Angiopoietin-1 and -2 in Infectious Diseases Associated with Endothelial Cell Dysfunction

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Master of Science
Institute of Medical Science
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2012

Abstract

Normal endothelial cell function is controlled in part by a tightly regulated balance between angiopoietin-1 and -2 (Ang-1 and Ang-2). Angiopoietin dysregulation (decreased Ang-1 and increased Ang-2) leads to an activated endothelium that is contractile, adhesive, and prothrombotic. Since an activated endothelial phenotype is seen in invasive group A streptococcal infection, E. coli O157:H7-induced hemolytic-uremic syndrome (HUS), and sepsis, we hypothesized that angiopoietin dysregulation might also be present in these syndromes, and to that end, measured angiopoietin levels in several well-characterized patient cohorts. Decreased Ang-1 and/or increased Ang-2 were found in all three syndromes, and were predictive of clinical outcome in HUS and sepsis. The prognostic utility of Ang-2 in sepsis was further enhanced by combination with biomarkers of inflammation. Angiopoietin dysregulation may therefore represent a shared final common pathway to endothelial activation as well as a clinically useful prognostic biomarker in streptococcal toxic shock, HUS, and sepsis.
Acknowledgments

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<tr>
<td>ABC</td>
<td>Abacavir</td>
</tr>
<tr>
<td>ACCP</td>
<td>American College of Chest Physicians</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute Coronary Syndrome</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute Lung Injury</td>
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<tr>
<td>AMI</td>
<td>Acute Myocardial Infarction</td>
</tr>
<tr>
<td>Ang-1</td>
<td>Angiopoietin-1</td>
</tr>
<tr>
<td>Ang-2</td>
<td>Angiopoietin-2</td>
</tr>
<tr>
<td>Ang-3</td>
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</tr>
<tr>
<td>Ang-4</td>
<td>Angiopoietin-4</td>
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<tr>
<td>APACHE</td>
<td>Acute Physiology and Chronic Health Evaluation</td>
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<tr>
<td>ARDS</td>
<td>Acute Respiratory Distress Syndrome</td>
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<td>ART</td>
<td>Antiretroviral Therapy</td>
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<tr>
<td>AUC</td>
<td>Area under the Curve</td>
</tr>
<tr>
<td>CAP</td>
<td>Community-acquired Pneumonia</td>
</tr>
<tr>
<td>CaRT</td>
<td>Classification and Regression Tree</td>
</tr>
<tr>
<td>CHF</td>
<td>Congestive Heart Failure</td>
</tr>
<tr>
<td>Chi3L1</td>
<td>Chitinase-3-like protein-1</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CPAP</td>
<td>Continuous Positive Airway Pressure</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive Protein</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated Intravascular Coagulation</td>
</tr>
<tr>
<td>Dll4</td>
<td>Delta-like ligand 4</td>
</tr>
<tr>
<td>DMARDs</td>
<td>Disease-modifying Anti-rheumatic Drugs</td>
</tr>
<tr>
<td>ED</td>
<td>Emergency Department</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGR-1</td>
<td>Early Growth Response-1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte Sedimentation Rate</td>
</tr>
<tr>
<td>FDP</td>
<td>Fibrinogen Degradation Product</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast Growth Factor-2</td>
</tr>
<tr>
<td>GAS</td>
<td>Group A Streptococcus (<em>Streptococcus pyogenes</em>)</td>
</tr>
<tr>
<td>Gb3</td>
<td>Globotriaosylceramide</td>
</tr>
<tr>
<td>HAP</td>
<td>Hospital-acquired Pneumonia</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Hemoglobin A1c</td>
</tr>
<tr>
<td>HFO</td>
<td>High Frequency Oscillation</td>
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</tbody>
</table>
HIF-2α  Hypoxia-inducible Factor-2α
HIV  Human Immunodeficiency Virus
HMG-CoA  3-hydroxy-3-methyl-glutaryl-CoA (reductase inhibitor)
HUS  Hemolytic-uremic Syndrome
ICAM-1  Intercellular Adhesion Molecule-1
ICH  Intracranial Hemorrhage
ICU  Intensive Care Unit
IGRA  Interferon-γ Release Assay
IL  Interleukin
IP-10  Interferon-γ-inducible Protein - 10 kDa
IQR  Interquartile Range
LPS  Lipopolysaccharide
MAPK  Mitogen-Activated Protein Kinase
MCP-1  Monocyte Chemoattractant Protein-1
MEDS  Mortality in Emergency Department Sepsis (score)
MHC  Major Histocompatibility Complex
MMP  Matrix Metalloproteinase
mRNA  messenger Ribonucleic Acid
NF-κB  Nuclear Factor-κB
NIHSS  National Institutes of Health Stroke Scale
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<tr>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NS</td>
<td>Non-significant</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>OSA</td>
<td>Obstructive Sleep Apnea</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>Partial pressure of Carbon Dioxide (arterial)</td>
</tr>
<tr>
<td>PAH</td>
<td>Pulmonary Arterial Hypertension</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor-1</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial pressure of Oxygen (arterial)</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PCT</td>
<td>Procalcitonin</td>
</tr>
<tr>
<td>PF4</td>
<td>Platelet Factor 4</td>
</tr>
<tr>
<td>PI3k</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>POCT</td>
<td>Point-of-care Test</td>
</tr>
<tr>
<td>PROWESS</td>
<td>recombinant human activated Protein C Worldwide Evaluation in Severe Sepsis</td>
</tr>
<tr>
<td>PSI</td>
<td>Pneumonia Severity Index</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver Operating Characteristic (curve)</td>
</tr>
<tr>
<td>RR</td>
<td>Relative Risk</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SAPS</td>
<td>Simplified Acute Physiology Score</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>SCCM</td>
<td>Society of Critical Care Medicine</td>
</tr>
<tr>
<td>sFlk-1</td>
<td>soluble Fms-like tyrosine kinase receptor-1 (VEGFR2 - Vascular Endothelial Growth Factor Receptor 2)</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>soluble Fms-like tyrosine kinase receptor-1 (VEGFR1 - Vascular Endothelial Growth Factor Receptor 1)</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic Inflammatory Response Syndrome</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SN</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>SOFA</td>
<td>Sequential Organ Failure Assessment</td>
</tr>
<tr>
<td>SP</td>
<td>Specificity</td>
</tr>
<tr>
<td>STEC</td>
<td>Shiga Toxin-producing <em>Escherichia coli</em> (<em>E. coli</em>)</td>
</tr>
<tr>
<td>sTie-2</td>
<td>soluble Tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-2</td>
</tr>
<tr>
<td>sTREM-1</td>
<td>soluble Triggering Receptor Expressed on Myeloid cells-1</td>
</tr>
<tr>
<td>STSS</td>
<td>Streptococcal Toxic Shock Syndrome</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell, subset 1</td>
</tr>
<tr>
<td>Tie-1</td>
<td>Tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-1</td>
</tr>
</tbody>
</table>
Tie-2 Tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-2

