a valuable biomarker in sepsis and DIC. HMGB-1 was also significantly elevated in non-survivors compared to survivors, although the predictive value was lower than procalcitonin (AUC = 0.67). This is similar to the observations of the association of infection biomarkers with DIC. HMGB-1 may contribute to both thrombosis and inflammation (Ito 2006; Stark 2016) and is a potential therapeutic target in DIC (Suda 2006; H. Yang 2004). While the associations of procalcitonin, nucleosomes, and HMGB-1 with severity of coagulopathy and patient mortality indicate the relevance of infection response to the pathophysiology of sepsis-associated DIC, further questions are raised regarding the specific roles of HMGB-1, nucleosomes, and other nuclear components and the optimal application of this information. Additionally, this suggests that special attention should be paid to the potential interactions of therapeutics for DIC with these nuclear materials.

Several endothelial markers also emerged as strong predictors of mortality. Protein C was significantly reduced in non-survivors compared to survivors. Protein C is the most studied endothelial factor in sepsis-associated DIC, and reductions in protein C levels have previously been associated with poor outcome in patients with sepsis and DIC (Bouchard 2015; Collins 2006; LaRosa 2006a, 2006b; Macias & Nelson, 2004; Shorr 2010; Shorr 2008). Furthermore, the protein C pathway is a promising therapeutic target. Protein C functions as an endogenous anticoagulant as well as performing other anti-inflammatory functions, including the destruction of extracellular histones. Protein C depletion leads in the loss of these antithrombotic and cytoprotective functions, resulting in increased severity of coagulopathy and increased mortality. Surprisingly, Ang-2

demonstrated the highest predictive value for mortality of the measured endothelial markers, superior to protein C (AUC = 0.71). Ang-2 is predominately involved in the maintenance of endothelial cell barrier function. Increased Ang-2 is associated with increased intracellular gap formation. Sepsis patients already suffer from hypotension, shock, and impaired perfusion; increased loss of fluid into the intravascular space further impairs perfusion and increases mortality. Elevated Ang-2 has been implicated in the development of respiratory dysfunction, another contributor to mortality. The mechanisms by which Ang-2 may contribute to hemostatic dysfunction remain unclear. This suggests Ang-2 and the Ang-Tie system is a new avenue for investigation in the molecular pathophysiology of sepsis and DIC. A third endothelial marker, endocan, was also elevated in non-survivors compared to survivors, underscoring the importance of endothelial function to this disease.

Despite the accepted association between the development of DIC and increased mortality in sepsis patients, none of the markers used in the diagnosis of DIC (INR, platelet count, D-Dimer, or fibrinogen) demonstrated significant association with mortality. The only hemostatic marker to differ between survivors and non-survivors was PAI-1. Unlike fibrinogen, platelets, and INR, which are modified in disease due to consumption, or D-Dimer, which is produced as a consequence of thrombus breakdown, PAI-1 may be a cause rather than an effect of DIC. When released from platelets or the endothelium, PAI-1 inhibits fibrinolysis by inhibiting tissue plasminogen activator. Significantly elevated PAI-1 may prevent the breakdown of thrombi in the

microcirculation, magnifying the end-organ damage resulting from even mild coagulopathy. PAI-1 is reduced by treatment of DIC patients with rTM (Saito 2007).

Differences between survivors and non-survivors were also present in the inflammatory markers IL-6 and IL-8 and the platelet marker PF-4 (AUC = 0.70 for all). Although the predictive values for these markers are weaker than those seen for infection and endothelial markers, this supports the hypothesis that all systems contribute to the development of sepsis and DIC and may provide significant insight into disease pathophysiology. The strong predictive values present in markers of endothelial function and infection response indicate that these systems should be a focus for the development of therapeutics. However, no single biomarker demonstrated exceptionally strong predictive value for outcome in this complex and heterogeneous disease. Procalcitonin demonstrated the best predictive value for outcome in this patient population and, measurement of procalcitonin is therefore useful as a single rapid indicator of patient prognosis. However, improved information is provided when a combination of biomarkers are used. The predictive algorithm developed in this study incorporated biomarkers of infection (procalcitonin), inflammation (VEGF and the IL-6:IL-10 ratio), endothelial function (endocan) and platelet function (PF-4) and provided improved predictive value over procalcitonin alone.

The development of a predictive algorithm employing a combination of biomarkers may provide improved predictive ability. Alternatively, separate analysis of the association of biomarkers with outcome based on patient DIC status may provide improved predictive ability in conjunction with a deeper understanding of the underlying

pathology associated with different levels of coagulopathy. Due to the small number of non-survivors, particularly amongst patients with sepsis and no DIC, this analysis was not performed in this patient cohort.

**Association of Biomarkers with Platelet Count**

Platelet-related biomarkers demonstrated the least degree of association of with severity of illness and outcome of any biomarker category. However, platelet depletion is a major characteristic of sepsis-associated DIC, as circulating platelets are reduced due consumptive coagulopathy. Reduced platelet count is included in the ISTH algorithm for the diagnosis of DIC, and thrombocytopenia in conjunction with elevated INR is commonly used as a screening test for sepsis-associated coagulopathy. Analysis of the association of the level of platelet biomarkers with platelet count revealed that the circulating levels of these biomarkers are largely controlled by platelet depletion.

The interpretation of levels of factors released by platelets in patients with sepsis and DIC is complicated by the dual processes of platelet activation and platelet consumption. Activated platelets may secrete factors at high levels. However, this effect may be cancelled out by sufficient reduction in platelet number. However, it is important to understand platelet function in sepsis and DIC, as platelets are involved not only in hemostasis but also in the response to infection. Previous analyses (Claushuis 2016) have shown that sepsis patients with severe thrombocytopenia demonstrate a different molecular profile than those with platelet counts within the normal range. Accordingly, patients were divided into groups of normal platelet count (≥150 K/µl), mild

thrombocytopenia (100-149 K/μl), and moderate to severe thrombocytopenia (<100 K/μl).

Procalcitonin displayed the most striking association with platelet count of any biomarker. The elevation of PCT in patients with platelets of <100 K/μl or 100-149 K/μl compared to those within the normal range highlights the association of infection with platelet depletion. Ang-2 also displayed a dramatic increase in patients` with reduced platelet counts compared to those within normal limits. This emphasizes the importance of endothelial function and the response to infection in sepsis-associated coagulopathy.

Platelet depletion was also reflected in the association with hemostatic markers. The increases in INR and decrease in fibrinogen observed in patients with reduced platelet counts are reflective of the same consumptive process.

The association of platelet count with classical markers of inflammation was relatively limited. The clearest associations with platelet count were VEGF and EGF, both of which were higher in patients with normal platelet counts than those with thrombocytopenia. As both of these factors are released by activated platelets, this pattern is also likely reflective of platelet depletion.

The relationship of platelet biomarkers to platelet count was largely dictated by platelet depletion. CD40L and MP were both significantly reduced in patients with reduced platelet counts compared to those within the normal range. The reduction of these markers in patients with dramatically reduced platelet counts indicates that the degree of platelet consumption in these patients overcomes the increased secretion of these markers by activated platelets. The relatively limited association of platelet

biomarkers with organ failure, severity of DIC, and mortality is attributable to this same process rather than to a lack of involvement of platelet-related factors in disease pathophysiology. Measurement of platelet factors at an earlier time point may provide insight into the disease progression of patients who are likely to develop coagulopathy, as dramatic platelet activation precedes platelet depletion. Measurement of platelet count at both early and late time points in disease would also allow for analysis of not only platelet count but change in a patient's platelet count over time. Due to the wide range of normal platelet counts (150-400 K/µl), some patients who have experienced a drop in platelet count of more than 50% may still be classified as having a normal platelet count, whereas some patients with only minor drops in platelet count may be classified as thrombocytopenic. Further study of this phenomenon should occur in patients with more detailed information available regarding the timing of the onset of illness.

**Biomarkers on Days 4 and 8 and Changes Over Time**

Analysis of the relationships of biomarkers at ICU days 4 and 8 to DIC score and mortality revealed few associations. Protein C showed persistent associations with DIC score on days 4 and 8. Furthermore, changes in the level of protein C over time were also associated with survival. This supports the relevance of protein C to disease pathophysiology and the potential utility of this biomarker as a means to assess response to therapy.

Persistent elevations in IL-6 and IL-8 on days 4 and 8 were seen in non-survivors compared to survivors. This highlights the relevance of ongoing pro-inflammatory processes to mortality in this patient cohort.

**Potential Confounding Factors**

Prior to selection of a biomarker panel for use in animal studies or incorporation into a predictive algorithm, it was important to understand the influence of potential confounding factors on these biomarkers. No known comorbidity interferes with the utility of any of the biomarkers analyzed in this study in the evaluation and understanding of sepsis-associated DIC.

Special attention was paid to age as a potential confounding factor, as age can significantly change both baseline characteristics and response in disease. Furthermore, previous studies have demonstrated that age may change not only the biomarker profile seen in patients but also the association of this profile with patient outcome (Rondina 2015). This information is particularly relevant in sepsis and DIC, which affects patients of all ages. In this patient cohort, correlations between age and biomarker level were relatively week, although associations were present for several biomarkers. The strongest correlation (r=0.35) was observed for endocan, which was also significantly elevated in patients with age ≥65 compared to younger patients. When analyzed via two-way ANOVA, procalcitonin, IL-8, and Ang-2 showed significant differences based on both age and survival as well as a significant interaction factor. For these factors, the increases in non-survivors compared to survivors was more pronounced in younger patients than those with age ≥65. Additionally, this blunting of the increase in these markers in older patients may have implications for disease outcome in elderly patients and interfere with the use of some of these biomarkers.

Common and significant medical conditions were also analyzed for association with biomarker levels. History of pulmonary disease, which was present in 17 patients, was associated with the highest number of biomarkers. Compared to patients without a history of pulmonary disease, these patients had reduced D-Dimer, IFNγ, IL-10, nucleosomes, and TNFα. However, this should not interfere with the use of these markers.

**Correlations Between Biomarkers**

Many correlations were seen between the analyzed biomarkers. From this analysis, several groups of biomarkers emerge as potentially independently regulated although associated with patient outcome. For example, PAI-1, which varied significantly based on mortality, did not correlate with other hemostatic biomarkers and showed no strong correlation with any measured biomarker.

The highest concentration of correlations was among the inflammatory markers. While inflammation is critical to the pathogenesis of sepsis and to the development of DIC in sepsis patients, this suggests that inflammation may be monitored effectively through a small number of biomarkers.

Through this analysis, biomarkers of infection emerge as particularly important parameters and a new direction for research in the field of sepsis-associated DIC. While these factors were strongly associated with both mortality and the severity of coagulopathy, nucleosomes and HMGB-1 were relatively independent of the inflammatory processes. In contrast, procalcitonin, an indicator of infection but not a mediator of infection response, showed the most correlations and the strongest

correlations overall with any biomarkers. Procalcitonin showed associations with hemostatic markers, including a strong correlation with D-Dimer, strong correlations with several inflammatory markers including IL-6, IL-8, IL-10, TNFα and MCP-1, and correlations with endothelial markers including a strong correlation with Ang-2. However, procalcitonin showed no association with platelets; the only association between infection biomarkers and platelets was a weak correlation between nucleosomes and MP-TF. Infection may lead to generalized inflammation which in turn contributes bidirectionally to coagulopathy; however, infection was more strongly associated with endothelial function supporting the involvement of these factors not only with infection and inflammation but also with the development of the endothelial and coagulation dysfunction.

Platelet biomarkers demonstrated relatively limited relationships with other biomarkers with the exception of strong correlation with platelet counts. While platelets certainly play a role in the ongoing processes, this suggests that monitoring platelet count alone may provide sufficient information about this status in sepsis-associated DIC patients.

A specific association that may be of note for the biomarkers is the one seen between nucleosomes and HMGB-1. Both nucleosomes and HMGB-1 may be released into circulation as a part of NETosis; however, this is not the only mechanism of release for HMGB-1, which may also be released by endothelial cells. Patients with the highest levels of HMGB-1 using tertile analysis also had elevated levels of nucleosomes compared to patients with lower HMGB-1 levels. The platelet factors are more complex

in this patient population due to the consumption of platelets in DIC. Platelets may become strongly activated by the ongoing infection and release several factors, but may also be depleted to such an extent that this ability becomes exhausted. Additional information regarding the time course of illness is required to facilitate analysis of the relationship of platelet activation and depletion with disease development.

**Stepwise Linear Regression Modeling**

The aim of this analysis was to develop an algorithm based on a combination of biomarkers to predict clinical outcome in patients with sepsis-associated DIC. This aim was based on the hypothesis that a combination of biomarkers representative of multiple physiological processes would provide better predictive ability for outcome in sepsis patients than a single biomarker. A predictive equation for outcome was developed, incorporating procalcitonin, VEGF, IL-6:IL-10 ratio, endocan, and PF-4. As hypothesized, biomarkers representative of multiple physiological systems were incorporated into this algorithm, which exhibited a predictive value for mortality superior to that of any individual biomarker.

The stepwise linear regression modeling approach used in this aim was valuable because it provided an unbiased method to select the optimal biomarkers for the prediction of outcome and did not rely on preconceived ideas about the potential utility of each biomarker. This unbiased approach is valuable in a complex pathophysiological scenario such as sepsis-associated DIC, as the model is developed to be mathematically optimal rather than to conform to current knowledge about the utility of each parameter. This approach has been used successfully to predict outcome in other complex disease

processes such as pediatric intensive care unit patients (Hon 2017), kidney transplant patients (Dahle 2015), and in patients following the hip fracture repair (Durand 2018).

The models developed in this study represent an improvement over many previously developed models as they incorporate markers not only of a single aspect of disease, such as inflammatory cytokines (Andaluz-Ojeda 2012) but of multiple pathophysiological processes.

The models generated using stepwise linear regression are summarized in Table 36. These models support the hypothesis that multiple markers representative of different physiological systems are required predict outcome in patients with sepsis and DIC.

The most successful and robust model generated using this approach is that incorporating biomarkers only (excluding clinical data) and based on a constant starting assumption. This model incorporated 5 variables representative of 6 biomarkers (procalcitonin, VEGF, the IL-6:IL-10 ratio, endocan, and PF-4) and had an overall AUC for prediction of mortality of 0.87, greater than the value of any individual biomarker. Although other models generated based on a linear starting assumption achieved a greater AUC value (0.95), inclusion of 12 terms representing 13 biomarkers in this model is prohibitive for practical implementation in either the clinical or research setting.

The incorporation of clinical data into the predictive model did not yield an improvement in predictive ability compared to models incorporating biomarkers alone. The model generated using both biomarkers and clinical data and a constant starting assumption displayed the weakest predictive value of the four models (AUC=0.84).

Additionally, the APACHE-II score incorporated in this model is itself a complicated parameter representing the measurement of numerous laboratory values.

Procalcitonin emerged as the most useful marker (highest predictive value) in the analysis of the individual biomarkers, and was included in all four models developed for the total sepsis patient population, further emphasizing the importance of infection response to disease pathophysiology. Although the procalcitonin itself is not an antimicrobial factor, procalcitonin release has been shown in animal models to be proportional to bacterial load (Becker 2010; Nylen 1998; Steinwald 1999) and is widely accepted as a biomarker of bacterial infection. Notably, the most commonly included clinical parameter was white blood cell count (WBC). Elevated WBC is often used clinically as an indicator of infection. This further emphasizes the importance of infection response in the pathophysiology of DIC.

**Table 36. Summary of Stepwise Linear Regression Model Results**

| Marker | Included in Predictive Model | | | |
|---|---|---|---|---|
| | Biomarkers Constant AUC=0.87 | Biomarkers Linear AUC=0.95 | Clinical Constant AUC=0.84 | Clinical Linear AUC=0.89 |
| D-Dimer | No | Yes | No | Yes |
| F1.2 | No | No | No | No |
| PAI-1 | No | Yes | No | Yes |
| INR | No | No | No | No |
| Platelets | No | No | No | No |
| Fibrinogen | No | No | No | No |
| Nucleosomes | No | No | No | No |
| HMGB-1 | No | Yes | No | No |
| Procalcitonin | Yes | Yes | Yes | Yes |
| IL-2 | No | No | No | No |
| IL-4 | No | No | No | No |
| IL-6 | No | No | No | No |
| IL-8 | No | No | No | No |
| IL-10 | No | No | No | No |
| VEGF | Yes | No | No | No |
| IFNγ | No | No | No | No |
| TNFα | No | No | No | No |
| IL-1α | No | No | No | No |
| IL-1β | No | No | No | No |
| MCP-1 | No | Yes | No | No |
| EGF | No | Yes | No | Yes |
| IL-6:IL-10 | Yes | Yes | No | Yes |
| TFPI | No | No | No | No |
| Protein C | No | Yes | No | No |
| Endocan | Yes | Yes | No | Yes |
| Ang-2 | No | No | No | No |
| vWF | No | No | No | No |
| CD40L | No | Yes | No | Yes |
| MP | No | Yes | No | Yes |
| MP-TF | No | No | No | No |
| PF-4 | Yes | Yes | No | No |
| APACHE II | N/A | N/A | Yes | No |
| WBC | N/A | N/A | Yes | Yes |

Sepsis is a heterogeneous disease, and biomarker levels vary greatly between patients. Therefore, it is probable that different model components contribute to model output to different degrees for different patient depending on individual pathophysiology. By incorporating markers representative of different aspects of pathophysiology, the model may be applicable to the broadest set of patients with reasonable accuracy.

Different factors may contribute to mortality in patients who develop severe coagulopathy compared to those who do not develop significant coagulopathy. Therefore, the development of separate predictive algorithms for outcome based on the severity of coagulation disorder as defined by DIC score is a rational approach to model improvement. In this study, the number of patients in each DIC score subgroup present a significant limitation to this line of inquiry, and models produced using these small sample sizes are unlikely to be fully generalizable. However, extremely accurate (AUC=1) algorithms for the prediction of mortality were developed when patients were divided on the basis of DIC score. Although the practicality of these specific models may be limited**,** this study provides a proof of concept for the development of separate predictive algorithms for patients with different severities of coagulopathy.

Although these models do not provide information about the role of any marker in the molecular pathogenesis of sepsis or DIC, identification of markers commonly included in the models may provide insight into processes contributing substantially to patient outcome. The importance of infection response was further emphasized in models generated for patients with different severities of DIC by the common inclusion of HGMB-1.