TLR4 Toll-like Receptor 4

TNF Tumour Necrosis Factor

tPA tissue Plasminogen Activator

UC Ulcerative Colitis

VAP Ventilator-associated Pneumonia

VCAM-1 Vascular Cell Adhesion Molecule-1

VE-cadherin Vascular Endothelial-cadherin

VEGF Vascular Endothelial Growth Factor

VILI Ventilator-induced Lung Injury

vWF von Willebrand Factor

WPB Weibel-Palade Body
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Chapter 1
Literature Review

1.1 The Vascular Endothelium

The vascular endothelium, once conceptualized as little more than the inert and passive lining of blood vessels, is now known to be a complex organ responsible for interacting with, and responding to, its environment. As reviewed by Aird, bloodborne mediators (including cytokines, pro-inflammatory molecules, and growth factors), surrounding and supporting cells, mechanical shear stress, and physico-chemical factors (including temperature and pH) can all act on endothelial cells to influence their function. This influence is revealed in the endothelial cell phenotype, typically dichotomized as either activated or resting/quiescent, although intermediary states are also possible. Quiescent endothelial cells are anticoagulant, anti-adhesive, and vasodilatory, while activated endothelial cells are pro-coagulant, adhesive, and contractile. These properties are reflected in cell surface marker expression, secreted molecules, and the integrity of the endothelial cell barrier.

As reviewed by Levi and van der Poll, as well as Schouten et al, resting endothelial cells express thrombomodulin and the endothelial protein C receptor on their surface, both of which facilitate the generation of the crucial anticoagulant molecule, activated protein C. Downregulation of thrombomodulin expression, as occurs in activated endothelial cells, leads to reduced protein C generation and subsequently, increased thrombin generation. Likewise, tissue factor pathway inhibitor, tissue-type plasminogen activator, heparan sulfates, prostacyclin, and antithrombin are either expressed or released by the anticoagulant, resting endothelium, while tissue factor, plasminogen-activator inhibitor (PAI)-1 and von Willebrand Factor (vWF) are upregulated in the procoagulant, activated endothelium. Differential cell surface molecule expression between quiescent and activated endothelial cells influences not only the relative balance between pro- and anti-coagulant activity, but also the degree of adhesion of circulating hematologic cells. E-selectin is expressed on activated endothelial cells, where, in combination with P-selectin, it facilitates rolling of leukocytes along the endothelial layer as a prelude to leukocyte adhesion (facilitated by the upregulation of ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) to activated endothelial cells and subsequent transmigration across the endothelial barrier to a site of injury or inflammation. It should be noted that although
this is the classical paradigm of endothelial activation and leukocyte response, there is some organ-specific variability as described in a second review by Aird.\textsuperscript{4}

In addition to cell surface marker expression, activation of the endothelium also influences its secretory function. As reviewed by Lowenstein \textit{et al}, endothelial cells contain Weibel-Palade bodies (WPBs), intracellular structures that store a variety of molecules for rapid release or externalization upon exposure of the cell to inflammatory or noxious stimuli, including mechanical factors (hypoxia, trauma, or shear stress), lipids (sphingosine-1-phosphate, ceramide, and oxidized low-density lipoprotein), inflammatory mediators (thrombin, fibrin, terminal complement components, and leukotrienes), and angiogenic factors (VEGF).\textsuperscript{5} Nitric oxide (NO) impairs or inhibits WPB release.\textsuperscript{6} WPB exocytosis occurs within minutes, in contrast to the transcriptional response which requires hours, and liberates the following: vWF, P-selectin, tissue plasminogen activator, Interleukin (IL)-8, endothelin-1, and angiopoietin-2 (Ang-2), among others. Consistent with the opposing actions of certain of these molecules, differential regulation of WPB contents and exocytosis is known to exist.\textsuperscript{7} Nonetheless, the majority of the molecules contained in WPBs are prothrombotic, inflammatory, or vasoconstrictive, characteristics associated with an activated endothelium.

Finally, endothelial cell activation may manifest as abnormal barrier function. The resting endothelium relies on stable endothelial cell morphology and intact inter-endothelial cell adherens junctions (comprised largely of the protein vascular endothelial (VE)-cadherin) and tight junctions (composed of the occludin, claudin, and junctional adhesion molecule proteins) to limit vascular leak.\textsuperscript{8} Junctional protein stability is maintained in part by close association with actin bundles. Under the various conditions which activate the endothelium (described in the preceding paragraphs), junctional proteins may be downregulated, mis-located, or phosphorylated, all leading to loss of junctional integrity. Furthermore, many vaso-active agents activate the Rho GTPases (RhoA or Rac1), which in turn activate Rho kinase, leading to myosin light chain phosphorylation, stress fibre (as opposed to the usual actin fibre) formation, and increased cell contractility. Ultimately, this sequence of events produces inter-endothelial cell gaps, and consequently, vascular leak.\textsuperscript{9}

Under normal circumstances, all of the above responses are appropriately adaptive in the setting of tissue inflammation or injury. However, these responses can be detrimental when they are
exaggerated or excessive, as is the case in certain infectious disease syndromes associated with prominent endothelial cell activation.

1.1.1 Streptococcal Toxic Shock Syndrome

According to the Working Group on Severe Streptococcal Infections, Group A Streptococcal (GAS; *Streptococcus pyogenes*) toxic shock (STSS) is defined by the isolation of Group A streptococcus from a normally sterile site and hypotension (systolic blood pressure ≤ 90 mmHg), as well as at least 2 of the following 6 signs of major organ dysfunction: acute kidney injury, coagulopathy, necrotizing fasciitis, erythematous rash that may desquamate, elevated liver enzymes, or acute respiratory distress syndrome (ARDS). STSS results largely from the action of a bacterial-derived exotoxin that functions as a superantigen to activate a polyclonal subset of T cells far in excess of that recruited during a normal immune response. Massive pro-inflammatory cytokine release follows. Ultimately, endothelial activation leads to profound hypotension and vascular leak, however, the mechanism by which this occurs has not yet been fully explored.

Antibiotics are administered in STSS but have little effect on pre-formed toxin. Therefore, patients require aggressive supportive care, commonly in the intensive care unit (ICU). In addition, mortality from STSS remains high, reported at 43% in the most recent epidemiologic study. As a result, detailed exploration of all potential mediators of endothelial activation in STSS will be crucial to the discovery of the new therapeutic targets necessary to improve patient outcome.

1.1.2 Hemolytic-Uremic Syndrome

Shiga (or vero-) toxin-producing *Escherichia coli* (STEC) are a potentially serious cause of foodborne infectious diarrhea and were responsible for a recent and well-publicized European epidemic centered in Germany in 2011. During that outbreak, at least 4075 patients were infected, and 908 subsequently developed the hemolytic uremic syndrome (HUS). Thirty-four of those patients died.

HUS is defined by the triad of non-immune hemolytic anemia, thrombocytopenia, and acute renal failure. Clinical signs and symptoms result from circulating bacterial-derived Shiga toxin, since STEC infections are rarely associated with frank bacteremia. Shiga toxin binds to its
receptor, globotriaosylceramide (Gb3), which is concentrated on renal glomerular endothelial cells, and induces the characteristic pathophysiologic changes associated with HUS, namely endothelial cell swelling and detachment from the basement membrane. Endothelial dysfunction, characterized by increased procoagulant activity, vWF release, and leukocyte adhesion, is present even earlier in the disease process, before the clinical signs and symptoms of HUS are manifest. Whether this occurs directly as a result of toxin interaction with the endothelial cells or secondarily as a result of elevated pro-inflammatory cytokines is not clear, as both mechanisms have been documented. Furthermore, although the inciting factors (Shiga toxin and/or cytokines) and sequelae (procoagulant and adhesive endothelium) are well-described, the intervening mediators of endothelial dysfunction have not been determined.

1.1.3 Sepsis

Prior to 1992, proposed definitions of sepsis were often vague, contradictory, and fluid. With the publication of consensus definitions by the American College of Chest Physicians (ACCP) and Society of Critical Care Medicine (SCCM), early patient diagnosis and treatment, as well as clinical trial enrolment or inclusion criteria, could be standardized. The Systemic Inflammatory Response Syndrome (SIRS) was defined as the existence of two or more of: temperature >38°C or < 36°C, heart rate > 90 beats per minutes, respiratory rate > 20 breaths per minute or PaCO₂ (partial pressure of carbon dioxide) < 32 mmHg, and leukocyte count > 12 x 10⁹ cells/L, < 4000 x 10⁹ cells/L, or > 10% immature (band) forms. SIRS may result from a variety of both infectious and non-infectious conditions, with pancreatitis, ischemia, trauma, and burns being among the more common causes in the latter group. Sepsis was then defined as SIRS occurring as a result of infection. As elegantly illustrated in a Venn diagram from the original manuscript, not all patients with infection have SIRS, and not all patients with SIRS have infection, however, all patients with sepsis have both (suspected infection and SIRS). Bacteremia was recognized as the most common, but not sole, cause of sepsis. Severe sepsis was defined as sepsis associated with organ dysfunction, hypoperfusion, or hypotension, while septic shock was defined as sepsis-induced hypotension, hypoperfusion, or need for inotropic or vasopressor support despite adequate fluid resuscitation. Upon re-evaluation in 2001, the basic concepts behind each definition were maintained, and more specific guidance provided regarding vital sign, inflammatory, hemodynamic, organ dysfunction, and perfusion parameters that might be used to
make the diagnosis of sepsis.\textsuperscript{25} Despite concerns about specificity, the original definitions of SIRS and sepsis remain in common use.

In 2009, the Canadian Institute for Health Information released updated statistics on the epidemiology of sepsis in Canada (excluding Quebec).\textsuperscript{26} In 2008-2009, there were more than 30,000 sepsis-related hospitalizations in Canada, an increase of more than 4,000 in 4 years, with an associated mortality rate of 30.5%. Severe sepsis accounted for 39% of those admissions, and 45% of the deaths. Patients with sepsis remained in hospital for a median of 9 days longer than did patients hospitalized for other indications, and 45% of patients with sepsis required ICU care, further adding to the healthcare cost. This problem is mirrored in other high-income countries in which sepsis remains a leading cause of healthcare expenditure and patient mortality.\textsuperscript{27-30}

As recently reviewed by Lee and Liles, the clinical manifestations of sepsis ultimately derive from diffuse and profound endothelial activation, with all of its associated characteristics: hypercoagulability, inflammation, and vascular leak.\textsuperscript{31} In a mouse model of sepsis, disruption of endothelial-specific NF-κB reduced thrombin-antithrombin complex formation (a marker of coagulation), diminished neutrophil infiltration, attenuated pulmonary edema and major organ dysfunction, and reversed hypotension, all without enhancing bacterial clearance, suggesting that the documented benefits were a result of improved endothelial function.\textsuperscript{32} \textit{In vivo} studies of sepsis have documented impaired endothelium-dependent microvascular reactivity and elevated levels of circulating markers of endothelial activation (including E-selectin, soluble ICAM-1, soluble VCAM-1, and PAI-1), directly proportional to disease severity.\textsuperscript{33, 34} The stimuli for endothelial activation in sepsis are bacterial products, such as lipopolysaccharide (LPS), detected by pattern recognition molecules, such as toll-like receptor 4 (TLR4) on endothelial cells and monocytes and macrophages of the innate immune system. Endothelial activation via an LPS/TLR4 signalling pathway has been shown to augment neutrophil recruitment and extravasation, endothelial contractility and vascular leak, and upregulation of procoagulant genes, while activation of the innate immune system leads to production of pro-inflammatory cytokines and subsequent activation of endothelial cells.\textsuperscript{35-38} Typically, such responses are appropriate, adaptive, and localized, and the inciting infection is cleared. However, when these responses are exaggerated, excessive, or prolonged, sepsis and critical illness result.
1.2 Angiogenesis

Angiogenesis, the development and maturation of new blood vessels or their repair following injury, is a critical function of the healthy endothelium and is mediated by both positive and negative regulatory factors. Given the complexity of the process (reviewed in detail by Carmeliet and Jain) and the intent of this review to focus specifically on the angiopoietins, only a limited discussion of angiogenesis will be included here.

The quiescent endothelium is maintained in a resting state by cell survival signals produced by the pericytes that underlie and support the endothelial cell monolayer. These signals include VEGF, which is anti-apoptotic via the phosphatidylinositol-3-kinase (PI3k)/Akt pathway, as well as Dll4/Notch, and angiopoietin-1 (Ang-1). Ang-1 has been shown to upregulate Delta-like ligand 4 (Dll4) in endothelial cells to facilitate basal signalling through the Notch receptor and promote continued endothelial quiescence. In response to hypoxic (hypoxia-inducible factor (HIF)-2α), inflammatory, or mechanical stimuli, angiopoietin-2 (Ang-2) is released from endothelial cells, and mediates endothelial cell detachment from the basement membrane via matrix metalloproteinases. Endothelial permeability is further increased by VEGF, which allows plasma proteins to establish a subendothelial extracellular matrix. The sprouting angiogenesis that follows requires the migration of Dll4-expressing “tip” endothelial cells in response to a concentration gradient of VEGF on the extracellular matrix, followed by the proliferation of Notch1-expressing “stalk” endothelial cells in response to high levels of the Notch1 ligand Jagged 1. Akin to its function in resting endothelial cells, Dll4 on the tip cell is thought to interact with Notch on the stalk cells to prevent concurrent sprouting or branching of the stalk cells, thereby facilitating vessel growth in a single direction. Following migration, the endothelial cells form tubules and return to quiescence to complete maturation of the new vessel, a process guided in part by Ang-1.
1.3 Molecular Structure and Function of the Angiopoietins and their Receptor(s)

Ang-1 and Ang-2 are glycoproteins with a similar size of 70 kDa and coiled-coil domains at the amino termini that facilitate oligomerization. Ang-1 must, at minimum, form a tetramer in order to activate its receptor, but is in fact usually found in superclusters of oligomers. Ang-2, in contrast, typically exists in dimeric form in vivo. The carboxy terminus of both molecules consists of a fibrinogen-related domain that regulates binding to their shared receptor, Tie-2. Traditionally, Ang-1 has been considered an agonist, and Ang-2 an antagonist, at the Tie-2 receptor, inducing opposite effects on endothelial cell function.