**Limitations**

Several limitations were observed in the analysis of the Utah patient cohort, primarily with regards to sample size and availability of clinical information for the Utah patient cohort.

A cohort of approximately 100 patients is typical in many published studies of sepsis patients, with the exception of large clinical trials (Angus 2007; Chen 2012; Davis 2010; Delabranche 2013; Gogos 2000; Kranidioti 2009). However, this may provide limitations in statistical power when patients are subdivided into multiple groups. In particular, this limited the ability to analyze associations with mortality separately for patients with sepsis + no DIC, sepsis + non-overt DIC, and sepsis + overt DIC. Furthermore, the number of available patient samples decreased dramatically between day 0 (n=103) and day 4 (n=57), with further decreases by day 8 (n=30). This reduction in sample size reduces the statistical power for identifying differences in biomarker level on the basis of DIC score or patient outcome. Furthermore, patients were lost to follow-up due to both death and recovery, changing the overall characteristics of this cohort and limiting the utility of the analysis of change in biomarker levels over time.

A second limitation of this cohort is the lack of certain types of clinical information. Insufficient information was available to permit analysis of the prophylactic dose of heparin administered, type and dose of vasopressor, other treatments (i.e. antibiotics), length of hospitalization prior to either discharge or death, and pathogen type. Precise information regarding the time course of disease development relative to the timing of blood draw placed limitations on the analysis of time-dependent phenomena,

such as the activation and subsequent depletion of platelets or the changes in biomarker levels over time.

With the exception of protein C, protein antigen levels, not functional levels, were measured as a part of this study. Accordingly, only quantitative changes in biomarker level, not qualitative changes in protein function, were analyzed.

## *In vitro* Coagulation Profiles of Thrombomodulin, Antithrombin, and Heparin

Currently, no specific treatments are available for DIC in the United States. Therapy is limited to supportive care and treatment (i.e. antibiotics) to eliminate the underlying infection. Safe and effective treatments for DIC represent an unmet therapeutic need. Recombinant thrombomodulin (rTM), antithrombin (AT) and unfractionated heparin (UFH) represent three potential therapeutic approaches for this patient population. Prior to *in vivo* examination of the mechanism of action of drug mechanism of action, *in vitro* comparison of the relative anticoagulant activity of these drugs is required in order to address potential safety concerns. In contrast to the strong anticoagulant activity displayed by UFH, rTM and AT displayed mild and moderate anticoagulant activity, respectively. This indicates that rTM and AT may display an improved safety profile in humans and may act substantially via non-anticoagulant mechanisms.

Treatment with an anticoagulant or antithrombotic drug is associated with a risk of bleeding. Under normal circumstances, this can range from mild (i.e. epistaxis or menorrhagia) to severe and potentially fatal (i.e. intracranial hemorrhage). Patients with

DIC are at an elevated risk of bleeding in the absence of an anticoagulant drug, and therefore particular care must be taken when administering a drug with anticoagulant properties to this patient population. Accordingly, an understanding of the anticoagulant profiles of drugs for the treatment of DIC is important before *in vivo* administration. Direct comparison of drugs can be accomplished through *in vitro* coagulation testing. These experiments demonstrate that heparin is a strong anticoagulant while AT provides moderate anticoagulation *in vitro* and rTM possesses minimal anticoagulant properties *in vitro*.

The comparisons made between the three drugs in this study were all performed in whole blood at physiologically relevant concentrations. rTM was supplemented into human whole blood and rat plasma at concentrations from 0.625-10 µg/ml. This is representative of the circulating level of rTM in the management of patients with sepsis-associated DIC, which is typically within the range of 0.5-1.5 µg/ml (Moll 2004; Vincent 2013). Heparin was supplemented in human whole blood and rat plasma at concentrations of 0.0625-1 U/ml. For similar indications, therapeutic levels of heparin range from 1.5-5.0 µg/ml (0.15-0.5 U/ml) in blood (Jaimes 2009; Liu 2014). Antithrombin was used at concentrations of 0.0625-1 U/ml in human whole blood and rat plasma. In DIC, therapeutic blood levels of AT range from 1-2.5 U/ml (Choi 2014; Kienast 2006).

Similar results were obtained using PT and aPTT. aPTT is designed to monitor heparin therapy, and heparin produced maximal results on this test in human plasma at low concentrations. In comparison, the effects of rTM and AT on these assays was

minimal, even at supertherapeutic doses. The heparin-induced increase in PT was relatively low, as PT is not designed to monitor pathways affected by heparin. Neither rTM nor AT dramatically increased PT, demonstrating low anticoagulant effects for these drugs. Comparable results were obtained using both human blood and rat plasma, validating the use of these drugs in the rat model.

Unlike PT and aPTT, which assess specific components of the coagulation cascade, TEG provides a measure of the global effect of a drug on coagulation. The pattern of anticoagulation suggested by TEG is the same as that shown using PT and aPTT. Heparin strongly inhibits clot formation at low concentration while rTM displays a minimal anticoagulant effect throughout the analyzed coagulation range. AT displayed intermediate anticoagulant properties. This suggests that while antithrombotic activity may be important for the function of rTM, the mechanism of action of this drug is not limited to anticoagulation.

## Animal Models of Sepsis and DIC

### Validation of the CLP Model for Sepsis and DIC

In order to study the effects of rTM, AT, and UFH *in vivo¸* a physiologically relevant, validated animal model of sepsis-associated DIC was employed. A rat cecal ligation and puncture model (CLP) was standardized for use for this purpose. This model is appropriate for use in the study of drugs for the treatment of sepsis-associated DIC on the basis of its physiological relevance. Active bacterial infection and coagulation dysfunction were confirmed by significant elevations in procalcitonin and significant

reductions in platelet count, respectively. Furthermore, the elevations in IL-10 and nucleosomes detected in CLP rats are consistent with pathophysiological changes in human disease.

Several modeling approaches for sepsis exist and can be divided into two general categories: the injection of toxins (most commonly LPS) or the induction of systemic infection (i.e. CLP). The CLP model here replicated a clinical scenario of polymicrobial infection with gut flora, resulting in systemic inflammation and hemostatic dysfunction, and in which treatment was not administered until after the development of disease. The presence of active infection is important, as factors involved in the response to infection (i.e. nucleosomes) are important to the pathogenesis of sepsis-associated DIC and may be affected by treatment with rTM, AT, or UFH. In contrast, the injection of LPS produces only a transient sepsis-like scenario, which does not allow for administration of drugs after the development of disease or for the study of all drug mechanisms of action.

The rat CLP protocol used in this study led to the development of sepsis and coagulopathy while maintaining a sufficiently low mortality rate (9% in the untreated CLP group) to allow for study of the results of drug treatment. This is appropriate, as the aim of this study was not to evaluate changes in outcome but rather changes in pathophysiology secondary to drug treatment. The pathophysiological changes seen in the CLP rats were consistent with those observed in human sepsis patients and validate this model system for use in the study of drug mechanisms of action.

In the initial validation studies, procalcitonin was measured as an indicator of bacterial infection in order to validate the development of sepsis in CLP rats. Procalcitonin was undetectable in the majority of sham-operated rats and was significantly elevated in the CLP group. This indicates the presence of an active bacterial infection as a result of the CLP procedure. The rats in the CLP group also experienced significant weight loss compared to sham-operated controls, which is established as an indicator of illness in this model (Breuille 1999; Brooks 2007; Nemzeck 2004)

Platelets were counted as an indicator of hemostatic status. Significant reduction in platelets was observed in CLP rats compared to sham-operated controls, indicating the development of consumptive coagulopathy in these rats.

A limited number of additional biomarkers were selected for analysis due to the small blood volume of a rat and the availability of methods for the detection of rat proteins. The correlations between biomarkers observed in the Utah cohort patients justified the selection of a single biomarker or small number of biomarkers representative of each process. Although both infection response and endothelial function were identified as critical contributors to the pathogenesis of sepsis-associated DIC, this analysis was focused on infection response and inflammation. The traumatic nature of the cardiac blood puncture blood draw led to significant coagulation activation and prevented the accurate measurement of some hemostatic parameters. PT was measured and samples that failed to clot excluded from further analysis as serum. Nucleosomes, IL-10, and PAI-1 were selected for analysis in rat samples. IL-6 and histone H3 could not be detected in these samples. It is possible that these mediators do not persist in the blood to the 72 hour

time point (Miki 2015). Although TNFα is often measured in studies of sepsis, it was not included in this study as it peaks early and transiently in sepsis (Kinasewitz 2004) and therefore would not be expected to be detectable at the 72 hour time point.

Nucleosomes were selected for inclusion in this analysis not only as a biomarker of infection response but as a potential mediator of coagulation dysfunction in septic patients with therapeutic implications. rTM, AT, and UFH are hypothesized to have non-anticoagulant activity mediated through interactions with extracellular nuclear components, such as nucleosomes. Therefore, an elevation in nucleosomes consistent with that seen in human disease, such as that observed in CLP rats compared to sham-operated controls, is required for the study of the mechanism of action of these agents.

IL-10 was selected as a classic inflammatory marker. In the Utah cohort, IL-10 was significantly elevated in sepsis patients and correlated strongly with other notable inflammatory markers, including IL-6, IL-8, IFNγ, and MCP-1, making it appropriate for use in this analysis. The significant elevation in IL-10 in CLP rats compared to sham-operated controls is consistent with the pathophysiology seen in humans and further validates this model system.

PAI-1 was selected for inclusion in this study on the basis of reports suggesting PAI-1 as a potential means to monitor rTM therapy. However, although PAI-1 was detected in the rat plasma; the elevation of PAI-1 in the CLP group compared to sham operated controls was not statistically significant.

**Comparison of Heparin, rTM, and AT in Rats with CLP-Induced Sepsis and DIC**

This study compared the non-anticoagulant effects of rTM, AT, and UFH in rats with CLP-induced sepsis and coagulopathy. The underlying hypothesis was that each of the three drugs would exhibit a distinct pattern of non-anticoagulant effects, including ant-inflammatory effects and neutralization of circulating nuclear material. Therefore, the primary goal in this study was not to compare drug efficacy as quantified by reduction in mortality but to understand the interactions of these drugs with factors identified as important in human sepsis patients. Treatment with rTM, AT, and UFH resulted in a significant reduction in nucleosome levels, showing that interaction with extracellular nuclear material is a component of the mechanism of action of each of these agents. UFH displayed additional anti-inflammatory effects, with UFH treatment also resulting in a significant reduction in IL-10. AT treatment led to additional effects that were not quantified in terms of anti-inflammatory activity, resulting in a lessening of weight loss. An improved understanding of the non-anticoagulant mechanisms of rTM, AT, and UFH may have significant implications for the optimal clinical implementation of these drugs.

The approach taken in this study has several advantages. Models of sepsis associated DIC are highly variable, and thus a head-to-head comparison is required to gain accurate information regarding comparative effects of rTM, AT, and UFH. Furthermore, the approach used in this study is highly replicative of the clinical scenarios in which these drugs would be employed. Previous studies have demonstrated reduction in infection-related factors such as nucleosomes, histones, cfDNA, and HMGB-1 in response to treatment with rTM or AT in LPS injection models (Iba, Miki, Hashiguchi,

Yamada, & Nagaoka, 2014; Iba, Miki, hasiguchi, Tabe, & Nagaoka, 2014; Takehara 2017). While this provides *in vivo* support for the interactions of rTM or AT with these factors, it does not address the consequences of active bacterial infection on these interactions. NETosis, and therefore the release of nuclear material into blood, contributes to bacterial clearance (Araujo 2016; Czaikoski 2016; Yost 2016); modulation of nuclear factors by therapeutic agents may be significantly altered in the presence of live bacteria.

The drug administration protocol used in this study replicates the clinical scenario typical of sepsis-associated DIC. In numerous animal studies of treatments for sepsis and DIC, drugs are administered at the time of or prior to the induction of sepsis. However, in the clinical setting drugs for sepsis are administered after the development of disease. In this study, drugs were administered 24 hours after the CLP procedure, allowing time for the development of sepsis and DIC. Drug doses were also selected to balance clinical relevance with the potential to detect non-anticoagulant effects. The purpose of this study was not the generation of a dose-response curve for the non-anticoagulant effects of each drug but rather the comparison of the effects of rTM, AT, and UFH. Accordingly, a single drug dose was selected for comparison for each drug.

rTM was evaluated at a single dose of 1 mg/kg. This is higher than the dose of 60 μg/kg at which this drug is administered to humans. However, rats have been reported to require a higher dose of rTM than humans to elicit a comparable response. In rats, 1 mg/kg is a commonly used dose, both in the initial preclinical studies (Gonda 1993; Mohri 1994) and in more recent work (Hagiwara 2010; Iba 2013; Nagato 2009).

Antithrombin was administered at two doses; 50 IU/kg, replicating a clinically utilized dose of 3000 IU in a human, and a higher dose of 125 IU/kg, which is commonly used in studies of this drug in rats and may be required to achieve anti-inflammatory effects (Iba 2014; Iba 2014; Uchiba 1998; Yamashiro 2001; Yang 1994). Although it is hypothesized that higher doses of AT may be required to achieve anti-inflammatory effects, no significant differences were observed between the two doses in this study, and AT was administered at 125 IU/kg to the majority of rats. This high dose of AT did not result in any detectable bleeding.

UFH is used clinically at a wide range of doses dependent on indication. The range of doses that have been used in animal studies of sepsis is well reviewed by Li et. al. and ranges over several orders of magnitude. This high-dose heparin treatment of septic animals typically results in increased mortality (Li 2011). Low doses of UFH were selected for this study, as these doses may be more appropriate in this disease state. Of the 5 rats that received 70 IU/kg UFH, one died within 24 hours of drug administration. Blood was visible in the nares and necropsy revealed the presence of a significant amount of blood in the GI tract, suggesting that heparin-induced bleeding was the cause of death in this rat. All other rats were treated with UFH at 25 IU/kg, and no mortality was seen at this dose. No significant differences in biomarker level were apparent between rats on the basis of heparin dose. Treatment with heparin at 25 U/kg resulted in a significant decrease in both nucleosomes and IL-10 compared to untreated rats.

When directly compared, rTM, AT, and UFH displayed distinct anti-inflammatory profiles in rats with CLP-induced sepsis and coagulopathy. All three drugs

caused a significant reduction in nucleosomes compared to untreated rats. UFH treatment caused a reduction in IL-10, while AT led to a reduction in weight loss. The reduction in circulating nucleosomes caused by rTM, AT, and UFH treatment in a model involving active bacterial infection is the most notable finding of this study. The results of the biomarker profiling studies in the Utah cohort as well as a growing body of literature have identified circulating nuclear material as a contributor to the pathophysiology of sepsis-associated DIC and a potential therapeutic target.

This finding further validates the importance of extracellular nuclear material to the mechanism of action of rTM. *In vitro,* rTM has been shown to bind to histones (Nakahara 2013) and HMGB-1 (Abeyama 2005) in a protein C independent manner and to inhibit histone-induced platelet aggregation. rTM may also contribute to histone clearance through the activation of protein C, which degrades histones in its active form (Xu 2009). The effect of protein C on nucleosomes, however, are less clear; in a retrospective analysis of plasma from children with meningococcal sepsis treated with protein C, no reduction in nucleosome levels was observed in treated children (Zeerleder 2012). Interestingly, histones may also decrease the antithrombotic efficacy of rTM by inhibiting TM-dependent activation of protein C (Ammollo 2011). This study demonstrates that rTM administration leads to a reduction in circulating nucleosomes as well as circulating free histones.

Heparin may reduce circulating nucleosomes by binding to both nucleosomes and histones as well as inducing nucleosome breakdown by releasing histones from chromatin (Fuchs 2010; Napirei 2009). Histones bind to heparin as well as endogenous

glycosaminoglycans (GAGs) including heparin sulfate (Henriquez 2002). Heparin has also been shown to bind to histone-DNA complexes (van Bruggen 1996). The influence of heparin treatment on not only histones but also nucleosomes is important to establish, as different structures of histones, DNA, and nucleosomes may have different procoagulant and proinflammatory processes (Noubouossie 2017).

Heparin has been shown to inhibit histone-induced platelet interaction both in *in vitro* platelet aggregations and in a mouse model of histone-induced thrombocytopenia (Fuchs, Bhandari, & Wagner, 2011). A major concern with the use of heparin in patients with sepsis-associated DIC is the risk of bleeding, which was also apparent in the rat model. However, several non-anticoagulant heparins have also been shown to possess anti-histone or anti-nucleosome properties both *in vitro* and *in vivo* (Ammollo 2011; van Bruggen 1996; Wildhagen 2014; Zhang 2014). Non-anticoagulant heparins may provide an approach to target circulating nuclear material and other inflammatory factors in sepsis and DIC patients without causing a significant risk of bleeding.

In comparison to rTM and heparin, little is known about the potential interaction of AT with nucleosomes or other nuclear components. This study demonstrates that AT treatment also leads to a reduction in circulating nuclear material. A large proportion of the anti-inflammatory activity of AT is mediated through its anticoagulant activity, with reduced coagulation resulting in reduced inflammation (J. H. Levy, Sniecinski, Welsby, & Levi, 2016). As demonstrated in this experiment, AT does have additional activity including the reduction of circulating nucleosomes. In contrast to rTM and UFH, this is likely due to the regulation of processes upstream of NETosis. The coagulation

independent anti-inflammatory activities of AT are mediated largely through the upregulation of prostacyclin and the inhibition of leukocyte adhesion, migration, cytokine production, and chemotaxis (Hoffmann 2002; Levy 2016; Roemisch 2002; Souter 2001). As treatment of platelets with prostacyclin reduces platelet-induced NET formation (Carestia 2016), AT-induced prostacyclin release may reduce the amount of nucleosomes released into the circulation. AT also binds to the syndecan-4 receptors on neutrophils, reducing activity (Opal 2002).

The co-administration of low-dose heparin as DVT prophylaxis may explain the lack of anti-inflammatory effects in human trials of AT and may reduce the utility of these findings. Heparin is an AT-dependent anticoagulant; co-administration of heparin with AT results in enhanced heparin-AT binding and anticoagulant activity while inhibiting coagulation-independent actions of AT (Hoffmann 2002; Opal 2002; Roemisch 2002).