1.3.1 Regulation of Angiopoietin Expression and Release

Ang-1 is produced primarily in the pericytes and smooth muscle cells that surround the endothelial cell monolayer, but can also derive from platelets. More recent evidence suggests that Ang-1 is also expressed in neutrophils in vitro, but the in vivo significance of this finding is not clear. Ang-1 production by pericytes is constitutive, but can be upregulated by Sonic hedgehog (Shh) and downregulated by Fibroblast Growth Factor-2 (FGF-2). In contrast, FGF-2 upregulates Ang-2 expression in endothelial cells and VEGF expression in stromal cells, illustrating the positive/negative regulatory factor interplay common in vascular physiology.

Ang-2 is produced by endothelial cells and stored in the Weibel-Palade bodies for rapid release upon exposure to various physico-chemical, mechanical, and inflammatory stimuli (discussed in Chapter 1.1). Of particular relevance to the angiogenic system, WPB exocytosis can be induced by VEGF and sphingosine-1-phosphate (S1P). S1P is a phospholipid signalling molecule that is secreted from platelets, activated monocytes and mast cells, and vascular endothelial cells, providing evidence of an additional degree of interaction between the inflammatory and angiogenic pathways. The net effect of S1P on the vasculature may be context-dependent, as S1P not only has a role in Ang-2 release via WPB exocytosis, but is also implicated as a mediator of Ang-1 signalling. Ang-1 has been found to increase intra-endothelial cell concentrations of S1P and to be at least partly dependent on S1P for its effects on vascular permeability.
1.3.2 Angiopoietin Receptor(s) and Signalling

The primary angiopoietin receptor, Tie-2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-2), as well as Tie-1 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-1), both belong to a family of vascular tyrosine kinase receptors expressed primarily, but not exclusively, in endothelial cells.\(^{57}\) The extracellular domain of Tie-2 contains three immunoglobulin-like domains and three epidermal growth factor (EGF)-like domains.\(^ {58}\)

Binding of the Tie-2 agonist Ang-1 leads to phosphorylation of the receptor and downstream signalling. Ang-2 binds at the same site, but its effects may be agonistic or antagonistic depending on the microenvironment.\(^ {59}\) In cultured endothelial cells, Ang-2, in the absence of Ang-1, can act as a partial agonist at Tie-2, leading to phosphorylation of the receptor and to the downstream effects characteristic of Ang-1/Tie-2 signalling.\(^ {60}\) However, Yuan et al propose that since Ang-1 binds the Tie-2 receptor with 20 times the affinity of Ang-2 (perhaps due to the multimerization of Ang-1 which would support clustering and activation of multiple Tie-2 molecules on the cell surface), Ang-2 acts as a relative antagonist in the presence of Ang-1, leading to relatively weaker signalling when it (Ang-2) binds to Tie-2. Consistent with this, Ang-2 induces a lesser degree of signalling through Tie-2 than does Ang-1, with reduced downstream phosphorylation of Akt. Interestingly, release kinetics prior to receptor internalization also differ between the two molecules, with Ang-2 being released more rapidly than Ang-1.\(^ {61}\) Taken together, the above studies indicate that the degree of activation of Tie-2 is determined by the relative balance between Ang-1 and Ang-2.

Like other members of the tyrosine kinase receptor family, Tie-2 exists on the cell surface in pre-formed clusters of oligomers.\(^ {62}\) Since tetrameric Ang-1 is the smallest oligomer that can activate Tie-2, receptor grouping likely facilitates ligand binding. Tie-2 is located on both the apical and basolateral membranes, thereby allowing it to be bound by circulating Ang-1/Ang-2, as well as by Ang-1/Ang-2 secreted by pericytes and endothelial cells, respectively.\(^ {63}\) Upon activation by Ang-1, Tie-2 is rapidly internalized through clathrin-coated pits, ubiquitylated, and degraded, likely to regulate the duration of signalling, as is common within the receptor family.\(^ {62, 64}\) As noted above, ligands appear to be released prior to internalization.\(^ {61}\) In keeping with its largely antagonist activity and its rapid rate of release after binding, Ang-2 is only a weak stimulus for
the internalization and degradation of Tie-2. Tie-2 is also regulated by constitutive and VEGF-inducible ectodomain cleavage, leading to a soluble Tie-2 (sTie-2) fragment that can bind Ang-1 (and Ang-2) and inhibit signalling through intact, transmembrane Tie-2 receptors.\textsuperscript{65}

The function of the alternate angiopoietin receptor, Tie-1, has only recently been determined. Ang-1 can induce Tie-1 phosphorylation in a Tie-2-dependent manner, however, Ang-1-induced activation of Tie-1 downregulates the Akt and 42/44 MAPK (mitogen-activated protein kinase) pathways, resulting in inhibition of Tie-2-mediated effects.\textsuperscript{66} Tie-1 has been shown to form heterodimers with Tie-2 on the endothelial cell surface, and experiments using inactive forms of Tie-1 or Tie-2 tyrosine kinases suggest that it is Tie-2 tyrosine kinase activity that results in Tie-1 phosphorylation.\textsuperscript{67} In keeping with their opposing actions, Ang-1 has been found to preferentially bind non-Tie-1-associated Tie-2.\textsuperscript{68} In addition to acting as a regulator of Ang-1/Tie-2 signalling, Tie-1 has also been proposed as a second mechanism by which Ang-2 may exert its context-dependent agonist/antagonist effects. In the presence of Tie-1, Ang-2 functions as an antagonist, binding but not signalling at Tie-2, and in the absence of Tie-1, Ang-2 functions as an agonist, binding and signalling through Tie-2.\textsuperscript{69} Tie-1 also mediates, in part, the cross-talk between the angiopoietins and VEGF. VEGF indirectly phosphorylates Tie-2 by facilitating proteolytic cleavage of the extracellular domain of Tie-1, thereby enhancing access of Ang-1 to the binding site of Tie-2.\textsuperscript{70, 71} In addition to VEGF, inflammatory stimuli and shear stress can also rapidly induce cleavage of the Tie-1 ectodomain.\textsuperscript{72, 73} Taken together, these findings suggest that endothelial function can be precisely regulated in response to changes in the microenvironment, and that this regulation can occur by a variety of means, including changes in receptor cell surface expression, cleavage of the Tie-1 extracellular domain, and the relative agonist/antagonist concentrations.