Despite discussion in the literature of additional anti-inflammatory effects of rTM, none were observed in this study. Only heparin demonstrated significant reduction in generalized inflammation, as assessed through IL-10.

No drug significantly reduced procalcitonin, although a trend towards procalcitonin reduction with heparin was seen. The continued elevation of procalcitonin is likely a reflection of persistent bacterial infection; rTM, AT, and UFH do not have antibiotic properties. In this study, rats were not treated with antibiotics, and thus resolution of the bacterial infection was not anticipated.

AT treatment was also associated with reduced weight loss. Sepsis is associated with the development of a catabolic state, and weight loss in a CLP model is expected and an indicator of illness (Breuille 1999; Brooks 2007; Nemzeck 2004).

This study demonstrates that rTM, AT, and UFH have distinct non-anticoagulant activity profiles in sepsis-associated DIC. UFH demonstrated additional anti-inflammatory activity in comparison with rTM and AT, leading to a reduction in IL-10 levels as well as nucleosomes. However, UFH is a powerful anticoagulant associated with a high bleeding risk in DIC. Both animal studies and clinical trials have thus far failed to show a reduction in mortality with heparin treatment in DIC.

The reduction in circulating nucleosome levels induced by treatment with rTM, AT, and UFH underscores the importance of infection-related nuclear material in sepsis-associated DIC. This material should be included in future clinical and mechanistic studies in order to better understand the pathophysiology of DIC, develop improved therapeutic agents, and identify patients for treatment.

## Clinical Implications

The results presented in this dissertation have clinical implications with respect to both the diagnosis and treatment of patients with sepsis-associated DIC. These studies demonstrate that INR, the most commonly used parameter in the identification and classification of patients with sepsis-associated coagulopathy, does not provide a consistent description of the ongoing coagulation dysfunction in these patients. The results of the biomarker profiling in the Utah cohort patients show that alternative

approaches, such as measurement of extracellular nuclear material or factors associated with endothelial dysfunction, provide more pathophysiolgically relevant insight into disease progression. Furthermore, the results of the mathematical modeling in this patient cohort provide an approach to predict mortality in sepsis patients, which may be important to guide appropriate therapy.

The *in vitro* and *in vivo* studies of thrombomodulin, antithrombin, and heparin provide insight into the mechanisms of action of these potential therapeutic agents. The *in vitro* studies demonstrate that the anticoagulant activity, and therefore the anticipated associated bleeding risk, of rTM and AT is minimal in comparison to that of UFH. The results from the *in vivo* studies demonstrate that all three drugs act through non-anticoagulant mechanisms, including reducing the levels of circulating nuclear material. This may provide a means to target therapy with patients with the highest potential to respond to therapy.

## Conclusion

The aims of this dissertation were (1) to understand the molecular pathogenesis of sepsis-associated DIC by profiling plasma biomarkers of inflammation, infection, endothelial function, and platelet function as well as hemostatic dysregulation and assess their relevance to disease progression and outcome, (2), to develop and validate an *in vivo* animal model of sepsis-associated DIC, and (3) to assess the effects and mechanism of action of therapeutic modulation on the pathogenesis of sepsis-associated DIC.

Evaluation of biomarkers of hemostasis, inflammation, infection, endothelial function, and platelet function in the Utah patient cohort revealed that all systems are associated with organ failure, severity of coagulopathy, and mortality. However, inflammation and hemostasis are more strongly associated with organ failure, whereas infection (including extracellular nuclear material) and endothelial function are more strongly associated with severity of coagulopathy and outcome.

A predictive algorithm for outcome was developed which incorporated biomarkers of infection, inflammation, endothelial function, and platelet function and had superior predictive value to any individual marker. The rat cecal ligation and puncture model used in these studies included the infection and coagulation dysfunction that define sepsis-associated DIC, and further replicates the inflammatory and infection response profile observed in human patients. Finally, rTM, AT, and UFH were shown to have distinct activity profiles in terms of both in *in vitro* coagulation testing and *in vivo* modulation of disease pathophysiology.

Sepsis-associated DIC is a complex syndrome that involves not only hemostatic dysfunction but also inflammation, infection response, and endothelial and platelet function. The patient cohorts included in this dissertation demonstrated dysfunction of all aspects of hemostasis, including global coagulation testing, individual coagulation factors, endogenous anticoagulants, the fibrinolytic system, as well as the endothelium and platelets. Although DIC is defined on the basis of coagulopathy, the results presented in this dissertation demonstrate that other factors, including inflammation, infection response, and endothelial function are associated with both the severity of coagulopathy

and with patient mortality. Further study is warranted in order to elucidate the cause and effect relationship between coagulation, inflammation, infection response, and endothelial activation.

In conclusion, the results presented in this dissertation provide significant insight into the molecular pathogenesis of sepsis-associated disseminated intravascular coagulation and its pharmacologic modulation.

# CHAPTER SIX

# SUMMARY

This dissertation is focused on the molecular pathophysiology of sepsis-associated disseminated intravascular coagulation (DIC) and its pharmacologic modulation. These studies included extensive exploration of the hemostatic, inflammatory, infection response, endothelial function, and platelet function profiles of human sepsis patients. Three drugs—recombinant thrombomodulin (rTM), antithrombin (AT), and unfractionated heparin (UFH) were evaluated *in vitro* and *in vivo*, for their anticoagulant and anti-inflammatory effects in this disease. The results from the major experimental protocols included in this dissertation are summarized below.

## Coagulation Profiling in DIC vs. Warfarin Treated Patients

1. The international normalized ratio (INR) is commonly used to describe the coagulation status of both DIC patients and patients receiving warfarin for therapeutic anticoagulation. The underlying cause of the coagulation dysfunction is fundamentally different in these two patient populations; accordingly, INR may provide different information in these different patient populations. In order to analyze these differences, the global coagulation tests INR, aPTT, and fibrinogen as well as protein and functional levels of coagulation Factors VII, IX, and X were measured in a cohort of patients receiving warfarin anticoagulation and in a cohort of patients with sepsis and overt or

non-overt DIC defined by the ISTH scoring algorithm. **In order to better understand the pathophysiology underlying the elevation of INR in patients with sepsis-associated DIC, the relationship of individual coagulation factors and other global coagulation tests to INR was compared in patients with DIC to warfarin-treated patients.**

2. **In DIC patients, increased INR was associated with a progressive increase in aPTT, whereas no such pattern was apparent in warfarin-treated patients.** This suggests that an elevated INR in DIC patients is reflective of global coagulation dysfunction, while in warfarin-treated patients, this finding reflects a targeted dysfunction of the extrinsic pathway.

3. Functional and protein levels of Factors VII, IX, and X were reduced in both warfarin treated and DIC patients compared to healthy controls. **In warfarin treated patients, Factors VII, IX, and X decreased progressively with increasing INR, demonstrating a predictable and regular relationship between coagulation factor level and INR in warfarin-treated patients. In contrast, no predictable pattern of association between coagulation dysfunction as measured by INR and levels of individual coagulation factors was detected in DIC patients.**

4. **Distinct patterns of correlations were seen between coagulation factors and global coagulation tests in warfarin-treated patients compared to patients with sepsis-associated DIC.** In warfarin treated patients, strong correlations were present between the levels of individual coagulation factors and INR, but not with fibrinogen or

aPTT. In contrast, very few strong correlations were present in DIC patients in the levels of global coagulation tests as opposed to among individual coagulation factors. This confirms that the coagulation deficit described by an elevated INR in DIC patients is fundamentally different than that described by a comparable INR in warfarin-treated patients.

## Biomarker Profiling in Utah Cohort Patients

1. **A panel of biomarkers was evaluated in plasma samples collected from 103 patients with sepsis and well-defined DIC (the Utah cohort). These biomarkers were selected to represent several distinct physiological systems: hemostasis, infection response, inflammation, endothelial function, and platelet function.** Hemostatic function was represented by D-Dimer, F1.2, PAI-1, INR, platelet count, and fibrinogen. Infection response was represented by nucleosomes, HMGB-1, and procalcitonin. Inflammation was represented by IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFNγ, TNFα, IL-1α, IL-1β, MCP-1, EGF, and the IL-6:IL-10 ratio. Endothelial function was represented by TFPI, protein C, endocan, Ang-2, and vWF. Platelets were represented by CD40L, PF-4, MP, and MP-TF.

2. Organ failure was quantified using the SOA score. **Hemostatic and inflammatory biomarkers demonstrated strong associations with organ failure.** This supports the traditional model of sepsis as a syndrome in which organ failure occurs subsequent to overwhelming inflammation. Hemostatic biomarkers were also associated with organ failure, supporting the role of coagulation dysfunction in the development of

organ failure. The associations between endothelial or platelet function and organ failure were less pronounced.

3. In order to quantify the associations between the severity of coagulation function and the status of each physiological systems, patients were subdivided into three groups on the basis of the ISTH scoring algorithm for DIC. At baseline, 20 patients had sepsis + no DIC (ISTH score ≤2), 59 had sepsis + non-overt DIC (ISTH score 3-4), and 24 had sepsis + overt DIC (ISTH score ≥5). In addition to hemostatic parameters included in the DIC score (platelet count, INR and D-Dimer), **DIC status demonstrated strong associations with both infection response and endothelial function. This suggests that these processes play a critical role in the development of coagulation dysfunction in sepsis patients and should therefore be addressed with respect to the diagnosis, prognostic prediction, and response to therapy in these patients.** The established infection marker procalcitonin varied significantly based on the severity of coagulopathy. Additionally, nucleosomes, a component of the nuclear material released into the circulation due to NETosis, showed significant variation based on DIC status. Of the endothelial markers, both the endogenous anticoagulant protein C and the regulator of vascular permeability Ang-2 demonstrated significant variation based on DIC status. Only hemostatic markers used in the calculation of the DIC score (platelets, INR, and D-Dimer) showed significant variation based on DIC status. Fibrinogen, the fourth component of the DIC score, did not vary significantly based on DIC status, nor did F1.2 or PAI-1. Variations in inflammatory cytokines based on DIC status were limited to IL-8 and EGF, and no platelet biomarkers demonstrated significant association with the degree

of coagulation dysfunction. This demonstrates a contrast between the systems involved in organ failure and those associated with coagulopathy.

4. The primary outcome measure in this patient cohort was 28-day mortality. The overall mortality rate among the Utah cohort patients was 14.6%. Association of biomarkers with mortality was quantified in two ways; the difference between survivors and non-survivors, and predictive value as quantified by the area under the receiver operating curve (AUC). Association of biomarkers was quantified both in terms of difference between survivors and non-survivors as well as predictive value, quantified by the area under the receiver operating curve (AUC). Procalcitonin was significantly elevated in non-survivors compared to survivors and provided the strongest predictive value of any individual biomarker (AUC=0.77). **Infection markers demonstrated strong associations with mortality as well as with coagulation dysfunction.** In addition to procalcitonin, the nuclear protein HMGB-1 was significantly elevated in non-survivors compared to survivors (AUC=0.67). **Endothelial markers also demonstrated strong associations with mortality in addition to the severity of coagulation dysfunction.** Although the endogenous anticoagulant protein C was unsurprisingly reduced in non-survivors compared to survivors (AUC=0.81), the strongest predictive value for mortality among endothelial markers was found for Ang-2 (AUC=0.76). Endocan, an indicator of endothelial activation, was also significantly elevated in non-survivors compared to survivors. The only variation in hemostatic biomarkers identified on the basis of survival was PAI-1; none of the factors included in the DIC score demonstrated an association with survival. Limited relationships were observed between

mortality and inflammation or platelet function. The inflammatory cytokines IL-6 and IL-8 as well as the platelet factor PF-4 were significantly elevated in non-survivors compared to survivors. **Overall, this suggests that infection response and endothelial function are significant factors in both the development of coagulation dysfunction and in patient outcome.** However, inflammatory and coagulation factors more traditionally associated with sepsis are the key contributors to organ failure.

5. **A computational approach was used in order to generate an algorithm to predict mortality outcome in sepsis patients. This algorithm incorporated procalcitonin, VEGF, IL-6:IL-10 ratio, endocan, and PF-4, thereby representing infection, inflammation (including both endogenous pro- and anti- inflammatory processes), endothelial function, and platelet activation and had better predictive ability for outcome than any individual biomarker.** Stepwise linear regression modeling was used to generate models for outcome prediction in sepsis patients using an unbiased approach to biomarker selection. These models supported the hypothesis that sepsis is a complex disease best described not by a single biomarker but rather by a combination of biomarkers representative of multiple physiological systems. The best model created using this approach incorporated only biomarkers and was based on a constant starting assumption. Although the model generated using the linear starting assumption had a better predictive value (AUC=0.95 vs. 0.87 for the constant assumption model), the constant assumption model was judged to be optimal on the basis of the more limited number of biomarkers included.

## *In vitro* Coagulation Profiles of Thrombomodulin, Antithrombin, and Heparin

1. Recombinant thrombomodulin (rTM), antithrombin (AT) and unfractionated heparin (UFH) are all drugs of interest for the treatment of sepsis-associated DIC. These drugs were selected for use in this disease on the basis of their antithrombotic activity, mediated through distinct mechanisms. These drugs may also provide benefit through anti-inflammatory mechanisms in DIC. Patients with DIC experience an elevated risk of bleeding, especially when treated with anticoagulant agents. Accordingly, the understanding of the relative anticoagulant properties of drugs selected for use in this population is required. The anticoagulant profiles of rTM, AT, and UFH were compared *in vitro* at physiologically relevant concentrations in whole blood and citrated plasma acquired from healthy human volunteers.

2. In the aPTT and TT clotting tests, UFH demonstrated maximal anticoagulant activity even at low concentrations. In comparison, the clotting time prolongation by rTM and AT was minimal. The PT test is not designed to measure UFH activity; however, UFH still demonstrated greater anticoagulant activity in this test than either rTM or AT. **In comparison to UFH, a known strong anticoagulant, rTM and AT exhibited minimal overall anticoagulant activity in clotting tests.**

3. Thromboelastography was used to compare the global anticoagulant effects of rTM, AT, and UFH, with results similar to those seen in the clotting tests. **UFH demonstrated strong overall anticoagulant activity, even at low concentrations. rTM demonstrated minimal anticoagulant activity, even at supertherapeutic**

**concentrations**, indicating that while this drug possesses antithrombotic activity, it is not anticoagulant and therefore would not be expected to be associated with a markedly elevated risk of bleeding. **AT demonstrated intermediate anticoagulant activity, with marked alterations present in thromboelastographic parameters present at high concentrations. This indicates that mechanisms other than direct anticoagulation may be at play in the resolution of DIC seen clinically with treatment with rTM or AT.**

## *In vivo* Studies

1. A cecal ligation and puncture (CLP) model of sepsis and coagulopathy was used in rats. This model was designed to yield sepsis and coagulopathy with low 72 hour mortality to allow for effective analysis of the drug mechanism of action. **Elevated levels of procalcitonin in CLP rats compared to sham-operated controls confirmed the presence of active infection. The significant reduction in platelet count in CLP-operated rats compared to sham-operated controls confirmed the presence of coagulation dysfunction. The significant increase in IL-10 demonstrated inflammation similar to that observed in human disease. Furthermore, nucleosomes were significantly elevated in CLP rats compared to sham-operated controls. This is consistent with the findings in human disease and further emphasizes the relevance of role of nuclear material to sepsis and DIC.**

2. The anti-inflammatory effects of rTM, AT, and UFH were compared using the rat CLP model of sepsis and coagulopathy described above. Drug treatment did not result

in a reduction in procalcitonin, indicating that treatment with these agent does not lead directly to infection resolution. **Treatment with rTM, AT, or UFH did lead to a significant reduction in nucleosome level. Due to the potential involvement of nuclear material including nucleosomes in the pathogenesis of DIC, this provides an anti-inflammatory mechanism by which rTM and AT may contribute the reduction in the pathophysiology observed in sepsis.** UFH demonstrated additional anti-inflammatory activities, also leading to a significant reduction in IL-10. However, UFH treatment was associated with the potential for fatal bleeding complications.

# CHAPTER SEVEN

# CONCLUSIONS

Sepsis-associated disseminated intravascular coagulation (DIC) is a complex clinical condition with high mortality. This disease is characterized by the overwhelming inflammatory response to infection characteristic of sepsis as well as the consumptive coagulopathy that defines DIC. Patients with DIC experience both thrombotic and bleeding complications. Treatments for DIC represent an unmet therapeutic need; currently, no treatments are approved in the United States for this indication.

**The purpose of this dissertation was twofold. In order to test the hypothesis that DIC would be best described by a combination of biomarkers representative of multiple physiological processes, a wide array of biomarkers, representative of hemostasis, inflammation, infection, endothelial function, and platelet function, were measured in the plasma of a cohort of patients with sepsis and strictly defined coagulopathy. The relationship of these markers, and the physiological systems they represent, with severity of illness, severity of coagulopathy, and patient outcome was assessed. Based on this data, an algorithm to predict mortality in sepsis patients was developed, incorporating parameters representative of inflammation, infection, endothelial function, and platelet function. Furthermore, the relationship of the status of the coagulation cascade to global coagulation function in DIC patients was**

**assessed. Secondly, thrombomodulin, antithrombin, and heparin were studied for use in DIC. *In vitro* coagulation testing was performed to determine the relative anticoagulant effects of these drugs. Furthermore, additional anti-inflammatory effects of these agents were studied in a rat cecal ligation and puncture model of sepsis-associated DIC in order to test the hypothesis that each of these drugs has a distinct set of anticoagulant and non-anticoagulant actives.**

The initial identification of patients with sepsis-associated DIC is often based on the presence of an elevated INR. Elevated INR is typically indicative of a hypocoagulable state; however, DIC patients with an elevated INR are at risk of complications due to both thrombosis and bleeding. Therefore, it was hypothesized that the coagulation dysfunction resulting in INR elevation is substantially different in DIC patients than in patients with other causes of INR elevation such as warfarin anticoagulation. **In order to better understand the appropriate interpretation of INR in DIC patients, the relationship between INR, other global coagulation tests, and coagulation factor levels were compared in patients with sepsis-associated DIC to patients receiving warfarin anticoagulation. In contrast to warfarin treated patients, where elevated INR was associated with a uniform and predictable reduction in coagulation factor levels, no predictable pattern of coagulation dysfunction was associated with INR elevation in sepsis-associated DIC patients.**

Sepsis is defined as an “overwhelming inflammatory response to infection”, and DIC is currently defined on the basis of reactive coagulation dysfunction. However,

inflammation and coagulation dysfunction are not the only factors at play in this disease. The response to infection, particularly the expulsion of nuclear material into the extracellular environment, endothelial function, including the production or loss of endogenous anticoagulants, and platelet function also contribute to the pathogenesis of DIC. It was hypothesized that assessment of biomarkers representative of the numerous processes underlying the development of DIC using plasma samples acquired from septic patients would provide greater insight into the molecular pathogenesis of DIC. **The results of the studies carried out in this dissertation supported the hypothesis that a combination of biomarkers representative of multiple physiological systems would provide greater insight into the pathophysiology of sepsis-associated DIC than markers of a single system. Inflammation and coagulation demonstrated the greatest degree of association with organ failure. However, infection response, including extracellular nuclear material, and endothelial function, were associated with the severity of coagulation dysfunction and patient mortality to a greater degree than inflammation, hemostatic markers, or platelet function.**

The identification of patients with the most severe disease is important to target treatments with potential associated risks to patients with the greatest potential for benefit. To this end, a method to predict mortality in patients with sepsis and DIC would be beneficial. Stepwise linear regression modeling was used to generate an algorithm to predict mortality in sepsis patients and to test the hypothesis that a combination of biomarkers representative of multiple physiological processes would provide better

predictive ability for outcome in sepsis patients than a single biomarker. **A predictive equation for mortality in sepsis patients was generated. This equation predicts mortality in sepsis patients based on levels of procalcitonin, VEGF, IL-6:IL-10 ratio, endocan, and PF-4. As hypothesized, this algorithm incorporated biomarkers representative of multiple physiological systems including infection response (procalcitonin), the balance between pro- and anti-inflammatory processes (IL-6:IL-10 ratio), endothelial function (endocan), and platelet function (PF-4). Furthermore, this equation demonstrated a better predictive value for mortality than any individual biomarker.**

Safe and effective treatments for DIC represent an unmet therapeutic need. **Recombinant thrombomodulin (rTM), antithrombin (AT) and unfractionated heparin (UFH) represent potential therapeutic approaches for this indication. An understanding of the antithrombotic, anti-inflammatory, and other mechanisms by which rTM, AT, and heparin may modulate the pathogenesis of sepsis-associated DIC may improve the use of this therapeutic agent, including targeting to the appropriate patient population, as well as lay groundwork for design and testing of future therapeutics for sepsis-associated DIC.**

The risk of bleeding associated with any anticoagulant treatment in DIC is of significant concern. Accordingly, the relative anticoagulant activity of rTM, AT, and UFH was compared *in vitro* in both human and rat plasma using both clotting tests and thromboelastography. **rTM exhibited minimal anticoagulant ability, in contrast to the**

**strong anticoagulation caused by UFH. AT exhibited intermediate anticoagulant activity.**

In order to better understand the mechanisms by which rTM, AT, and UFH exert their activities, the effects of these drugs were studied *in vivo.* A physiologically relevant rat cecal ligation and puncture (CLP) model was used, and drugs were administered to rats with established bacterial infections. **rTM, AT, and UFH all demonstrated anti-nucleosome effects in a rat model of sepsis-associated DIC. Furthermore, UFH exhibited additional anti-inflammatory activity, while AT therapy resulted in lessened weight loss.** These non-anticoagulant mechanisms may contribute substantially to the efficacy of these drugs in sepsis-associated DIC.

The work presented in this dissertation contribute to an improved understanding of the molecular pathogenesis of sepsis-associated DIC and its modulation by potential therapeutic agents**. This work included both focused analysis of the status of the coagulation system in patients with sepsis-associated DIC as well as integrated analysis of numerous biomarkers representative of multiple physiological systems. Furthermore, this dissertation presents a mathematical approach designed to predict mortality in patients with sepsis, which has clinical implications as a potential means to identify patients who will benefit most from treatment. The results in both human samples and animal models identify infection response, particularly the presence of nuclear material in the extracellular environment, as an important component of the pathophysiology of DIC. The demonstration of the**

**reduction in circulating nuclear material due to treatment with rTM, AT, and UFH has implications for the further clinical development of these drugs.**

# APPENDIX A

# IRB AND IACUC APPROVAL INFORMATION

**Acquisition of Utah DIC samples**

LU #207958
Approved 9/15/2015
University of Utah IRB Approval: IRB_00029495
Title: Novel Markers of Sepsis and Venous Thromboembolism in Patients with Sepsis-Associated Coagulopathy

**Acquisition of de-identified patent plasma samples**

IRB #9192051098
Approved 5/10/1998, continuing approval granted 5/24/2016
Continuing approval granted 5/24/2016
Title: Loyola Plasma Bank

**Blood Draw from Healthy Human Volunteers**

LU# 9191051098
Approved 5/1/1998, continuing approval granted 8/24/2017
Title: Normal Donor Blood Collection

**Rat Cecal Ligation and Puncture Model**

IACUC #2017-009, LU#209143
Approved 4/3/2017
175 rats approved

# APPENDIX B

## SUPPLEMENTAL DATA TABLES (TABLES 37-76)

**Table 37. Hemostatic Biomarker Data in Utah Cohort Patients Compared to Healthy Controls**

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **D-Dimer (ng/ml)** | Healthy Controls | 76 | 57 | 71 | 10 | 0-249 |
| | Day 0 | 6,398 | 5,153 | 5,870 | 578 | 0-36,567 |
| | Day 4 | 5,603 | 4,192 | 4,390 | 581 | 335-16,496 |
| | Day 8 | 5,879 | 3,681 | 4,285 | 782 | 758-15,283 |
| **F1.2 (pmol/l)** | Healthy Controls | 151 | 136 | 60 | 8.5 | 83-397 |
| | Day 0 | 365 | 232 | 556 | 55 | 39-5,285 |
| | Day 4 | 468 | 351 | 319 | 42 | 62-1,309 |
| | Day 8 | 296 | 253 | 190 | 35 | 76-746 |
| **PAI-1 (pg/ml)** | Healthy Controls | 7 | 0.025 | 13 | 1.9 | 0-54 |
| | Day 0 | 66 | 38 | 71 | 7 | 0-357 |
| | Day 4 | 60 | 45 | 63 | 8.4 | 0-325 |
| | Day 8 | 47 | 45 | 49 | 8.9 | 0-173 |
| **INR** | Day 0 | 1.6 | 1.4 | 0.55 | 0.054 | 1-5.2 |
| | Day 4 | 1.5 | 1.3 | 0.41 | 0.054 | 1-3.2 |
| | Day 8 | 1.3 | 1.3 | 0.18 | 0.033 | 1.1-2 |
| **Platelets (k/µl)** | Day 0 | 189 | 181 | 101 | 10 | 23-571 |
| | Day 4 | 218 | 180 | 137 | 18 | 21-680 |
| | Day 8 | 312 | 291 | 167 | 32 | 98-737 |
| **Fibrinogen (mg/dl)** | Day 0 | 638 | 616 | 254 | 25 | 133-1,449 |
| | Day 4 | 602 | 627 | 202 | 27 | 153-1,063 |
| | Day 8 | 635 | 609 | 203 | 37 | 252-1,269 |

**Table 38. Infection Biomarkers in Utah Cohort Patients Compared to Healthy Controls**

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Nucleosomes (Units)** | Healthy Controls | 7 | 4.8 | 6.4 | 0.93 | 3.7-36 |
| | Day 0 | 13 | 8.2 | 19 | 1.9 | 0-119 |
| | Day 4 | 23 | 13 | 26 | 3.5 | 0-136 |
| | Day 8 | 18 | 13 | 21 | 3.8 | 0-77 |
| **HMGB-1 (ng/ml)** | Healthy Controls | 1.4 | 0.13 | 4.9 | 0.69 | 0.04-23 |
| | Day 0 | 9.1 | 5.3 | 13 | 1.3 | 0.18-87 |
| | Day 4 | 8 | 6.3 | 7.9 | 1 | 0.44-44 |
| | Day 8 | 8.3 | 6.3 | 6.5 | 1.2 | 2.3-29 |
| **Procalcitonin (pg/ml)** | Healthy Controls | 18 | 11 | 23 | 3.2 | 0-98 |
| | Day 0 | 1,810 | 663 | 3,210 | 316 | 8-21,162 |
| | Day 4 | 1,266 | 243 | 3,111 | 412 | 7.6-22,162 |
| | Day 8 | 699 | 243 | 1,650 | 301 | 5.6-8,751 |

**Table 39. Inflammation Biomarkers in Utah Cohort Patients Compared to Healthy Controls**

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **IL-2 (pg/ml)** | Healthy Controls | 1.1 | 0 | 1.8 | 0.26 | 0-6.3 |
| | Day 0 | 3.4 | 2.5 | 3 | 0.29 | 0-22 |
| | Day 4 | 5.5 | 2.5 | 9.6 | 1.3 | 0-56 |
| | Day 8 | 3.6 | 2.5 | 2.6 | 0.47 | 1.6-12 |
| **IL-4 (pg/ml)** | Healthy Controls | 1.3 | 1.4 | 1.1 | 0.16 | 0-6.7 |
| | Day 0 | 2.8 | 2.6 | 1.4 | 0.13 | 0-9.7 |
| | Day 4 | 3.3 | 2.5 | 3 | 0.4 | 0.94-18 |
| | Day 8 | 2.7 | 2.5 | 1.6 | 0.29 | 1.2-10 |
| **IL-6 (pg/ml)** | Healthy Controls | 1.2 | 0.94 | 1.2 | 0.18 | 0.25-7.3 |
| | Day 0 | 165 | 44 | 252 | 25 | 0-857 |
| | Day 4 | 82 | 28 | 150 | 20 | 3.4-764 |
| | Day 8 | 78 | 28 | 157 | 29 | 0.63-764 |
| **IL-8 (pg/ml)** | Healthy Controls | 2.6 | 2.6 | 0.89 | 0.13 | 0.98-5.7 |
| | Day 0 | 41 | 12 | 106 | 10 | 0-708 |
| | Day 4 | 30 | 16 | 48 | 6.3 | 3.4-317 |
| | Day 8 | 39 | 16 | 60 | 11 | 3-278 |
| **IL-10 (pg/ml)** | Healthy Controls | 0.68 | 0.6 | 0.36 | 0.052 | 0-2.1 |
| | Day 0 | 16 | 2.8 | 78 | 7.7 | 0-758 |
| | Day 4 | 4.6 | 3 | 4.2 | 0.56 | 0.67-17 |
| | Day 8 | 5.7 | 3 | 13 | 2.3 | 0.75-72 |
| **VEGF (pg/ml)** | Healthy Controls | 4.8 | 4.6 | 1.3 | 0.19 | 2.1-7.8 |
| | Day 0 | 28 | 20 | 37 | 3.6 | 0-339 |
| | Day 4 | 39 | 29 | 35 | 4.6 | 3.2-188 |
| | Day 8 | 29 | 29 | 34 | 6.2 | 3.7-184 |
| **IFNγ (pg/ml)** | Healthy Controls | 0.17 | 0 | 0.31 | 0.045 | 0-1.8 |
| | Day 0 | 7.3 | 0.41 | 50 | 4.9 | 0-507 |
| | Day 4 | 1.2 | 0.42 | 2.3 | 0.3 | 0-12 |
| | Day 8 | 0.65 | 0.42 | 0.49 | 0.09 | 0-2 |
| **TNFα (pg/ml)** | Healthy Controls | 1.7 | 1.8 | 1 | 0.15 | 0-6.6 |
| | Day 0 | 6 | 4.1 | 7.2 | 0.71 | 0-57 |
| | Day 4 | 5.2 | 4.3 | 4.5 | 0.59 | 0.74-29 |
| | Day 8 | 4.6 | 4.3 | 3 | 0.54 | 0.84-15 |
| **IL-1α (pg/ml)** | Healthy Controls | 0.1 | 0.1 | 0.11 | 0.015 | 0-0.53 |
| | Day 0 | 0.76 | 0.31 | 3.6 | 0.36 | 0-37 |
| | Day 4 | 1.1 | 0.31 | 3.1 | 0.41 | 0.15-23 |
| | Day 8 | 1.1 | 0.31 | 3.4 | 0.62 | 0.1-19 |

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **IL-1β (pg/ml)** | Healthy Controls | 0.79 | 0 | 1.2 | 0.18 | 0-5.6 |
| | Day 0 | 2.3 | 1.3 | 2.3 | 0.23 | 0-11 |
| | Day 4 | 2.8 | 1.7 | 3.9 | 0.51 | 0.65-25 |
| | Day 8 | 2.2 | 1.7 | 1.6 | 0.3 | 0.6-6.6 |
| **MCP-1 (pg/ml)** | Healthy Controls | 89 | 90 | 27 | 3.9 | 30-150 |
| | Day 0 | 337 | 259 | 257 | 25 | 0-802 |
| | Day 4 | 304 | 226 | 212 | 28 | 61-802 |
| | Day 8 | 247 | 226 | 173 | 32 | 36-802 |
| **EGF (pg/ml)** | Healthy Controls | 1.4 | 1.3 | 1.2 | 0.17 | 0-5.4 |
| | Day 0 | 6.4 | 4.6 | 6.2 | 0.61 | 0-46 |
| | Day 4 | 9.5 | 5.6 | 10 | 1.4 | 1.3-50 |
| | Day 8 | 8.2 | 5.6 | 13 | 2.3 | 1.5-56 |
| **IL-6:IL-10 Ratio** | Healthy Controls | 1.8 | 1.3 | 2.1 | 0.31 | 0.38-15 |
| | Day 0 | 41 | 13 | 70 | 6.9 | 0-460 |
| | Day 4 | 27 | 9.1 | 52 | 6.9 | 0.55-301 |
| | Day 8 | 15 | 9.8 | 18 | 3.2 | 0.59-93 |

All biomarkers showed significant differences (Mann-Whitney t test, $p < 0.05$) from the healthy control population at each time point. Biomarkers were measured in 50 healthy controls, 103 patients on Day 0, 57 patients on Day 4, and 30 patients on Day 8.

**Table 40. Endothelial Biomarker Data in Utah Cohort Patients Compared to Healthy Controls**

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **TFPI (ng/ml)** | Healthy Controls | 61 | 59 | 19 | 2.7 | 24-106 |
| | Day 0 | 104 | 90 | 72 | 7.1 | 4.8-423 |
| | Day 4 | 89 | 65 | 88 | 12 | 24-671 |
| | Day 8 | 87 | 66 | 61 | 11 | 32-337 |
| **Protein C (%)** | Healthy Controls | 98 | 94 | 18 | 2.5 | 71-142 |
| | Day 0 | 61 | 52 | 49 | 4.8 | 0-309 |
| | Day 4 | 57 | 58 | 27 | 3.6 | 9.7-113 |
| | Day 8 | 77 | 58 | 35 | 6.4 | 32-147 |
| **Endocan (ng/ml)** | Healthy Controls | 1.9 | 0.85 | 4.5 | 0.63 | 0.17-25 |
| | Day 0 | 10 | 6.2 | 9.5 | 0.93 | 1.4-60 |
| | Day 4 | 9.1 | 5.3 | 11 | 1.4 | 1.4-50 |
| | Day 8 | 9.2 | 5.3 | 7.9 | 1.4 | 1.5-27 |
| **Ang-2 (pg/ml)** | Healthy Controls | 1869 | 1566 | 1070 | 151 | 503-5,538 |
| | Day 0 | 15,236 | 8,435 | 19,130 | 1,894 | 650-13,6317 |
| | Day 4 | 7,302 | 5,156 | 8,639 | 1,274 | 48-53,240 |
| | Day 8 | 10,065 | 5,156 | 13,454 | 2,936 | 448-61,010 |
| **vWF (%)** | Healthy Controls | 93 | 93 | 19 | 2.7 | 59-131 |
| | Day 0 | 246 | 251 | 71 | 7 | 107-379 |
| | Day 4 | 249 | 253 | 77 | 10 | 38-398 |
| | Day 8 | 255 | 253 | 65 | 12 | 154-374 |

All biomarkers showed significant differences (Mann-Whitney t test, $p < 0.05$) from the healthy control population at each time point. Biomarkers were measured in 50 healthy controls, 103 patients on Day 0, 57 patients on Day 4, and 30 patients on Day 8.

**Table 41. Platelet Biomarker Data in Utah Cohort Patients Compared to Healthy Controls**

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Platelets (k/µl)** | Day 0 | 189 | 181 | 101 | 10 | 23-571 |
| | Day 4 | 218 | 180 | 137 | 18 | 21-680 |
| | Day 8 | 312 | 291 | 167 | 32 | 98-737 |
| **CD40L (pg/ml)** | Healthy Controls | 75 | 70 | 95 | 13 | 0-625 |
| | Day 0 | 378 | 236 | 588 | 58 | 0-4756 |
| | Day 4 | 594 | 335 | 627 | 83 | 0-2733 |
| | Day 8 | 624 | 335 | 921 | 168 | 0-3796 |
| **MP (nM)** | Healthy Controls | 13 | 11 | 12 | 1.7 | 0-62 |
| | Day 0 | 38 | 31 | 29 | 2.9 | 2.4-159 |
| | Day 4 | 54 | 32 | 50 | 6.7 | 2.8-208 |
| | Day 8 | 49 | 32 | 50 | 9.1 | 11-225 |
| **MP-TF (pg/ml)** | Healthy Controls | 0.34 | 0.25 | 0.3 | 0.042 | 0-1.1 |
| | Day 0 | 1.1 | 0.84 | 0.94 | 0.092 | 0.06-7.4 |
| | Day 4 | 1.2 | 0.95 | 0.94 | 0.12 | 0-5.1 |
| | Day 8 | 1.1 | 0.95 | 0.87 | 0.16 | 0.12-4.6 |
| **PF4 (ng/ml)** | Healthy Controls | 18 | 18 | 5.9 | 0.91 | 8-35 |
| | Day 0 | 77 | 64 | 35 | 3.5 | 15-169 |

All biomarkers showed significant differences (Mann-Whitney t test, $p < 0.05$) from the healthy control population at each time point. Biomarkers were measured in 50 healthy controls, 103 patients on Day 0, 57 patients on Day 4, and 30 patients on Day 8, with the exception of PF4, which was not measured in patient samples from Day 4 or Day 8.