The function of Tie-1 independent of Tie-2 remains controversial. Disruption of the Tie-1 gene is embryonic lethal at day E13.5 due to edema, hemorrhage, and microvascular rupture.\textsuperscript{74} Prior to death, endothelial cells in these animals demonstrate increased filopodia and resulting enhanced capillary density, as well as abnormal lymphangiogenesis.\textsuperscript{75} Double Tie-1 and Tie-2 knockout (-/-, null) mice display a phenotype more severe than that seen with knockout of either gene alone.\textsuperscript{76} Beyond the developmental stage, however, the Tie-2-independent function of Tie-1 is less clear. In a mouse model of atherosclerosis, Tie-1 was overexpressed at regions of shear stress, while Tie-1 knockdown attenuated atherosclerotic changes.\textsuperscript{77} \textit{In vitro} studies have
suggested that Tie-1 may have its own function in the endothelial response to inflammation. Overexpression of Tie-1 in endothelial cells lead to auto-phosphorylation and subsequent upregulation of cell surface adhesion molecules, including E-selectin, VCAM-1, and ICAM-1, while knockdown of Tie-1 by siRNA (small interfering RNA) lead to downregulation of certain pro-inflammatory molecules, including IL-1β.\textsuperscript{78,79} However, these findings have yet to be duplicated in higher order models. Furthermore, other authors have not detected an effect of siRNA knockdown of Tie-1 on Ang-1 function.\textsuperscript{80}

In summary, the net signalling that occurs through Tie-2 is regulated by the balance in local concentrations of Ang-1 and Ang-2, as well as by the degree of co-localization and ectodomain cleavage of Tie-1. Through the latter mechanism, VEGF and inflammatory stimuli can modulate the magnitude of Ang-1 and Ang-2 signalling (or lack thereof) through Tie-2, and influence endothelial cell function.

1.3.3 Downstream effects of Angiopoietin-1 and -2

Consistent with their classic roles as competitive antagonists of the same receptor, Ang-1 and Ang-2 typically exert opposite effects on signalling through Tie-2. Via Tie-2, Ang-1 activates both the Erk and Akt pathways. Erk is thought to be important for cell migration and proliferation, while Akt modulates cell survival and inhibits apoptosis.\textsuperscript{81} Multimeric Ang-1 results in relocation of Tie-2 to areas of cell-to-cell contact, subsequently leading to Ang-1 bridging and \textit{trans}-activation of Tie-2, preferentially activating Akt, which subsequently activates endothelial Nitric Oxide Synthase (eNOS) and phosphorylates the forkhead transcription factor Foxo1.\textsuperscript{82} This results in Foxo1 exclusion from the nucleus, and reduced transcription of its dependent genes. The net result is vascular quiescence in the setting of cell-to-cell contact. Sensing of neighbouring endothelial cells may be facilitated through Tie-2 receptors clustered in lipid rafts that also contain VE-cadherin, and appears to hinge on the expression of the transcription factor Krüppel-like factor 2.\textsuperscript{83,84} In the absence of cell-to-cell contact, Ang-1 can be bound to fibronectin, collagen, vitronectin, and other components of the extracellular matrix to anchor Tie-2 to the cell surface in contact with the substratum. In this situation, signalling through Tie-2 results in preferential activation of the Erk pathway, resulting in increased cell mobility and enhanced likelihood of forming a functional endothelial cell monolayer.\textsuperscript{82} Also necessary for cell migration is Tie-2-dependent caveolin-1 polarization, and
Ang-1-induced Early Growth Response-1 (Egr-1).\textsuperscript{67} Egr-1 is a transcription factor that upregulates multiple growth factors, cytokines, adhesion molecules, and pro-angiogenic factors. In siRNA knockdown studies, loss of Egr-1 abolished Ang-1-induced endothelial cell migration.\textsuperscript{85} Gene expression profiling of Ang-1-activated endothelial cells reveals upregulation of genes involved in endothelial cell proliferation, differentiation, migration, and survival, and downregulation of those involved in apoptosis and inhibition of transcription.\textsuperscript{86}

Besides its anti-apoptotic effect, Ang-1 exerts an anti-permeability effect on the endothelium, at least partly through increasing the depth and glycosaminoglycan content of the endothelial glycocalyx.\textsuperscript{87} Ang-1 also activates Rac1 and phosphorylates RhoGAP, while at the same time inhibiting RhoA, which is responsible for Ang-2 mediated cytoskeletal rearrangements and vascular permeability (see below).\textsuperscript{88} Ang-1 also has anti-thrombotic properties: downregulation of Ang-1 leads to enhanced VEGF- and tumour necrosis factor (TNF)-induced tissue factor expression through loss of Ang-1 negative regulation.\textsuperscript{89,90} Finally, Ang-1 is anti-inflammatory. It has been shown to downregulate endothelial cell production of the pro-inflammatory molecule endothelin-1, as well as LPS- and VEGF-induced cell surface expression of the leukocyte adhesion markers E-selectin, ICAM-1, and VCAM-1.\textsuperscript{91-93}

Consistent with its role as a Tie-2 antagonist, Ang-2 typically opposes the effects of Ang-1 and therefore induces pro-apoptotic, pro-permeability, and pro-adhesive changes in the endothelium. Blockade of signalling through Tie-2 leads to endothelial cell apoptosis via inhibition of the Akt pathway, as well as increased expression of the antiangiogenic molecule thrombospondin.\textsuperscript{94} Ang-2 increases thrombin-induced vascular permeability by impairing VE-cadherin organization and increasing intercellular gap formation, possibly through actions on Rho kinase and myosin light chain phosphorylation, or by inducing αvβ3 integrin internalization and degradation.\textsuperscript{9,95,96} Tie-2 knockdown mimics the effect of Ang-2 on endothelial barrier function. Treatment with exogenous Ang-2 has been shown to result in endothelial detachment from the basement membrane within 4 hours.\textsuperscript{97} Finally, in a transgenic mouse model, inducible endothelial cell-specific Ang-2 expression enhanced myeloid cell recruitment, adhesion to ICAM-1 on the endothelial cell surface, and tissue infiltration.\textsuperscript{98}
A simplified summary of these effects casts Ang-1 as an effector of vascular quiescence, and Ang-2 as an effector of vascular activation, although there are certainly context-dependent nuances to this interpretation of the function of both molecules.
1.4 Angiopoietin-1 and -2 in Development

Disruption of the Ang-1 gene in mice is embryonic lethal at day E12.5. Prominent developmental defects include a simplified endocardium detached from the myocardial wall, loss of myocardial trabeculations (suggestive of diminished cross-talk between myocardial and endocardial cells), and generalized dilatation and loss of complexity (diminished branching) of vascular networks. Ultrastructural examination of these abnormal vessels reveals rounded endothelial cells separated from the underlying supportive cells. Disruption of Tie-2 is also embryonic lethal in mice, resulting in death by day E10.5. The Tie-2 null phenotype is one of large, unbranched blood vessels, and a simplified vascular network, similar to that seen with Ang-1 knockout.

Overexpression of Ang-2 in the vascular endothelium (transgenic mice created using Tie-2 transcriptional regulatory elements) is embryonic lethal at day E9.5-10.5. Pathology studied at day E9 revealed a disorganized and discontinuous vascular network, and confirmed the basic concept that Ang-2 antagonizes the effect of Ang-1; detachment of the endocardium from the underlying myocardium with overexpression of Ang-2 was identical to that seen in Tie-2 and Ang-1 knockout mice. Similarly, detachment of the endothelial layer and rounding of individual endothelial cells was seen in the vessel walls and was equivalent in Ang-2 overexpression and Ang-1 or Tie-2 underexpression.

In contrast to all of the above phenotypes, knockout of Ang-2 does not result in embryonic lethality. Instead of being a key component of vascular development, Ang-2 appears to be crucial for vascular remodelling. Using the retinal vasculature as a representative vascular bed, Ang-2 -/- mice were found to have grossly normal vessels at birth. However, they failed to show evidence of angiogenesis in response to hypoxia-induced VEGF, and similarly failed to demonstrate the usual and necessary hyaloid vascular regression. Similarly, in a rat pup model of angiogenesis, Ang-2 mediated new vessel sprouting in the presence of VEGF, and vessel regression and endothelial apoptosis in its absence. That Ang-2 is involved in the coordinated vascular sprouting and regression necessary to produce a mature vascular network is consistent with its known actions as a context-dependent antagonist/agonist at Tie-2. Of note, Ang-2 -/- mice typically die within two weeks of birth with profound chylous ascites ± pleural effusions. These mice were found to have a leaky and disorganized lymphatic network thought to result...
from the lack of Ang-2 agonist activity at Tie-2 in the presence of VEGF. This is consistent with more recent in vitro work that confirms a prominent agonist effect of Ang-2 on lymphatic endothelial cells.\textsuperscript{103} Finally, Ang-2 -/- mice are unable to mount an appropriate inflammatory response to either chemical or bacterial insult, partly as a result of the loss of Ang-2-induced sensitization of endothelial cells to the effects of TNF, in particular to TNF-induced expression of cell adhesion molecules.\textsuperscript{104}
1.5 Angiopoietin-1 and -2 in Mature Endothelial Cells

In healthy adult human tissue, Ang-1 is widely expressed in most organs while Ang-2 mRNA is detectable primarily in those anatomic sites with significant angiogenesis, namely the ovary (which must create a densely vascularized corpus luteum after follicular rupture), uterus, and placenta.\(^{100}\) It was early studies involving the rat ovary that characterized the influence of VEGF on the vascular action of Ang-2: in the presence of VEGF, as is found in the evolving corpus luteum, excess Ang-2 promoted vessel sprouting by blocking the stabilizing effect of Ang-1, while in the absence of VEGF, as is found in follicular atresia, excess Ang-2 promoted vessel regression.\(^{100}\) Reflecting its role in destabilizing the mature endothelium (whether physiologic, in the promotion of angiogenesis, or pathologic, in the initiation of endothelial dysfunction), administration of exogenous Ang-2 leads to severe vascular leak and pulmonary edema in mice.\(^9\)

In contrast, transgenic mice that overexpress Ang-1 were found to have leak-resistant vessels, even in the presence of inflammatory mediators that typically increase vascular permeability.\(^{105}\) Furthermore, while isolated VEGF overexpression enhances angiogenesis but produces pathologically permeable vessels, co-expression of VEGF with Ang-1 leads to the development of new, functional, appropriately permeable vessels. In addition, while adenoviral-delivered VEGF lead to diffuse edema and death in mice, this was blocked by the pre-administration of adenoviral-delivered Ang-1.\(^{106}\) Further parsing out the role of Ang-1 in the adult vasculature, a murine Cre-Lox model of conditional Ang-1 gene knockout demonstrated no obvious phenotype during vascular quiescence with Ang-1 gene disruption initiated after E13.5, but significantly altered vascular remodelling in response to injury.\(^{107}\)

Taken together, the studies of the angiopoietins in embryonic development and in the mature endothelium indicate that Ang-1 signalling through the Tie-2 receptor is indispensable for the formation of new blood vessels whether during embryogenesis or during repair of the adult vasculature. Although Ang-1 plays a role in the maintenance of vascular quiescence, it is not an absolute pre-requisite for the resting endothelial state. In contrast, Ang-2 is not absolutely required during embryogenesis, but is necessary for vascular remodelling thereafter, during which its function is context-dependent as per the presence or absence of VEGF.
1.6 Angiopoietin-3 and -4, and Angiopoietin-like proteins

While Ang-1 and Ang-2 are the best characterized, they are not the only members of the angiopoietin family. Angiopoietin-3 and -4 (Ang-3 and Ang-4) are Tie-2 receptor agonists, capable of inducing Tie-2 phosphorylation and subsequent angiogenesis.\textsuperscript{108} Both are species-specific, with Ang-3 limited to mice and Ang-4 to humans, and represent mouse-human orthologues based on equivalent chromosomal loci.\textsuperscript{109} The functional role of Ang-4 remains unclear: some \textit{in vitro} studies have found that Ang-4 inhibits endothelial cell migration and supports endothelial barrier function,\textsuperscript{110} while others have described increased angiogenesis and tumour progression in glioblastoma multiforme,\textsuperscript{111} suggesting that the effect of Ang-4 may be context-dependent.

The physiologic role of the angiopoietin-like proteins is currently under active study. Angiopoietin-like proteins 1-7 all have a coiled-coil domain at the N-terminus and a fibrinogen-like domain at the C-terminus, akin to the angiopoietins, but none bind Tie-2 or Tie-1, and most appear to have important \textit{in vivo} functions unrelated to angiogenesis.\textsuperscript{112} As an example of the pleiotropic effects of this family of molecules, Angiopoietin-like protein-4 participates both in increased endothelial permeability through direct interactions with integrin $\alpha5\beta1$ (and subsequent Rac signalling), VE-cadherin, and claudin-5, and in hypertriglyceridemia in response to glucocorticoids.\textsuperscript{113,114} Angiopoietin-like protein-2 is highly expressed in adipose tissue, increases in response to chronic hypoxia and inflammation, and is correlated with insulin resistance in obese patients.\textsuperscript{115} Angiopoietin-like protein-3 increases plasma levels of cholesterol and triglycerides, and mutations in its gene have recently been implicated in familial combined hypolipidemia.\textsuperscript{116} Angiopoietin-like protein-6 has also been implicated in obesity, although the literature on the subject is conflicting.\textsuperscript{117}
1.7 Dysregulation of Angiopoietin-1 and -2 in Human Disease

Because of the central role of angiogenesis in the development, proper function, defence, and subsequent repair of every organ system, it is not surprising that the angiopoietins, important regulators of angiogenesis, have been widely studied and implicated in a variety of human diseases and syndromes. Although the occasional study has attempted to explore angiopoietin levels in parenchymal tissue or non-hematogenous bodily fluids, the vast majority of the studies presented below have examined serum, plasma, or whole blood concentrations of Ang-1 and Ang-2, in keeping with their vascular origins and targets. Because of the breadth and depth of the literature in this field, the following review will focus primarily on clinical studies, with reference to in vitro or animal models where required to provide supporting evidence or rationale. It should be noted that, in healthy individuals, the serum/plasma concentration of Ang-1 is greater than that of Ang-2, and that angiopoietin dysregulation refers to a perturbation of this typical ratio (decreased Ang-1, increased Ang-2, and an increased Ang-2:Ang-1 ratio).