**Table 42. Baseline Hemostatic Biomarker Levels Stratified by DIC Score**

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **D-Dimer (ng/ml)** | Healthy Controls | 76 | 57 | 71 | 10 | 0-249 |
| | No DIC | 1,355 | 1,188 | 1,113 | 249 | 0-3,171 |
| | Non-Overt DIC | 6,972 | 6,312 | 4,987 | 649 | 423-27,871 |
| | Overt DIC | 9,189 | 6,232 | 7,615 | 1,554 | 1,041-36,567 |
| **F1.2 (pmol/l)** | Healthy Controls | 151 | 136 | 60 | 8.5 | 83-397 |
| | No DIC | 242 | 189 | 193 | 43 | 39-812 |
| | Non-Overt DIC | 335 | 256 | 276 | 36 | 47-1,699 |
| | Overt DIC | 540 | 232 | 1049 | 214 | 63-5,285 |
| **PAI-1 (pg/ml)** | Healthy Controls | 7 | 0.025 | 13 | 1.9 | 0-54 |
| | No DIC | 49 | 31 | 48 | 11 | 0-176 |
| | Non-Overt DIC | 70 | 39 | 67 | 8.7 | 6.1-256 |
| | Overt DIC | 70 | 30 | 95 | 19 | 0-357 |
| **INR** | No DIC | 1.5 | 1.2 | 0.96 | 0.21 | 1-5.2 |
| | Non-Overt DIC | 1.4 | 1.4 | 0.18 | 0.023 | 1.1-2.3 |
| | Overt DIC | 1.9 | 1.8 | 0.56 | 0.12 | 1.2-3.2 |
| **Platelets (K/µl)** | No DIC | 243 | 221 | 87 | 19 | 85-433 |
| | Non-Overt DIC | 205 | 185 | 95 | 12 | 59-571 |
| | Overt DIC | 104 | 81 | 71 | 15 | 23-295 |
| **Fibrinogen (mg/dl)** | No DIC | 662 | 650 | 269 | 60 | 221-1,449 |
| | Non-Overt DIC | 660 | 626 | 231 | 30 | 298-1,428 |
| | Overt DIC | 567 | 518 | 291 | 59 | 133-1,404 |

**Table 43. Baseline Infection Biomarker Levels Stratified by DIC Score**

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Nucleosomes (Units)** | Healthy Controls | 7 | 4.8 | 6.4 | 0.93 | 3.7-36 |
| | No DIC | 6.3 | 5.1 | 6.3 | 1.4 | 0-23 |
| | Non-Overt DIC | 11 | 8.6 | 14 | 1.8 | 0-86 |
| | Overt DIC | 23 | 9.7 | 31 | 6.3 | 0-119 |
| **HMGB-1 (ng/ml)** | Healthy Controls | 1.4 | 0.13 | 4.9 | 0.69 | 0.04-23 |
| | No DIC | 5.6 | 4.8 | 3.5 | 0.77 | 2.5-18 |
| | Non-Overt DIC | 9 | 5.1 | 15 | 1.9 | 0.18-87 |
| | Overt DIC | 12 | 7.5 | 14 | 2.8 | 3.3-66 |
| **Procalcitonin (pg/ml)** | Healthy Controls | 18 | 11 | 23 | 3.2 | 0-98 |
| | No DIC | 1,309 | 88 | 4,700 | 1,051 | 8-21,162 |
| | Non-Overt DIC | 1,484 | 687 | 1,743 | 227 | 18-6,493 |
| | Overt DIC | 3,028 | 1,182 | 4,214 | 860 | 83-18,965 |

**Table 44. Baseline Inflammatory Biomarker Levels Stratified by DIC Score**

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **IL-2 (pg/ml)** | Healthy Controls | 1.1 | 0 | 1.8 | 0.26 | 0-6.3 |
| | No DIC | 2.9 | 2.3 | 1.8 | 0.41 | 0-8.3 |
| | Non-Overt DIC | 3.4 | 2.6 | 3.4 | 0.44 | 0-22 |
| | Overt DIC | 3.5 | 2.3 | 2.7 | 0.55 | 0-11 |
| **IL-4 (pg/ml)** | Healthy Controls | 1.3 | 1.4 | 1.1 | 0.16 | 0-6.7 |
| | No DIC | 2.5 | 2.4 | 0.65 | 0.14 | 1.5-3.9 |
| | Non-Overt DIC | 3 | 2.7 | 1.6 | 0.21 | 0-9.7 |
| | Overt DIC | 2.7 | 2.5 | 1.1 | 0.22 | 1.1-5.5 |
| **IL-6 (pg/ml)** | Healthy Controls | 1.2 | 0.94 | 1.2 | 0.18 | 0.25-7.3 |
| | No DIC | 56 | 15 | 168 | 37 | 1.9-764 |
| | Non-Overt DIC | 197 | 72 | 271 | 35 | 0-857 |
| | Overt DIC | 176 | 80 | 244 | 50 | 0.27-764 |
| **IL-8 (pg/ml)** | Healthy Controls | 2.6 | 2.6 | 0.89 | 0.13 | 0.98-5.7 |
| | No DIC | 11 | 5.1 | 17 | 3.8 | 1.7-70 |
| | Non-Overt DIC | 43 | 13 | 103 | 13 | 0-708 |
| | Overt DIC | 61 | 16 | 147 | 30 | 0.51-708 |
| **IL-10 (pg/ml)** | Healthy Controls | 0.68 | 0.6 | 0.36 | 0.052 | 0-2.1 |
| | No DIC | 2.5 | 1.8 | 2.4 | 0.53 | 0.69-11 |
| | Non-Overt DIC | 7.9 | 2.9 | 17 | 2.2 | 0-101 |
| | Overt DIC | 47 | 4.3 | 159 | 32 | 0.45-758 |
| **VEGF (pg/ml)** | Healthy Controls | 4.8 | 4.6 | 1.3 | 0.19 | 2.1-7.8 |
| | No DIC | 24 | 18 | 19 | 4.2 | 5.5-92 |
| | Non-Overt DIC | 33 | 23 | 45 | 5.9 | 0-339 |
| | Overt DIC | 20 | 13 | 17 | 3.4 | 3.2-65 |
| **IFNγ (pg/ml)** | Healthy Controls | 0.17 | 0 | 0.31 | 0.045 | 0-1.8 |
| | No DIC | 0.6 | 0.41 | 0.59 | 0.13 | 0-2.7 |
| | Non-Overt DIC | 3.7 | 0.45 | 8.4 | 1.1 | 0-38 |
| | Overt DIC | 22 | 0.4 | 103 | 21 | 0.24-507 |
| **TNFα (pg/ml)** | Healthy Controls | 1.7 | 1.8 | 1 | 0.15 | 0-6.6 |
| | No DIC | 5.7 | 2.6 | 12 | 2.7 | 0.74-57 |
| | Non-Overt DIC | 6.3 | 4.7 | 6.3 | 0.82 | 0-36 |
| | Overt DIC | 5.5 | 6.2 | 2.5 | 0.5 | 0.76-9.4 |
| **IL-1α (pg/ml)** | Healthy Controls | 0.1 | 0.1 | 0.11 | 0.015 | 0-0.53 |
| | No DIC | 0.29 | 0.28 | 0.084 | 0.019 | 0.16-0.49 |
| | Non-Overt DIC | 1.1 | 0.28 | 4.8 | 0.62 | 0-37 |
| | Overt DIC | 0.42 | 0.35 | 0.29 | 0.06 | 0.15-1.6 |

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **IL-1β (pg/ml)** | Healthy Controls | 0.79 | 0 | 1.2 | 0.18 | 0-5.6 |
| | No DIC | 1.2 | 1 | 0.56 | 0.12 | 0.6-2.9 |
| | Non-Overt DIC | 2.8 | 1.5 | 2.7 | 0.35 | 0-11 |
| | Overt DIC | 2.2 | 1.5 | 1.9 | 0.39 | 0.65-7.5 |
| **MCP-1 (pg/ml)** | Healthy Controls | 89 | 90 | 27 | 3.9 | 30-150 |
| | No DIC | 185 | 166 | 122 | 27 | 7.6-523 |
| | Non-Overt DIC | 380 | 310 | 279 | 36 | 0-802 |
| | Overt DIC | 360 | 288 | 244 | 50 | 1.3-802 |
| **EGF (pg/ml)** | Healthy Controls | 1.4 | 1.3 | 1.2 | 0.17 | 0-5.4 |
| | No DIC | 7.5 | 7.7 | 5.1 | 1.1 | 1.6-23 |
| | Non-Overt DIC | 7.3 | 5.7 | 7.2 | 0.93 | 0-46 |
| | Overt DIC | 3.2 | 2 | 2.1 | 0.44 | 1.4-10 |
| **IL-6:IL-10 Ratio** | Healthy Controls | 1.8 | 1.3 | 2.1 | 0.31 | 0.38-15 |
| | No DIC | 13 | 7 | 16 | 3.7 | 0.97-69 |
| | Non-Overt DIC | 52 | 17 | 78 | 10 | 0-460 |
| | Overt DIC | 36 | 12 | 72 | 15 | 0.11-344 |

**Table 45. Baseline Endothelial Biomarker Levels Stratified by DIC Score**

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **TFPI (ng/ml)** | Healthy Controls | 61 | 59 | 19 | 2.7 | 24-106 |
| | No DIC | 95 | 81 | 58 | 13 | 32-285 |
| | Non-Overt DIC | 104 | 94 | 69 | 8.9 | 4.8-423 |
| | Overt DIC | 110 | 89 | 90 | 18 | 11-407 |
| **Protein C (%)** | Healthy Controls | 98 | 94 | 18 | 2.5 | 71-142 |
| | No DIC | 80 | 71 | 62 | 14 | 0-309 |
| | Non-Overt DIC | 59 | 51 | 40 | 5.3 | 2.5-309 |
| | Overt DIC | 48 | 41 | 52 | 11 | 2.7-277 |
| **Endocan (ng/ml)** | Healthy Controls | 1.9 | 0.85 | 4.5 | 0.63 | 0.17-25 |
| | No DIC | 7 | 5.1 | 6.4 | 1.4 | 1.4-24 |
| | Non-Overt DIC | 9.8 | 7.4 | 7.3 | 0.95 | 2-34 |
| | Overt DIC | 14 | 6.5 | 14 | 3 | 1.9-60 |
| **Ang-2 (pg/ml)** | Healthy Controls | 1,869 | 1,566 | 1,070 | 151 | 503-5,538 |
| | No DIC | 8,343 | 5,754 | 11,187 | 2,501 | 961-53,612 |
| | Non-Overt DIC | 11,736 | 82,74 | 10,563 | 1,387 | 650-44,167 |
| | Overt DIC | 29,440 | 17,618 | 30,732 | 6,273 | 1,816-136,317 |
| **vWF (%)** | Healthy Controls | 93 | 93 | 19 | 2.7 | 59-131 |
| | No DIC | 205 | 183 | 63 | 14 | 107-349 |
| | Non-Overt DIC | 260 | 271 | 69 | 9 | 111-370 |
| | Overt DIC | 247 | 251 | 73 | 15 | 122-379 |

**Table 46. Baseline Platelet Biomarker Levels Stratified by DIC Score**

| Biomarker | Patient Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Platelets (k/µl)** | No DIC | 243 | 221 | 87 | 19 | 85-433 |
| | Non-Overt DIC | 205 | 185 | 95 | 12 | 59-571 |
| | Overt DIC | 104 | 81 | 71 | 15 | 23-295 |
| **CD40L (pg/ml)** | Healthy Controls | 75 | 70 | 95 | 13 | 0-625 |
| | No DIC | 476 | 197 | 1,032 | 231 | 5-4,756 |
| | Non-Overt DIC | 412 | 259 | 478 | 62 | 0-2,923 |
| | Overt DIC | 213 | 148 | 187 | 38 | 13-659 |
| **MP (nM)** | Healthy Controls | 13 | 11 | 12 | 1.7 | 0-62 |
| | No DIC | 47 | 36 | 35 | 7.7 | 16-159 |
| | Non-Overt DIC | 39 | 34 | 29 | 3.9 | 2.4-138 |
| | Overt DIC | 28 | 23 | 20 | 4.2 | 2.8-93 |
| **MP-TF (pg/ml)** | Healthy Controls | 0.34 | 0.25 | 0.3 | 0.042 | 0-1.1 |
| | No DIC | 0.79 | 0.74 | 0.58 | 0.13 | 0.06-2.3 |
| | Non-Overt DIC | 1.1 | 0.87 | 0.93 | 0.12 | 0.24-7.4 |
| | Overt DIC | 1.4 | 1.1 | 1.1 | 0.23 | 0.41-5.2 |
| **PF4 (ng/ml)** | Healthy Controls | 18 | 18 | 5.9 | 0.91 | 8-35 |
| | No DIC | 82 | 65 | 38 | 8.4 | 38-169 |
| | Non-Overt DIC | 78 | 66 | 35 | 4.7 | 31-164 |
| | Overt DIC | 68 | 56 | 32 | 6.7 | 15-137 |

**Table 47. Association of Baseline Hemostatic Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range | AUC |
|---|---|---|---|---|---|---|---|
| **D-Dimer (ng/ml)** | Survivors | 6,210 | 4,781 | 6,090 | 652.9 | 0-36,567 | 0.60 |
| | Non-Survivors | 7,211 | 5,499 | 4,593 | 1,186 | 181-15,752 | |
| **F1.2 (pmol/l)** | Survivors | 371 | 223 | 598.8 | 64.2 | 39-5,285 | 0.54 |
| | Non-Survivors | 311.9 | 235 | 198.8 | 51.34 | 96-825 | |
| **PAI-1 (pg/ml)** | Survivors | 55.53 | 35.54 | 59.24 | 6.351 | 0-252.4 | 0.70 |
| | Non-Survivors | 114.3 | 106.8 | 97.81 | 25.25 | 7.53-357.5 | |
| **INR** | Survivors | 1.563 | 1.42 | 0.577 | 0.06186 | 1-5.22 | 0.60 |
| | Non-Survivors | 1.594 | 1.5 | 0.379 | 0.09475 | 1.17-2.77 | |
| **Platelets (k/µl)** | Survivors | 194.7 | 182 | 103.7 | 11.12 | 23-571 | 0.61 |
| | Non-Survivors | 153.1 | 140 | 72.79 | 18.8 | 36-272 | |
| **Fibrinogen (mg/dl)** | Survivors | 628 | 619 | 236.8 | 25.54 | 133-1,404 | 0.51 |
| | Non-Survivors | 693.5 | 574 | 338.1 | 84.51 | 324-1,449 | |

**Table 48. Association of Baseline Infection Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range | AUC |
|---|---|---|---|---|---|---|---|
| **Nucleosomes (Units)** | Survivors | 11.25 | 8 | 16.7 | 1.795 | 0-118.9 | 0.58 |
| | Non-Survivors | 22.93 | 15.6 | 27.3 | 7.038 | 0-85.6 | |
| **HMGB-1 (ng/ml)** | Survivors | 8.35 | 4.775 | 12.3 | 1.325 | 0.18-86.77 | 0.67 |
| | Non-Survivors | 13.36 | 7.185 | 16.3 | 4.063 | 2.87-65.67 | |
| **Procalcitonin (pg/ml)** | Survivors | 1213 | 433.7 | 1,708 | 183.1 | 8-9,083 | 0.77 |
| | Non-Survivors | 5031 | 2425 | 6,550 | 1691 | 93.5-21,162 | |

**Table 49. Association of Baseline Inflammatory Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range | AUC |
|---|---|---|---|---|---|---|---|
| **IL-2 (pg/ml)** | Survivors | 3.374 | 2.43 | 3.104 | 0.3328 | 0-21.61 | 0.52 |
| | Non-Survivors | 3.091 | 2.49 | 2.093 | 0.5403 | 1.6-9.83 | |
| **IL-4 (pg/ml)** | Survivors | 2.763 | 2.56 | 1.35 | 0.1447 | 0-9.71 | 0.55 |
| | Non-Survivors | 2.946 | 2.49 | 1.193 | 0.3081 | 1.15-5.5 | |
| **IL-6 (pg/ml)** | Survivors | 135.4 | 41.61 | 225 | 24.12 | 0-857.1 | 0.70 |
| | Non-Survivors | 294.3 | 150 | 319.3 | 82.45 | 0.27-764 | |
| **IL-8 (pg/ml)** | Survivors | 25.92 | 9.98 | 49.42 | 5.298 | 0-273 | 0.70 |
| | Non-Survivors | 83.62 | 36.06 | 176.9 | 45.66 | 0.51-708 | |
| **IL-10 (pg/ml)** | Survivors | 14.18 | 2.8 | 81.25 | 8.711 | 0-758 | 0.58 |
| | Non-Survivors | 20.26 | 2.97 | 61.09 | 15.77 | 0.45-240.8 | |
| **VEGF (pg/ml)** | Survivors | 25.37 | 20.51 | 18.97 | 2.034 | 0-91.55 | 0.57 |
| | Non-Survivors | 43.32 | 14.06 | 85.05 | 21.96 | 6.11-338.8 | |
| **IFNγ (pg/ml)** | Survivors | 8.219 | 0.41 | 54.5 | 5.843 | 0-507 | 0.54 |
| | Non-Survivors | 2.108 | 0.37 | 6.41 | 1.655 | 0.24-25.26 | |
| **TNFα (pg/ml)** | Survivors | 5.825 | 4.09 | 7.005 | 0.7511 | 0-56.54 | 0.52 |
| | Non-Survivors | 5.109 | 6.11 | 2.933 | 0.7573 | 0.76-9.73 | |
| **IL-1α (pg/ml)** | Survivors | 0.8346 | 0.28 | 3.952 | 0.4237 | 0-37.11 | 0.60 |
| | Non-Survivors | 0.3727 | 0.36 | 0.1347 | 0.03478 | 0.16-0.69 | |
| **IL-1β (pg/ml)** | Survivors | 2.19 | 1.3 | 2.183 | 0.234 | 0-10.95 | 0.58 |
| | Non-Survivors | 2.635 | 1.37 | 2.318 | 0.5985 | 0.91-8.83 | |
| **MCP-1 (pg/ml)** | Survivors | 322.9 | 224.8 | 249.2 | 26.71 | 0-802 | 0.53 |
| | Non-Survivors | 390.2 | 302.1 | 284 | 73.33 | 1.31-802 | |
| **EGF (pg/ml)** | Survivors | 6.297 | 4.91 | 4.947 | 0.5304 | 0-27.53 | 0.58 |
| | Non-Survivors | 7.443 | 2.63 | 11.22 | 2.897 | 1.4-45.86 | |
| **IL-6:IL-10 Ratio** | Survivors | 33.96 | 12.8 | 49.11 | 5.265 | 0-234.8 | 0.61 |
| | Non-Survivors | 81.31 | 16.61 | 136.2 | 35.18 | 0.6-460.3 | |