1.7.1 Angiopoietins in Critical Illness

The majority of work in this area has focused on the association of the angiopoietins with sepsis and its diagnosis, treatment, complications, and outcome. Angiopoietin dysregulation, and in particular a net excess of Ang-2, would be predicted to create a pro-inflammatory, pro-coagulant, and pro-adhesive endothelium that would in turn manifest as tissue inflammation, microvascular thrombosis, and hypotension, all frequently observed in septic patients. In healthy volunteers who received an infusion of LPS, serum Ang-2 levels began to rise 2 hours after the infusion and peaked at 4.5 hours, while neither sTie-2 nor Ang-1 was appreciably different from baseline. The increase in Ang-2 occurs coincident with that of IL-6, 30 minutes after that of TNF, and 30 minutes before that of IL-8. Of note, Ang-2 levels declined more slowly than did those of the cytokines, yet faster than both E-selectin and ICAM-1. An accompanying study of 21 septic patients found higher Ang-2 levels in non-survivors than survivors (28-day mortality), both on admission and at 72 hours. Likewise, in a study of 50 critically ill patients, plasma Ang-2 levels taken within 12 hours of the diagnosis of sepsis were predictive of ICU and 28-day mortality. Ang-2 levels on the day of enrolment and throughout the ICU admission correlated with fluid balance, with standard indices of pulmonary dysfunction, and with mortality, suggesting a potential role for serial measurements of Ang-2. vWF was not as uniformly associated with
illness severity, prompting the authors to speculate that, despite co-storage in the Weibel-Palade bodies, differential regulation might be relevant.\textsuperscript{7} Other studies have found a similar relationship between Ang-2 concentration and the severity of illness in sepsis: Orfanos \textit{et al} described a correlation between serum Ang-2, severe sepsis, and TNF,\textsuperscript{121} Siner \textit{et al}, Davis \textit{et al}, and Kümpers \textit{et al} between serum Ang-2, Sequential Organ Failure Assessment (SOFA) and Acute Physiology And Chronic Health Evaluation (APACHE) II scores, and mortality,\textsuperscript{122-124} and Parikh \textit{et al} between serum Ang-2 (but not Ang-1) and impaired pulmonary gas exchange, but not with survival or APACHE II score.\textsuperscript{9} Ang-2 has been found to be similarly predictive of mortality in certain subsets of critically ill patients, including those requiring renal replacement therapy and in children with septic shock.\textsuperscript{125, 126}

In contrast, decreased plasma Ang-1 at ICU admission predicted 28-day mortality in 70 patients with severe sepsis, while decreased Ang-1 levels were also associated with mortality in a study of Malawian children with severe bacterial infection.\textsuperscript{127, 128} Furthermore, activated protein C, which until recently was approved for the treatment of severe sepsis, has been found to upregulate both Ang-1 and Tie-2, thereby improving endothelial barrier function in a manner which may have been responsible for some of the therapeutic benefit documented in early clinical trials.\textsuperscript{129} Importantly, the clinical utility of Ang-1 as a prognostic biomarker may be limited by the frequent fluctuations in circulating levels seen in individual patients with hourly sampling over a 24-hour time period.\textsuperscript{118} The Ang-2:Ang-1 ratio, reflecting perturbations in one or both of the angiopoietins, has been found to be predictive of the eventual development of septic shock at the time of fever onset in neutropenic patients.\textsuperscript{130} Finally, although the soluble Tie-2 receptor does increase in sepsis, it has no apparent effect on Ang-1/2 function or circulating levels, and was not found to be a significant indicator of pulmonary edema nor a significant predictor of mortality in a multivariate analysis.\textsuperscript{131}

\textbf{1.7.2 Angiopoietins in Malaria}

In malaria, endothelial activation leads to adherence of parasitized erythrocytes, microvascular obstruction, and tissue ischemia. Ang-2, but not VEGF, was found to be elevated in severe malaria (and a better predictor of outcome than lactate) in association with reduced NO bioavailability.\textsuperscript{132} Subsequent studies, however, have all identified Ang-1 as a more consistent biomarker in malaria, discriminating between cerebral malaria and uncomplicated malaria in
Thai adults and Ugandan children, between cerebral, severe, and uncomplicated malaria in Thai adults, and between cerebral malaria with retinopathy and febrile, non-malarial decreased level of consciousness in Malawian children. Furthermore, decreased Ang-1 was associated with *Plasmodium falciparum* malaria in pregnancy, while an increased Ang-2:Ang-1 ratio was associated with both placental malaria and low birth weight infants.

**1.7.3 Angiopoietins in Cardiovascular Disease**

Given the prominence of the angiopoietin system in vascular remodelling, Ang-1 and Ang-2 have been proposed as both mediators and potential therapeutic targets for a variety of primary cardiovascular diseases. Higher levels of Ang-2 were associated with systolic blood pressure, diabetes, and the metabolic syndrome in 3778 third-generation cohort participants of the Framingham Heart Study. Likewise, David et al found a higher level of plasma Ang-2 in hypertensive patients as compared to healthy controls, and in those patients with atherosclerotic disease as compared to those without. Despite purported anti-inflammatory effects separate from their lipid-lowering properties, a six-week course of therapy with a 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase inhibitor (“statin”) did not lower Ang-2 levels in this population. It should be noted that other studies have documented an elevation in plasma angiogenic factors, including VEGF, Ang-1, Ang-2, and sTie-2, in treated hypertensive patients with high Framingham risk scores, attributed to the relative hypoxia resulting from rarefaction or loss of the microcirculation that occurs in hypertension.

As noted in the Framingham study, angiopoietin dysregulation has also been associated with disorders of glucose and lipid metabolism. *In vitro* studies involving human cardiac microvascular endothelial cells and human macrovascular endothelial cells have demonstrated that exposure to high concentrations of glucose leads to specific inhibition of Ang-1/Tie-2-induced Akt phosphorylation, resulting in a predicted loss of cell survival signalling. The Ang-1/Tie-2-induced phosphorylation of Erk 1/2 was not affected, leading to a predicted net signal favouring endothelial cell activation and migration. In clinical studies, Ang-2 is higher in patients with diabetes than in healthy control subjects, and elevated Ang-2 is independently associated with an elevated hemoglobin A1c (HbA1c), a marker of longterm glycemic control, as well as with diabetic retinopathy and a composite marker of macrovascular disease that included cardiovascular and cerebrovascular disease as well as neuropathy.
Given its association with multiple cardiovascular risk factors, it is not surprising that elevated levels of Ang-2 have been associated with coronary artery disease. Plasma levels of Ang-2 at presentation are significantly higher in patients with acute myocardial infarction (AMI) than in patients with unstable or stable angina, in whom Ang-2 levels are significantly higher than in healthy controls. Microarray analysis of myocardial biopsies from patients undergoing urgent coronary artery bypass grafting for AMI confirms significant upregulation of the Ang-2 gene. Ang-2 levels are also elevated in acute, versus chronic, congestive heart failure (CHF), and at a baseline routine health examination in patients who experienced AMI within the next ten years. The latter association was independent of the traditional cardiovascular risk factors.