**Table 50. Association of Baseline Endothelial Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range | AUC |
|---|---|---|---|---|---|---|---|
| **TFPI (ng/ml)** | Survivors | 96.2 | 87.5 | 57.1 | 6.1 | 4.8-407.4 | 0.55 |
| | Non-Survivors | 141.3 | 94.9 | 121.9 | 31.5 | 39.1-422.6 | |
| **Protein C (%)** | Survivors | 56.5 | 53.1 | 26.1 | 2.8 | 0-128 | 0.71 |
| | Non-Survivors | 37.2 | 34.4 | 19.5 | 5.2 | 2.7-67.1 | |
| **Endocan (ng/ml)** | Survivors | 9.0 | 5.5 | 7.9 | 0.8 | 1.4-37.6 | 0.58 |
| | Non-Survivors | 16.5 | 13.1 | 14.8 | 3.8 | 2.3-59.7 | |
| **Ang-2 (pg/ml)** | Survivors | 12,539 | 7,413 | 14,277 | 1,540 | 650-66,180 | 0.76 |
| | Non-Survivors | 30,165 | 19,300 | 33,385 | 8,620 | 1,812-136,317 | |
| **vWF (%)** | Survivors | 246.5 | 251 | 70.17 | 7.523 | 107-379 | 0.58 |
| | Non-Survivors | 239.7 | 219 | 79.38 | 20.5 | 122-345 | |

**Table 51. Association of Baseline Platelet Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range | AUC |
|---|---|---|---|---|---|---|---|
| **Platelets (k/µl)** | Survivors | 194.7 | 182 | 103.7 | 11.12 | 23-571 | 0.61 |
| | Non-Survivors | 153.1 | 140 | 72.79 | 18.8 | 36-272 | |
| **CD40L (pg/ml)** | Survivors | 380.3 | 243 | 604 | 64.76 | 0-4,756 | 0.55 |
| | Non-Survivors | 385.6 | 163 | 518.3 | 133.8 | 3-1,931 | |
| **MP (nM)** | Survivors | 38.61 | 30.92 | 29.85 | 3.277 | 2.75-159.4 | 0.53 |
| | Non-Survivors | 34.19 | 28.07 | 25.3 | 6.325 | 2.35-93.4 | |
| **MP/TF (pg/ml)** | Survivors | 1.062 | 0.83 | 0.9971 | 0.1069 | 0.06-7.4 | 0.62 |
| | Non-Survivors | 1.153 | 1.13 | 0.5411 | 0.1397 | 0.41-2.1 | |
| **PF4 (ng/ml)** | Survivors | 79.55 | 64.96 | 36.33 | 3.964 | 15.37-169.3 | 0.70 |
| | Non-Survivors | 58.93 | 55.55 | 19.22 | 5.136 | 41.43-119.1 | |

**Table 52. Day 4 Hemostatic Biomarker Levels Stratified by DIC Score**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **D-Dimer (ng/ml)** | No DIC | 3,057 | 1,819 | 3,455 | 1,042 | 335-12,509 |
| | Non-Overt DIC | 5,837 | 4,193 | 4,440 | 740 | 787-16,496 |
| | Overt DIC | 7,562 | 8,302 | 4,193 | 1,326 | 581-12,993 |
| **F1.2 (pmol/l)** | No DIC | 679 | 518 | 428 | 129 | 103-1,309 |
| | Non-Overt DIC | 441 | 359 | 281 | 47 | 62-1,029 |
| | Overt DIC | 329 | 271 | 207 | 65 | 80-657 |
| **PAI-1 (pg/ml)** | No DIC | 86 | 45 | 95 | 29 | 0-272 |
| | Non-Overt DIC | 46 | 37 | 32 | 5.3 | 0-120 |
| | Overt DIC | 81 | 54 | 94 | 30 | 14-325 |
| **INR** | No DIC | 1.3 | 1.2 | 0.12 | 0.035 | 1.2-1.5 |
| | Non-Overt DIC | 1.4 | 1.3 | 0.39 | 0.066 | 1-3.2 |
| | Overt DIC | 1.8 | 1.7 | 0.48 | 0.15 | 1.3-2.8 |
| **Platelets (k/µl)** | No DIC | 214 | 183 | 140 | 42 | 21-478 |
| | Non-Overt DIC | 222 | 182 | 119 | 20 | 72-534 |
| | Overt DIC | 209 | 131 | 199 | 63 | 63-680 |
| **Fibrinogen (mg/dl)** | No DIC | 612 | 657 | 231 | 70 | 233-972 |
| | Non-Overt DIC | 593 | 597 | 189 | 31 | 153-975 |
| | Overt DIC | 620 | 576 | 234 | 74 | 286-1,063 |

**Table 53. Day 4 Infection Biomarker Levels Stratified by DIC Score**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Nucleosomes (Units)** | No DIC | 30 | 20 | 25 | 7.5 | 6.2-79 |
| | Non-Overt DIC | 22 | 13 | 29 | 4.8 | 0-136 |
| | Overt DIC | 18 | 12 | 17 | 5.4 | 1.5-59 |
| **HMGB-1 (ng/ml)** | No DIC | 6.7 | 6.6 | 3 | 0.96 | 2.8-13 |
| | Non-Overt DIC | 8 | 5.4 | 9.5 | 1.6 | 0.44-44 |
| | Overt DIC | 9.2 | 8.1 | 4.3 | 1.4 | 4.8-18 |
| **Procalcitonin (pg/ml)** | No DIC | 827 | 137 | 1,424 | 429 | 28-4,813 |
| | Non-Overt DIC | 850 | 241 | 1,266 | 211 | 7.6-4,801 |
| | Overt DIC | 3,249 | 705 | 6,814 | 2155 | 77-22,162 |

**Table 54. Day 4 Inflammatory Biomarker Levels Stratified by DIC Score**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **IL-2 (pg/ml)** | No DIC | 3.2 | 2.1 | 3.5 | 1.1 | 0-13 |
| | Non-Overt DIC | 6.6 | 2.7 | 12 | 1.9 | 0-56 |
| | Overt DIC | 4 | 2.3 | 4.6 | 1.5 | 1.7-17 |
| **IL-4 (pg/ml)** | No DIC | 2.9 | 2.7 | 0.96 | 0.29 | 2.2-5.3 |
| | Non-Overt DIC | 3.7 | 2.5 | 3.7 | 0.61 | 1-18 |
| | Overt DIC | 2.6 | 2.5 | 1.5 | 0.47 | 0.94-6.5 |
| **IL-6 (pg/ml)** | No DIC | 100 | 23 | 184 | 56 | 3.4-626 |
| | Non-Overt DIC | 67 | 29 | 135 | 22 | 4.7-764 |
| | Overt DIC | 118 | 73 | 168 | 53 | 5.4-565 |
| **IL-8 (pg/ml)** | No DIC | 52 | 10 | 95 | 29 | 4.6-317 |
| | Non-Overt DIC | 23 | 15 | 23 | 3.9 | 3.4-100 |
| | Overt DIC | 29 | 18 | 34 | 11 | 4.7-124 |
| **IL-10 (pg/ml)** | No DIC | 5.2 | 2.4 | 5.6 | 1.7 | 1.1-17 |
| | Non-Overt DIC | 4.5 | 3.1 | 3.8 | 0.63 | 0.97-16 |
| | Overt DIC | 4.3 | 2.7 | 4.5 | 1.4 | 0.67-14 |
| **VEGF (pg/ml)** | No DIC | 55 | 46 | 53 | 16 | 9-188 |
| | Non-Overt DIC | 37 | 30 | 27 | 4.6 | 3.7-126 |
| | Overt DIC | 29 | 17 | 33 | 10 | 3.2-114 |
| **IFNγ (pg/ml)** | No DIC | 0.52 | 0.27 | 0.62 | 0.19 | 0-2.2 |
| | Non-Overt DIC | 1.5 | 0.51 | 2.8 | 0.46 | 0-12 |
| | Overt DIC | 0.9 | 0.46 | 1.3 | 0.4 | 0-3.8 |
| **TNFα (pg/ml)** | No DIC | 5.9 | 3.6 | 7.8 | 2.4 | 1.8-29 |
| | Non-Overt DIC | 5 | 4.5 | 3.6 | 0.6 | 0.74-21 |
| | Overt DIC | 4.9 | 4.9 | 2.3 | 0.74 | 1.6-8.5 |
| **IL-1α (pg/ml)** | No DIC | 0.36 | 0.29 | 0.27 | 0.08 | 0.2-1.2 |
| | Non-Overt DIC | 0.81 | 0.33 | 1.3 | 0.22 | 0.18-5.1 |
| | Overt DIC | 2.8 | 0.26 | 7 | 2.2 | 0.15-23 |
| **IL-1β (pg/ml)** | No DIC | 2.2 | 1.9 | 1.5 | 0.47 | 0.76-5.1 |
| | Non-Overt DIC | 3.2 | 1.6 | 4.6 | 0.77 | 0.68-25 |
| | Overt DIC | 2.2 | 1.4 | 2.4 | 0.77 | 0.65-8.9 |
| **MCP-1 (pg/ml)** | No DIC | 318 | 239 | 200 | 60 | 156-802 |
| | Non-Overt DIC | 290 | 222 | 219 | 36 | 61-802 |
| | Overt DIC | 335 | 272 | 218 | 69 | 113-802 |
| **EGF (pg/ml)** | No DIC | 12 | 6.3 | 12 | 3.8 | 1.3-44 |
| | Non-Overt DIC | 10 | 6.1 | 11 | 1.8 | 1.4-50 |
| | Overt DIC | 4.5 | 2.2 | 5 | 1.6 | 1.3-17 |

**Table 55. Day 4 Endothelial Biomarker Levels Stratified by DIC Score**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **TFPI (ng/ml)** | No DIC | 70 | 64 | 27 | 8 | 42-129 |
| | Non-Overt DIC | 81 | 70 | 42 | 6.9 | 27-192 |
| | Overt DIC | 136 | 63 | 194 | 61 | 24-671 |
| **Protein C (%)** | No DIC | 76 | 69 | 25 | 7.8 | 46-113 |
| | Non-Overt DIC | 58 | 61 | 24 | 4 | 9.7-105 |
| | Overt DIC | 33 | 26 | 19 | 6.1 | 13-68 |
| **Endocan (ng/ml)** | No DIC | 4.5 | 3.9 | 2.4 | 0.73 | 1.4-8.3 |
| | Non-Overt DIC | 8.7 | 5.3 | 9.4 | 1.6 | 1.4-44 |
| | Overt DIC | 16 | 6.7 | 17 | 5.3 | 2.6-50 |
| **Ang-2 (pg/ml)** | No DIC | 4,688 | 4,404 | 3,248 | 1,027 | 48-11,316 |
| | Non-Overt DIC | 7,886 | 6,806 | 9,099 | 1,517 | 436-53,240 |
| | Overt DIC | 7,829 | 6,680 | 7,185 | 2,272 | 1,593-24,532 |
| **vWF (%)** | No DIC | 203 | 213 | 99 | 31 | 38-353 |
| | Non-Overt DIC | 249 | 248 | 69 | 12 | 123-398 |
| | Overt DIC | 291 | 308 | 62 | 20 | 174-380 |

**Table 56. Day 4 Platelet Biomarker Levels Stratified by DIC Score**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Platelets (k/µl)** | No DIC | 214 | 183 | 140 | 42 | 21-478 |
| | Non-Overt DIC | 222 | 182 | 119 | 20 | 72-534 |
| | Overt DIC | 209 | 131 | 199 | 63 | 63-680 |
| **CD40L (pg/ml)** | No DIC | 459 | 140 | 684 | 206 | 0-2,043 |
| | Non-Overt DIC | 672 | 523 | 555 | 93 | 0-2,150 |
| | Overt DIC | 461 | 208 | 815 | 258 | 0-2,733 |
| **MP (nM)** | No DIC | 73 | 57 | 67 | 21 | 5.4-208 |
| | Non-Overt DIC | 53 | 33 | 43 | 7.1 | 7.6-167 |
| | Overt DIC | 43 | 24 | 58 | 18 | 2.8-205 |
| **MP/TF (pg/ml)** | No DIC | 0.99 | 0.86 | 0.63 | 0.19 | 0-2.3 |
| | Non-Overt DIC | 1.3 | 0.95 | 1.1 | 0.18 | 0-5.1 |
| | Overt DIC | 1.2 | 1.1 | 0.65 | 0.21 | 0.28-2.4 |

**Table 57. Day 8 Hemostatic Biomarker Levels Stratified by DIC Score**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **D-Dimer (ng/ml)** | No DIC | 1,988 | 2,121 | 1,105 | 391 | 758-3,625 |
| | Non-Overt DIC | 7,293 | 7,105 | 4,134 | 881 | 1,197-15,283 |
| **F1.2 (pmol/l)** | No DIC | 222 | 231 | 83 | 29 | 87-351 |
| | Non-Overt DIC | 322 | 307 | 212 | 45 | 76-746 |
| **PAI-1 (pg/ml)** | No DIC | 20 | 18 | 20 | 7.2 | 0-50 |
| | Non-Overt DIC | 57 | 41 | 53 | 11 | 0-173 |
| **INR** | No DIC | 1.2 | 1.2 | 0.073 | 0.026 | 1.1-1.3 |
| | Non-Overt DIC | 1.4 | 1.4 | 0.18 | 0.039 | 1.1-2 |
| **Platelets (k/µl)** | No DIC | 375 | 327 | 203 | 77 | 98-737 |
| | Non-Overt DIC | 291 | 250 | 153 | 33 | 102-731 |
| **Fibrinogen (mg/dl)** | No DIC | 580 | 520 | 172 | 61 | 443-961 |
| | Non-Overt DIC | 655 | 620 | 214 | 46 | 252-1,269 |

**Table 58. Day 8 Infection Biomarker Levels Stratified by DIC Score**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Nucleosomes (Units)** | No DIC | 9.1 | 8.5 | 7.5 | 2.6 | 0.8-26 |
| | Non-Overt DIC | 21 | 12 | 23 | 5 | 0-77 |
| **HMGB-1 (ng/ml)** | No DIC | 8.6 | 6.3 | 8.2 | 2.9 | 2.3-28 |
| | Non-Overt DIC | 8.2 | 6.2 | 6 | 1.3 | 3-29 |
| **Procalcitonin (pg/ml)** | No DIC | 82 | 77 | 60 | 21 | 5.6-177 |
| | Non-Overt DIC | 923 | 314 | 1887 | 402 | 6.9-8,751 |

**Table 59. Day 8 Inflammatory Biomarker Levels Stratified by DIC Score**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **IL-2 (pg/ml)** | No DIC | 6.1 | 5.8 | 3.9 | 1.4 | 1.7-12 |
| | Non-Overt DIC | 2.7 | 2.5 | 0.96 | 0.2 | 1.6-4.8 |
| **IL-4 (pg/ml)** | No DIC | 3.6 | 2.9 | 2.8 | 0.99 | 1.5-10 |
| | Non-Overt DIC | 2.3 | 2.2 | 0.68 | 0.14 | 1.2-3.7 |
| **IL-6 (pg/ml)** | No DIC | 5.3 | 4.5 | 3.9 | 1.4 | 0.63-12 |
| | Non-Overt DIC | 104 | 49 | 177 | 38 | 2-764 |
| **IL-8 (pg/ml)** | No DIC | 6.6 | 5 | 3.8 | 1.4 | 3-13 |
| | Non-Overt DIC | 50 | 24 | 66 | 14 | 3.1-278 |
| **IL-10 (pg/ml)** | No DIC | 2.2 | 1.5 | 1.7 | 0.62 | 0.75-5.8 |
| | Non-Overt DIC | 7 | 2.8 | 15 | 3.2 | 0.97-72 |
| **VEGF (pg/ml)** | No DIC | 17 | 15 | 4.2 | 1.5 | 12-25 |
| | Non-Overt DIC | 33 | 20 | 39 | 8.3 | 3.7-184 |
| **IFNγ (pg/ml)** | No DIC | 0.88 | 0.62 | 0.6 | 0.21 | 0.3-1.7 |
| | Non-Overt DIC | 0.57 | 0.42 | 0.44 | 0.093 | 0-2 |
| **TNFα (pg/ml)** | No DIC | 3.5 | 3.5 | 1.6 | 0.56 | 0.84-5.8 |
| | Non-Overt DIC | 5 | 4.4 | 3.3 | 0.7 | 1.5-15 |
| **IL-1α (pg/ml)** | No DIC | 0.49 | 0.39 | 0.3 | 0.11 | 0.22-0.92 |
| | Non-Overt DIC | 1.3 | 0.28 | 4 | 0.85 | 0.1-19 |
| **IL-1β (pg/ml)** | No DIC | 2.8 | 2.6 | 1.6 | 0.57 | 0.6-5 |
| | Non-Overt DIC | 1.9 | 1.5 | 1.6 | 0.34 | 0.6-6.6 |
| **MCP-1 (pg/ml)** | No DIC | 143 | 134 | 85 | 30 | 36-245 |
| | Non-Overt DIC | 285 | 259 | 183 | 39 | 40-802 |
| **EGF (pg/ml)** | No DIC | 6.7 | 5.5 | 4.3 | 1.5 | 2.1-13 |
| | Non-Overt DIC | 8.7 | 3 | 15 | 3.1 | 1.5-56 |

**Table 60. Day 8 Endothelial Biomarker Levels Stratified by DIC Score**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **TFPI (ng/ml)** | No DIC | 62 | 63 | 17 | 6 | 36-90 |
| | Non-Overt DIC | 97 | 73 | 69 | 15 | 32-337 |
| **Protein C (%)** | No DIC | 111 | 121 | 32 | 11 | 65-147 |
| | Non-Overt DIC | 65 | 58 | 28 | 5.9 | 32-136 |
| **Endocan (ng/ml)** | No DIC | 3.2 | 2.7 | 1.6 | 0.56 | 1.5-6.4 |
| | Non-Overt DIC | 11 | 9.3 | 8.2 | 1.8 | 1.5-27 |
| **Ang-2 (pg/ml)** | No DIC | 2,154 | 2,042 | 737 | 261 | 1,072-3,180 |
| | Non-Overt DIC | 10,750 | 8,159 | 12,888 | 2,748 | 448-61,010 |
| **vWF (%)** | No DIC | 246 | 251 | 54 | 19 | 182-309 |
| | Non-Overt DIC | 258 | 239 | 70 | 15 | 154-374 |

**Table 61. Day 8 Platelet Biomarker Levels Stratified by DIC Score**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Platelets (k/µl)** | No DIC | 375 | 327 | 203 | 77 | 98-737 |
| | Non-Overt DIC | 291 | 250 | 153 | 33 | 102-731 |
| **CD40L (pg/ml)** | No DIC | 400 | 347 | 235 | 83 | 91-809 |
| | Non-Overt DIC | 706 | 226 | 1,061 | 226 | 0-3,796 |
| **MP (nM)** | No DIC | 38 | 32 | 23 | 8.3 | 13-78 |
| | Non-Overt DIC | 53 | 35 | 56 | 12 | 11-225 |
| **MP/TF (pg/ml)** | No DIC | 0.7 | 0.56 | 0.35 | 0.13 | 0.48-1.5 |
| | Non-Overt DIC | 1.2 | 0.97 | 0.96 | 0.21 | 0.12-4.6 |

**Table 62. Association of Day 4 Hemostatic Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **D-Dimer (ng/ml)** | Survivors | 5,171 | 4,079 | 4,160 | 613 | 462-16,496 |
| | Non-Survivors | 7,238 | 6,652 | 5,300 | 1,676 | 335-16,325 |
| **F1.2 (pmol/l)** | Survivors | 456 | 339 | 326 | 48 | 62-1,309 |
| | Non-Survivors | 479 | 419 | 288 | 91 | 156-1,107 |
| **PAI-1 (pg/ml)** | Survivors | 57 | 40 | 62 | 9.1 | 0-325 |
| | Non-Survivors | 68 | 47 | 75 | 24 | 18-272 |
| **INR** | Survivors | 1.5 | 1.3 | 0.4 | 0.059 | 1-3.2 |
| | Non-Survivors | 1.5 | 1.3 | 0.49 | 0.16 | 1.1-2.8 |
| **Platelets (k/µl)** | Survivors | 230 | 197 | 141 | 21 | 21-680 |
| | Non-Survivors | 162 | 130 | 102 | 32 | 63-397 |
| **Fibrinogen (mg/dl)** | Survivors | 625 | 646 | 195 | 29 | 153-1,063 |
| | Non-Survivors | 491 | 456 | 218 | 69 | 233-902 |

**Table 63. Association of Day 4 Infection Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Nucleosomes (Units)** | Survivors | 22 | 13 | 27 | 4 | 0-136 |
| | Non-Survivors | 20 | 17 | 20 | 6.4 | 0-59 |
| **HMGB-1 (ng/ml)** | Survivors | 6.5 | 6.1 | 3.9 | 0.59 | 0.44-24 |
| | Non-Survivors | 11 | 8 | 13 | 4 | 0.52-44 |
| **Procalcitonin (pg/ml)** | Survivors | 1,134 | 189 | 3,333 | 491 | 7.6-22,162 |
| | Non-Survivors | 1,624 | 767 | 1,936 | 612 | 53-4,987 |

**Table 64. Association of Day 4 Inflammatory Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **IL-2 (pg/ml)** | Survivors | 5.4 | 2.5 | 9.6 | 1.4 | 0-56 |
| | Non-Survivors | 5.9 | 2.5 | 10 | 3.3 | 1.7-35 |
| **IL-4 (pg/ml)** | Survivors | 3.6 | 2.6 | 3.3 | 0.49 | 0.94-18 |
| | Non-Survivors | 2.3 | 2.1 | 0.86 | 0.27 | 1-4.1 |
| **IL-6 (pg/ml)** | Survivors | 62 | 23 | 123 | 18 | 3.4-764 |
| | Non-Survivors | 170 | 65 | 230 | 73 | 17-626 |
| **IL-8 (pg/ml)** | Survivors | 21 | 14 | 28 | 4.1 | 3.4-126 |
| | Non-Survivors | 62 | 29 | 92 | 29 | 13-317 |
| **IL-10 (pg/ml)** | Survivors | 4 | 2.9 | 3.5 | 0.51 | 0.67-15 |
| | Non-Survivors | 6 | 3.2 | 5.7 | 1.8 | 1.6-17 |
| **VEGF (pg/ml)** | Survivors | 41 | 30 | 37 | 5.5 | 3.2-188 |
| | Non-Survivors | 23 | 19 | 14 | 4.4 | 5.8-44 |
| **IFNγ (pg/ml)** | Survivors | 1.4 | 0.46 | 2.5 | 0.37 | 0-12 |
| | Non-Survivors | 0.33 | 0.3 | 0.28 | 0.09 | 0-0.87 |
| **TNFα (pg/ml)** | Survivors | 5.3 | 4.2 | 4.8 | 0.71 | 1.5-29 |
| | Non-Survivors | 4.2 | 4.2 | 2.3 | 0.73 | 0.74-8.5 |
| **IL-1α (pg/ml)** | Survivors | 1.2 | 0.31 | 3.4 | 0.51 | 0.15-23 |
| | Non-Survivors | 0.41 | 0.25 | 0.31 | 0.098 | 0.22-1.2 |
| **IL-1β (pg/ml)** | Survivors | 3 | 1.6 | 4.3 | 0.63 | 0.65-25 |
| | Non-Survivors | 1.9 | 1.6 | 0.87 | 0.28 | 0.91-3.6 |
| **MCP-1 (pg/ml)** | Survivors | 284 | 222 | 199 | 29 | 65-802 |
| | Non-Survivors | 366 | 271 | 262 | 83 | 61-802 |
| **EGF (pg/ml)** | Survivors | 11 | 6.7 | 11 | 1.6 | 1.3-50 |
| | Non-Survivors | 5.1 | 3.3 | 4.7 | 1.5 | 1.4-17 |

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**Table 65. Association of Day 4 Endothelial Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **TFPI (ng/ml)** | Survivors | 75 | 63 | 39 | 5.8 | 24-192 |
| | Non-Survivors | 152 | 80 | 187 | 59 | 42-671 |
| **Protein C (%)** | Survivors | 58 | 58 | 28 | 4.1 | 9.7-113 |
| | Non-Survivors | 53 | 58 | 23 | 7.2 | 21-86 |
| **Endocan (ng/ml)** | Survivors | 7.5 | 5 | 8.4 | 1.2 | 1.4-44 |
| | Non-Survivors | 17 | 12 | 16 | 5.1 | 2.5-50 |
| **Ang-2 (pg/ml)** | Survivors | 6,706 | 4,443 | 8,338 | 1,243 | 48-53,240 |
| | Non-Survivors | 9,817 | 9,407 | 6,662 | 2,107 | 1,317-24,532 |
| **vWF (%)** | Survivors | 243 | 248 | 73 | 11 | 38-380 |
| | Non-Survivors | 264 | 259 | 93 | 29 | 130-398 |

**Table 66. Association of Day 4 Platelet Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Platelets (k/µl)** | Survivors | 230 | 197 | 141 | 21 | 21-680 |
| | Non-Survivors | 162 | 130 | 102 | 32 | 63-397 |
| **CD40L (pg/ml)** | Survivors | 666 | 381 | 672 | 99 | 0-2,733 |
| | Non-Survivors | 302 | 269 | 230 | 73 | 0-777 |
| **MP (nM)** | Survivors | 60 | 38 | 54 | 8.1 | 2.8-208 |
| | Non-Survivors | 34 | 27 | 20 | 6.2 | 20-86 |
| **MP/TF (pg/ml)** | Survivors | 1.2 | 0.95 | 0.99 | 0.15 | 0-5.1 |
| | Non-Survivors | 1 | 0.81 | 0.67 | 0.21 | 0.28-2.3 |

**Table 67. Association of Day 8 Hemostatic Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **D-Dimer (ng/ml)** | Survivors | 4,837 | 3,468 | 3,467 | 708 | 758-13,167 |
| | Non-Survivors | 10,044 | 10,822 | 5,021 | 2,050 | 1,197-15,283 |
| **F1.2 (pmol/l)** | Survivors | 286 | 240 | 190 | 39 | 76-746 |
| | Non-Survivors | 334 | 329 | 204 | 83 | 132-683 |
| **PAI-1 (pg/ml)** | Survivors | 40 | 32 | 40 | 8.3 | 0-173 |
| | Non-Survivors | 75 | 48 | 72 | 29 | 0-165 |
| **INR** | Survivors | 1.3 | 1.3 | 0.19 | 0.038 | 1.1-2 |
| | Non-Survivors | 1.4 | 1.4 | 0.14 | 0.058 | 1.3-1.7 |
| **Platelets (k/µl)** | Survivors | 314 | 311 | 156 | 33 | 98-737 |
| | Non-Survivors | 306 | 244 | 220 | 90 | 139-731 |
| **Fibrinogen (mg/dl)** | Survivors | 641 | 609 | 195 | 40 | 443-1,269 |
| | Non-Survivors | 611 | 583 | 254 | 104 | 252-947 |

**Table 68. Association of Day 8 Infection Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Nucleosomes (Units)** | Survivors | 16 | 9.2 | 18 | 3.6 | 0-71 |
| | Non-Survivors | 26 | 13 | 31 | 13 | 0-77 |
| **HMGB-1 (ng/ml)** | Survivors | 8.8 | 6.3 | 7.1 | 1.4 | 2.3-29 |
| | Non-Survivors | 6.4 | 5.8 | 2.9 | 1.2 | 3-9.9 |
| **Procalcitonin (pg/ml)** | Survivors | 646 | 126 | 1,776 | 363 | 5.6-8,751 |
| | Non-Survivors | 909 | 654 | 1,103 | 450 | 31-3,052 |

**Table 69. Association of Day 8 Inflammatory Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **IL-2 (pg/ml)** | Survivors | 3.9 | 2.7 | 2.8 | 0.58 | 1.6-12 |
| | Non-Survivors | 2.7 | 2.6 | 0.76 | 0.31 | 1.6-3.7 |
| **IL-4 (pg/ml)** | Survivors | 2.8 | 2.6 | 1.7 | 0.35 | 1.4-10 |
| | Non-Survivors | 2.4 | 2.2 | 0.95 | 0.39 | 1.2-3.7 |
| **IL-6 (pg/ml)** | Survivors | 37 | 30 | 41 | 8.4 | 0.63-185 |
| | Non-Survivors | 243 | 66 | 308 | 126 | 29-764 |
| **IL-8 (pg/ml)** | Survivors | 21 | 16 | 24 | 4.9 | 3-116 |
| | Non-Survivors | 109 | 78 | 102 | 42 | 9.8-278 |
| **IL-10 (pg/ml)** | Survivors | 3.3 | 2.7 | 2 | 0.42 | 0.75-8.5 |
| | Non-Survivors | 15 | 2.5 | 28 | 11 | 1.2-72 |
| **VEGF (pg/ml)** | Survivors | 23 | 15 | 18 | 3.6 | 3.7-83 |
| | Non-Survivors | 53 | 30 | 65 | 27 | 8.3-184 |
| **IFNγ (pg/ml)** | Survivors | 0.71 | 0.42 | 0.53 | 0.11 | 0.24-2 |
| | Non-Survivors | 0.43 | 0.53 | 0.25 | 0.1 | 0-0.65 |
| **TNFα (pg/ml)** | Survivors | 4.1 | 3.9 | 2.2 | 0.44 | 0.84-10 |
| | Non-Survivors | 6.7 | 5.1 | 4.8 | 2 | 1.5-15 |
| **IL-1α (pg/ml)** | Survivors | 1.3 | 0.32 | 3.8 | 0.78 | 0.1-19 |
| | Non-Survivors | 0.36 | 0.26 | 0.21 | 0.084 | 0.2-0.64 |
| **IL-1β (pg/ml)** | Survivors | 2.1 | 1.6 | 1.5 | 0.31 | 0.6-6.2 |
| | Non-Survivors | 2.4 | 1.7 | 2.2 | 0.89 | 0.84-6.6 |
| **MCP-1 (pg/ml)** | Survivors | 220 | 231 | 120 | 24 | 36-461 |
| | Non-Survivors | 358 | 281 | 300 | 122 | 40-802 |
| **EGF (pg/ml)** | Survivors | 7.4 | 3.5 | 11 | 2.3 | 1.5-56 |
| | Non-Survivors | 11 | 2.7 | 18 | 7.4 | 1.6-47 |

**Table 70. Association of Day 8 Endothelial Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **TFPI (ng/ml)** | Survivors | 89 | 65 | 67 | 14 | 32-337 |
| | Non-Survivors | 83 | 76 | 32 | 13 | 48-132 |
| **Protein C (%)** | Survivors | 84 | 76 | 36 | 7.3 | 32-147 |
| | Non-Survivors | 50 | 46 | 17 | 7 | 34-82 |
| **Endocan (ng/ml)** | Survivors | 8 | 5.5 | 7.3 | 1.5 | 1.5-25 |
| | Non-Survivors | 14 | 15 | 9.4 | 3.8 | 2.8-27 |
| **Ang-2 (pg/ml)** | Survivors | 7,640 | 3,180 | 12,044 | 2,458 | 934-61,010 |
| | Non-Survivors | 11,730 | 10,977 | 10,087 | 4,118 | 448-26,908 |
| **vWF (%)** | Survivors | 8.1 | 6.3 | 6.3 | 1.5 | 3.1-29 |
| | Non-Survivors | 6.4 | 5.8 | 2.9 | 1.2 | 3-9.9 |

**Table 71. Association of Day 8 Platelet Biomarker Levels with Survival**

| Biomarker | Group | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **Platelets (k/µl)** | Survivors | 314 | 311 | 156 | 33 | 98-737 |
| | Non-Survivors | 306 | 244 | 220 | 90 | 139-731 |
| **CD40L (pg/ml)** | Survivors | 523 | 260 | 734 | 150 | 0-3,468 |
| | Non-Survivors | 1,028 | 412 | 1,481 | 605 | 10-3,796 |
| **MP (nM)** | Survivors | 48 | 35 | 49 | 10 | 11-225 |
| | Non-Survivors | 54 | 34 | 58 | 23 | 14-164 |
| **MP/TF (pg/ml)** | Survivors | 1.1 | 0.76 | 0.96 | 0.2 | 0.12-4.6 |
| | Non-Survivors | 1.1 | 0.97 | 0.42 | 0.17 | 0.76-1.7 |

**Table 72. Association of Baseline Hemostatic Biomarker Levels with Platelet Count**

| Biomarker | Platelets (K/µl) | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **D-Dimer (ng/ml)** | < 100 | 8,945 | 6,438 | 8,497 | 1,854 | 150-36,567 |
| | 100-149 | 6,908 | 5,818 | 3,838 | 858 | 305-15,918 |
| | ≥ 150 | 5,285 | 3,793 | 5,093 | 652 | 0-27,871 |
| **F1.2 (pmol/l)** | < 100 | 605 | 257 | 1,110 | 242 | 63-5,285 |
| | 100-149 | 223 | 221 | 117 | 26 | 78-512 |
| | ≥ 150 | 325 | 223 | 283 | 36 | 39-1,699 |
| **PAI-1 (pg/ml)** | < 100 | 78 | 30 | 100 | 22 | 0-357 |
| | 100-149 | 41 | 25 | 46 | 10 | 1.9-191 |
| | ≥ 150 | 67 | 44 | 61 | 7.8 | 0-252 |
| **INR** | < 99 | 1.6 | 1.5 | 0.32 | 0.07 | 1.2-2.5 |
| | 140-149 | 2 | 1.6 | 0.99 | 0.22 | 1.2-5.2 |
| | ≥ 150 | 1.4 | 1.4 | 0.3 | 0.039 | 1-3 |
| **Fibrinogen (mg/dl)** | < 100 | 505 | 487 | 222 | 48 | 133-1,152 |
| | 100-149 | 656 | 619 | 277 | 64 | 249-1,404 |
| | ≥ 150 | 666 | 650 | 228 | 29 | 298-1,449 |

**Table 73. Association of Baseline Infection Biomarker Levels with Platelet Count**

| Biomarker | Platelets (K/µl) | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| Nucleosomes (Units) | < 100 | 21 | 9.4 | 29 | 6.3 | 0-119 |
| | 100-149 | 15 | 7 | 21 | 4.7 | 0-88 |
| | ≥ 150 | 9.8 | 7.8 | 12 | 1.6 | 0-86 |
| HMGB-1 (ng/ml) | < 100 | 13 | 6.4 | 15 | 3.3 | 3.2-66 |
| | 100-149 | 7.7 | 6.3 | 5.9 | 1.3 | 0.18-21 |
| | ≥ 150 | 8.3 | 4.9 | 14 | 1.8 | 0.65-87 |
| Procalcitonin (pg/ml) | < 100 | 3,217 | 1,995 | 4,296 | 937 | 189-18,965 |
| | 100-149 | 1,718 | 627 | 2,312 | 517 | 70-8,123 |
| | ≥ 150 | 102 | 86 | 71 | 9.1 | 4.8-423 |

**Table 74. Association of Inflammatory Biomarkers with Platelet Count**

| Biomarker | Platelets (K/µl) | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **IL-2 (pg/ml)** | < 100 | 4.7 | 2.7 | 5.3 | 1.1 | 0-22 |
| | 100-149 | 2.6 | 2.3 | 1.5 | 0.33 | 0-7.6 |
| | ≥ 150 | 3.1 | 2.4 | 2 | 0.26 | 0-11 |
| **IL-4 (pg/ml)** | < 100 | 3.1 | 2.5 | 2 | 0.43 | 1.1-9.7 |
| | 100-149 | 2.9 | 2.8 | 0.86 | 0.19 | 1.5-4.7 |
| | ≥ 150 | 2.6 | 2.5 | 1.2 | 0.15 | 0-9.7 |
| **IL-6 (pg/ml)** | < 100 | 131 | 52 | 218 | 48 | 1.3-764 |
| | 100-149 | 204 | 51 | 276 | 62 | 0.27-764 |
| | ≥ 150 | 154 | 42 | 246 | 32 | 0-857 |
| **IL-8 (pg/ml)** | < 100 | 63 | 16 | 158 | 35 | 4.1-708 |
| | 100-149 | 20 | 15 | 23 | 5.2 | 0.51-110 |
| | ≥ 150 | 29 | 7.5 | 51 | 6.5 | 0-238 |
| **IL-10 (pg/ml)** | < 100 | 52 | 4.3 | 170 | 37 | 0.92-758 |
| | 100-149 | 4.4 | 2.7 | 5.7 | 1.3 | 0.45-27 |
| | ≥ 150 | 5.7 | 2.6 | 11 | 1.4 | 0-65 |
| **VEGF (pg/ml)** | < 100 | 16 | 12 | 12 | 2.5 | 4.3-57 |
| | 100-149 | 20 | 18 | 14 | 3 | 3.2-57 |
| | ≥ 150 | 35 | 25 | 45 | 5.8 | 0-339 |
| **IFNγ (pg/ml)** | < 100 | 25 | 0.71 | 110 | 24 | 0.24-507 |
| | 100-149 | 2.1 | 0.38 | 6.7 | 1.5 | 0-30 |
| | ≥ 150 | 2.8 | 0.37 | 7.5 | 0.96 | 0-38 |
| **TNFα (pg/ml)** | < 100 | 7.1 | 7.3 | 3.4 | 0.73 | 1.5-18 |
| | 100-149 | 5 | 4.3 | 3.4 | 0.75 | 0.74-15 |
| | ≥ 150 | 5.5 | 3.2 | 8 | 1 | 0-57 |
| **IL-1α (pg/ml)** | < 100 | 0.5 | 0.36 | 0.52 | 0.11 | 0.15-2.5 |
| | 100-149 | 2.2 | 0.32 | 8.2 | 1.8 | 0.18-37 |
| | ≥ 150 | 0.37 | 0.27 | 0.3 | 0.039 | 0-1.6 |
| **IL-1β (pg/ml)** | < 100 | 2.6 | 1.6 | 2.8 | 0.61 | 0.65-11 |
| | 100-149 | 2.1 | 1.5 | 1.8 | 0.41 | 0.78-7.8 |
| | ≥ 150 | 2.2 | 1.3 | 2.1 | 0.27 | 0-9 |
| **MCP-1 (pg/ml)** | < 100 | 356 | 315 | 223 | 49 | 104-802 |
| | 100-149 | 328 | 291 | 248 | 56 | 1.3-802 |
| | ≥ 150 | 326 | 207 | 269 | 34 | 0-802 |
| **EGF (pg/ml)** | < 100 | 3.2 | 2 | 2.4 | 0.53 | 1.3-12 |
| | 100-149 | 4.4 | 4 | 2.6 | 0.59 | 1.6-11 |
| | ≥ 150 | 8.3 | 6.6 | 7.2 | 0.92 | 0-46 |
| **IL-6:IL-10 Ratio** | < 100 | 19 | 8.4 | 27 | 5.9 | 0.11-100 |
| | 100-149 | 67 | 18 | 94 | 21 | 0.6-344 |
| | ≥ 150 | 40 | 15 | 69 | 8.9 | 0-460 |

**Table 75. Association of Baseline Endothelial Biomarker Levels with Platelet Count**

| Biomarker | Platelets (K/µl) | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| **TFPI (ng/ml)** | < 100 | 106 | 92 | 68 | 15 | 11-346 |
| | 100-149 | 101 | 85 | 79 | 18 | 39-407 |
| | ≥ 150 | 102 | 86 | 71 | 9.1 | 4.8-423 |
| **Protein C (%)** | < 100 | 58 | 51 | 55 | 12 | 2.7-277 |
| | 100-149 | 54 | 44 | 64 | 14 | 0-309 |
| | ≥ 150 | 64 | 59 | 41 | 5.2 | 2.5-309 |
| **Endocan (ng/ml)** | < 100 | 12 | 7.5 | 10 | 2.2 | 1.9-38 |
| | 100-149 | 13 | 6.8 | 13 | 3 | 2.5-60 |
| | ≥ 150 | 8.5 | 5.3 | 7.3 | 0.94 | 1.4-34 |
| **Ang-2 (pg/ml)** | < 100 | 26,696 | 18,060 | 30,975 | 6,759 | 1,816-13,6317 |
| | 100-149 | 19,897 | 12,530 | 19,229 | 4,300 | 650-61,808 |
| | ≥ 150 | 9,538 | 6,892 | 9,692 | 1,251 | 961-53,612 |
| **vWF (%)** | < 100 | 256 | 260 | 74 | 16 | 128-379 |
| | 100-149 | 274 | 297 | 73 | 16 | 111-349 |
| | ≥ 150 | 232 | 244 | 67 | 8.6 | 107-370 |

**Table 76. Association of Baseline Platelet Biomarker Levels with Platelet Count**

| Biomarker | Platelets (K/µl) | Mean | Median | SD | SEM | Range |
|---|---|---|---|---|---|---|
| CD40L (pg/ml) | < 100 | 150 | 97 | 152 | 33 | 0-659 |
| | 100-149 | 274 | 249 | 164 | 37 | 57-643 |
| | ≥ 150 | 496 | 275 | 730 | 94 | 0-4756 |
| MP (nM) | < 100 | 20 | 17 | 20 | 4.3 | 2.8-93 |
| | 100-149 | 29 | 24 | 18 | 4.3 | 2.4-60 |
| | ≥ 150 | 47 | 42 | 31 | 4 | 5.6-159 |
| MP-TF (pg/ml) | < 100 | 1.2 | 0.93 | 0.87 | 0.19 | 0.41-3.9 |
| | 100-149 | 1.3 | 1.1 | 1 | 0.23 | 0.4-5.2 |
| | ≥ 150 | 0.97 | 0.82 | 0.93 | 0.12 | 0.06-7.4 |
| PF4 (ng/ml) | < 100 | 51 | 47 | 19 | 4.3 | 15-107 |
| | 100-149 | 81 | 67 | 36 | 8.2 | 31-142 |
| | ≥ 150 | 84 | 66 | 35 | 4.6 | 38-169 |

# APPENDIX C

# CLINICAL SCORING SYSTEMS (TABLES 77-82)

**Table 77. SCCM Definitions for Sepsis and Associated Conditions**

| Condition | Definition |
|---|---|
| **Bacteremia** | The presence of viable bacteria in the blood |
| **Systemic inflammatory response syndrome (SIRS)** | Systemic inflammatory response to a variety of severe clinical insults, manifested by two or more of the following conditions:<br>• Temperature >38ºC or <36ºC<br>• Heart rate >90 BPM<br>• Respiratory rate >20 breaths per minute or $PaCO_2$ <32 mmHg<br>• White blood cell count >12,000/mm$^3$, <4,000/mm$^3$, or >10% immature forms |
| **Sepsis** | The systemic response to infection, manifested by two or more of the following as a result of the infection:<br>• Temperature >38ºC or < 36ºC<br>• Heart rate >90 BPM<br>• Respiratory rate >30 breaths per minute or $PaCO_2$ <32 mmHg<br>• White blood cell count >12,000/mm$^3$ <4,000/mm$^3$, or >10% immature (band) forms |
| **Severe sepsis** | Sepsis associated with organ dysfunction, hypoperfusion, or hypotension, including but not limited to lactic acidosis, oliguria, or acute alteration in mental status |
| **Septic shock** | Sepsis-induced hypotension despite adequate fluid resuscitation along with perfusion abnormalities including but not limited to lactic acidosis, oliguria, or acute alteration in mental status. Patients receiving inotropic or vasopressive agents may not be hypotensive at the time perfusion abnormalities are measured |
| **Sepsis-induced hypotension** | Systolic blood pressure <90 mmHg or a reduction of ≥40 mmHg from baseline in the absence of other causes of hypotension |
| **Multiple organ dysfunction syndrome (MODS)** | Presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention |

Table adapted from (Bone et al., 1992; Levy et al., 2003)

**Table 78. 2016 SEPSIS-3 Diagnostic Scheme for Sepsis and Septic Shock**

| Condition | Requirements |
|---|---|
| **Sepsis** | 1. qSOFA ≥2<br>a. Respiratory rate ≥ 22/minute<br>b. Altered mentation<br>c. Systolic blood pressure ≤100 mmHg<br>2. SOFA or change in SOFA≥ 2 |
| **Septic Shock** | With a diagnosis of sepsis and despite adequate fluid resuscitation:<br>1. Vasopressors required to maintain MAP ≥ 65 mmHg<br>2. Serum lactate level > 2 mmol/L |

Table adapted from (Singer et al., 2016)

**Table 79. Sequential Organ Failure Assessment (SOFA) Score**

| SOFA Score | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| ***Respiration*** **$PaO_2/FiO_2$, mmHg** | <400 | <300 | <200 (with respiratory support) | <100 (with respiratory support |
| ***Coagulation*** **Platelets, $10^3/mm^3$** | <150 | <100 | <50 | <20 |
| ***Liver*** **Bilirubin, mg/dl (µmol/l)** | 1.2-1.9 (20-32) | 2.0-5.9 (33-101) | 6.0-11.9 (102-204) | >12.0 (>204) |
| ***Cardiovascular*** **Hypotension (Doses given in µg/kg*min)** | MAP < 70 mmHg | Dopamine ≤ 5 Or dobutamine, any dose | Dopamine > 5 Or epinephrine ≤ 0.1 Or norepinephrine ≤ 0.1 | Dopamine > 15 Or epinephrine > 0.1 Or norepinephrine > 0.1 |
| ***Central nervous system*** **Glasgow Coma Score** | 13-14 | 10-12 | 6-9 | <6 |
| ***Renal*** **Creatinine, mg/dl (µmol/l) or urine output** | 1.2-1.9 (110-170) | 2.0-3.4 (171-299) | 3.5-4.9 (300-440) Or <500 ml/day | >5 (>440) Or <200 ml/day |

Table adapted from (Vincent et al., 1996)

**Table 80. International Society of Thrombosis and Hemostasis (ISTH) Scoring System for DIC**

| Variable | Value | Points |
|---|---|---|
| **Platelets (K/µL)** | >100 | 0 |
| | 50-100 | 1 |
| | <50 | 2 |
| **INR** | <1.3 | 0 |
| | 1.3-1.7 | 1 |
| | >1.7 | 2 |
| **D-Dimer (ng/mL)** | <400 | 0 |
| | 400-4000 | 2 |
| | >4000 | 3 |
| **Fibrinogen (mg/dL)** | >100 | 0 |
| | <100 | 1 |

Table adapted from (Taylor et. al, 2001). A score of 5 or more is indicative of overt DIC.

**Table 81. Japanese Association for Acute Medicine (JAAM) Scoring System for Overt DIC**

| Variable | Value | Points |
|---|---|---|
| **Systemic Inflammatory Response Syndrome (SIRS) Criteria** | ≥ 3 | 1 |
| | 0-2 | 0 |
| **Platelet Count ($10^9$/L)** | < 80 or 50% decrease in 24 hours | 3 |
| | 81-120 or 30% decrease in 24 hours | 1 |
| | ≥ 120 | 0 |
| **Prothrombin Time (Patient Value/Normal Value)** | ≥ 1.2 | 1 |
| | < 1.2 | 0 |
| **Fibrinogen/fibrin degradation products (mg/L)** | ≥ 25 | 3 |
| | 10-24 | 1 |
| | < 10 | 0 |

Table adapted from (Gando et al., 2006). A score of 4 or more is indicative of DIC. For the purposes of this algorithm, SIRS criteria include (1) temperature > 38ºC or < 36ºC, (2) heart rate > 90 beats per minute, (3) Respiratory rate > 20 breaths per minute or $PaCo_2$ < 4.3 kPa (4) white blood cell count > 12,000 cells/$mm^3$, <4,000 cells/$mm^3$, or 10% bands

**Table 82. Acute Physiology and Chronic Health Evaluation (APACHE) II Scoring System**

| Physiological Variable | Points | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **+4** | **+3** | **+2** | **+1** | **0** | **+1** | **+2** | **+3** | **+4** |
| **Temperature, (°C)** | ≥ 41 | 39-40.9 | -- | 38.5-38.9 | 36-38.4 | 34-35.9 | 32-33.9 | 30-31.9 | ≤ 29.9 |
| **Mean Arterial Pressure (mmHg)** | ≥ 160 | 130-159 | 110-129 | -- | 70-109 | -- | 50-69 | -- | ≤ 49 |
| **Heart Rate (BPM)** | ≥ 180 | 140-179 | 110-139 | -- | 70-109 | -- | 55-69 | 40-54 | ≤ 39 |
| **Respiratory Rate** | ≥ 50 | 35-49 | -- | 25-34 | 12-24 | 10-11 | 6-9 | -- | ≤ 5 |
| **Oxygenation** ($FiO_2 \geq 0.5$; use A-a $DO_2$) | ≥ 500 | 350-499 | 200-349 | -- | < 200 | -- | -- | -- | -- |
| **Oxygenation** ($FiO_2 < 0.5$; use $PaO_2$, mm Hg) | -- | -- | -- | -- | > 70 | 61-70 | -- | 55-60 | < 55 |
| **Arterial pH** | ≥ 7.7 | 7.6-7.69 | -- | 7.5-7.59 | 7.33-7.49 | -- | 7.25-7.32 | 7.15-7.24 | < 7.15 |
| **Serum Na (mmol/L)** | ≥ 180 | 160-179 | 155-159 | 150-154 | 130-149 | -- | 120-129 | 111-119 | ≤ 110 |
| **Serum K (mmol/L)** | ≥ 7 | 6-6.9 | -- | 5.5-5.9 | 3.5-5.4 | 3-3.4 | 2.5-2.9 | -- | < 2.5 |
| **Serum creatinine (mg/dL)** Double score for acute renal failure | ≥ 3.5 | 2-3.4 | 1.5-1.9 | -- | 0.6-1.4 | -- | < 0.6 | -- | -- |
| **Hematocrit (%)** | ≥ 60 | -- | 50-59.9 | 46-49.9 | 30-45.9 | -- | 20-29.9 | -- | < 20 |

| Physiological Variable | Points | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **+4** | **+3** | **+2** | **+1** | **0** | **+1** | **+2** | **+3** | **+4** |
| **White Blood Count (1000s)** | ≥ 50 | -- | 20-39.9 | 15-19.9 | 3-14.9 | -- | 1-2.9 | -- | < 1 |
| **Glasgow Coma Score** | Score = 15 minus Glasgow Coma Score | | | | | | | | |
| **Age Points** | 0 points for age < 44 years; 2 points 45-54 years; 3 points 55-64 years; 6 points ≥ 75 years | | | | | | | | |
| **Chronic Health Status Points** | If immunocompromised or history of severe organ insufficiency: 2 points for elective postoperative patients; 5 points for non-operative patients or emergency postoperative patients | | | | | | | | |

Table adapted from (Knaus et. al. 1985)

# APPENDIX D

# MATLAB CODE FOR STEPWISE LINEAR REGRESSION MODELING

```
% Clear all clears all variables in workspace.
% Close all closes all open figures
clear all; close all;

% Set this up the first time you use the program.  Afterwards, you
% shouldn't have to change these lines again.
cd('C:\users\scott\desktop\');      % Root directory for patient data

try
  [filename, pathname] = uigetfile('*.xlsx;*.xls','Select Patient Data
File','multiselect','off');
  if isnumeric(filename)
    ME1 = MException('Filename:NoFileSelected','No excel file
selected');
    throw(ME1)
  end

% Imports Excel file containing patient data in following format:
% Top row - header with column names(biomarkers / clinical variables)
% First column - Individual sample names
% Second column - Outcome variable (i.e. mortality)
% Each column represents a biomarker, each row represents a patient

  fullpath = fullfile(pathname,filename);
  T = readtable(fullpath,'readrownames',true);
  [nPatients,nBiomarkers] = size(T);

% Runs the stepwise linear modeling function (output variable mdl)
% Can specify starting model as 'linear' or 'constant' (2nd term)
% Can also specify other constraints: categorical variables, variables
% to exclude, changes to PEnter/PRemove values, etc.

  mdl = stepwiselm(T,'linear','responsevar',1,'upper','linear');

% Records the names of the markers included in the model
% (IncludedMarkers), the coefficient table produced by the stepwiselm
% function (ModelSummary), the estimated coefficients to be used in
% calculation (CoefficientsEstimate), and the values of the model for
% each of the patients (PtValues)

  IncludedMarkers = mdl.CoefficientNames;
  ModelSummary = mdl.Coefficients;
  CoefficientsEstimate = mdl.Coefficients.Estimate;
  PtValues = mdl.Fitted;

% Exports model data and results to Excel file with user-determined
% name. Includes model results with included variables coefficients
% (Sheet 1)and results table including patient ID, mortality, and
% calculated value of the model (Sheet 2)

  PtID = T.Properties.RowNames;          % Patient ID Identifiers
  PtMortality = T.Mortality;             % Mortality value as 1 or 0
  ResultsTable = table(PtID,PtMortality,PtValues);
  defaultfilename = 'output.xlsx';
```

```
  filename = uiputfile(defaultfilename, 'Specify Output File Name');
  writetable(ModelSummary,filename,'Sheet',1,'WriteRowNames',true);
  writetable(ResultsTable,filename,'Sheet',2);

catch ME1
  if     strcmp(ME1.identifier,'MATLAB:dlmread:InvalidInputType')
      error('MATLAB:dlmread:InvalidInputType','Must select at least
one file');
  elseif strcmp(ME1.identifier,'Filename:NoFileSelected')
      error('Filename:NoFileSelected','Must select at least one
file');
  else
  disp('The program encountered an error')
  disp(ME1.identifier);
  disp(ME1.message);
  disp(' ');
  disp([ME1.stack(1).name, ', line: ', num2str(ME1.stack(1).line)]);
  disp('Type ''dbquit'' or ''return'' to exit debug mode.');
  keyboard;
  error(ME1);
  end
end
```

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# VITA

The author, Amanda Temple Walborn, was born on June 25, 1991 in Glen Ellyn, Illinois, and is the oldest daughter of Mr. Hugh Walborn and Mrs. Melinda Post.

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