Since vascular stabilization, repair, and regeneration are important prerequisites for functional recovery following stroke, high post-event Ang-1 levels in the cerebral vasculature would be predicted to have a beneficial effect. Indeed, in experimental animal models and in one prospective clinical trial involving 95 patients, elevated Ang-1 levels were associated with improved outcome after intracerebral hemorrhage (ICH). A serum Ang-1 level of ≥ 35 ng/mL at 72 hours post-ICH yielded an odds ratio (OR) of 14.7 for the occurrence of a good outcome at three months, defined as an improvement in the NIHSS (National Institutes of Health Stroke Scale) of ≥ 50%. Consistent with these observations, patients with subarachnoid hemorrhage who exhibited post-event cerebral vasospasm on transcranial Doppler ultrasound were found to have more prolonged and severe suppression of serum Ang-1 levels when compared with both healthy controls and patients with SAH but without cerebral vasospasm. Higher Ang-1 levels likewise correlate with more rapid middle cerebral artery recanalization after tPA (tissue plasminogen activator) therapy, while higher Ang-2 levels have been associated with risk of recurrence following lacunar infarct. Elevated levels of Ang-2 have also been implicated in post-stroke breakdown of the blood-brain barrier and subsequent cerebral edema.

1.7.4 Angiopoietins in Chronic Kidney Disease

A role for the angiopoietins in chronic kidney disease (CKD) was first suggested by animal studies. Podocyte-specific overexpression of Ang-2 lead to albuminuria and apoptosis of the glomerular endothelial cells, and to decreased expression of VEGF, an important local regulator of the antagonist/agonist effects of Ang-2. In contrast, exogenous Ang-1 has been found to attenuate renal dysfunction in a variety of models of acute and chronic kidney
Angiopoietin dysregulation appears to be greatest in patients on hemodialysis (although the comparison with those on peritoneal dialysis trended towards but did not reach statistical significance), and resolves with renal transplantation. Neither time of sampling (pre- or post-dialysis) nor transition to nocturnal hemodialysis for a period of 6 months altered angiopoietin dysregulation. Furthermore, in patients with CKD on dialysis (either hemo- or peritoneal), serum Ang-2 levels correlated with the presence of coronary heart disease and peripheral arterial disease. The same authors described a progressive increase in serum Ang-2 with each successive stage of chronic kidney disease, and attributed angiopoietin dysregulation in this setting to the reduction in nitric oxide, a known inhibitor of Weibel-Palade body exocytosis, seen in CKD. Given the relatively high molecular weight of the angiopoietins, and their existence as multimers, they are not candidates for glomerular filtration, nor are they detectable in urine (Page AV, unpublished observations). Therefore, elevated serum levels of Ang-2 in CKD are likely related to increased release, rather than decreased renal clearance. Further work by Kümpers et al determined that elevated Ang-2 was an independent predictor of mortality in CKD.

1.7.5 Angiopoietins in Pregnancy and Preeclampsia

The angiopoietins are frequently implicated in the vascular complications of pregnancy, but published reports differ with regard to the direction of change of Ang-1, Ang-2, and soluble Tie-2. Serum Ang-2 levels are higher in healthy pregnant women than in non-pregnant or post-partum women. Circulating Ang-2 levels were initially reported to be lower, and Ang-1 levels higher, in women with preeclampsia than in those with normal pregnancies. Further study revealed that this difference might be present in the third trimester only: the two groups of patients are indistinguishable in the first trimester, and those patients who will subsequently develop preeclampsia typically have higher serum Ang-2 concentrations in the second trimester. More recently, several authors reported a high Ang-2:Ang-1 ratio, primarily as a result of lower Ang-1, in women in the second trimester of pregnancy who would later develop preeclampsia, and in those with established preeclampsia. Low soluble Tie-2 levels have also been found in women with preeclampsia. In summary, while angiopoietin dysregulation as a marker or contributor to preeclampsia and other hypertensive disorders in pregnancy is certainly plausible, additional research is needed to clarify the relevant timing, direction, and magnitude of change of Ang-1, Ang-2, and soluble Tie-2.
Of note, Ang-2 has also been detected in the amniotic fluid of healthy women in the second and third trimesters of pregnancy, and is elevated in those with an intrauterine infection, consistent with its known links to inflammation.\textsuperscript{167}

**1.7.6 Angiopoietins in Malignancy**

Consistent with what is known about the role of Ang-2 in development, tumours from transgenic mice that overexpress Ang-2 have increased microvessel density, an immature vascular phenotype with a deficit of pericytes, and an enhanced rate of endothelial cell apoptosis.\textsuperscript{168} Various malignant cells, including chronic lymphocytic leukemia (CLL) cells, can secrete Ang-2.\textsuperscript{169} In addition, gastrointestinal stromal tumours, leiomyomas, schwannomas, colorectal adenocarcinomas, and to a lesser extent Kaposi’s sarcomas and cutaneous angiosarcomas, have all been shown to harbour Ang-1, Ang-2, Ang-4, Tie-1, and Tie-2 in the tumour cell cytoplasm.\textsuperscript{170-172} Although the clinical significance of this finding is not known, the presence of both the ligand and receptor in the same cell suggests that the tumour cells may be able to respond to autocrine or paracrine signalling through the angiopoietin/Tie system.

Further evidence of the interaction between the angiopoietin/Tie system and various malignancies is derived from cohort studies. Elevated circulating Ang-2 levels have been associated with reduced time to first treatment and higher mortality in CLL,\textsuperscript{169} increased mortality in acute myeloid leukemia (14.7\% three-year survival in patients with Ang-2 levels $\geq$ 1495.6 pg/mL versus 64.7\% in those with Ang-2 levels $<$ 1495.6 pg/mL),\textsuperscript{173} stage of disease and bone involvement in multiple myeloma,\textsuperscript{174} baseline disease and cardiac involvement in primary light chain (AL) amyloidosis,\textsuperscript{175} baseline disease and lymph node metastases in early gastric cancer,\textsuperscript{176} baseline disease in castration-resistant prostate cancer,\textsuperscript{177} reduced survival in pancreatic ductal adenocarcinoma,\textsuperscript{178} baseline disease, residual disease after debulking surgery, and poor overall and disease-free survival in ovarian carcinoma,\textsuperscript{179} increased mortality at the time of diagnosis of colorectal carcinoma,\textsuperscript{180} baseline disease, tumour burden, and prognosis in neuroendocrine tumours,\textsuperscript{181,182} disease stage, metastases, and overall survival in non-small cell lung cancer,\textsuperscript{183} survival and disease progression in metastatic malignant melanoma,\textsuperscript{184} baseline disease in liver cirrhosis and hepatocellular carcinoma,\textsuperscript{185} increased tumour size in soft tissue sarcoma,\textsuperscript{186} baseline disease in breast cancer,\textsuperscript{187} and inversely with tumour burden in response to treatment in metastatic renal cell carcinoma.\textsuperscript{188} Interestingly, high bone marrow concentrations
of Ang-1 (studied following reports of increased bone marrow microvessel density) have recently been reported to be independently associated with a higher rate of transformation to acute leukemia and with overall mortality in myelodysplastic syndromes.\textsuperscript{189}

Of note, the angiopoietin/Tie system has also been implicated in adverse effects associated with cancer therapy. Vascular leak syndrome occurs in more than half of the patients treated with high-dose IL-2 for metastatic renal cell carcinoma or melanoma, and in these patients, Ang-2 levels rose with each consecutive day of therapy and declined with discontinuation.\textsuperscript{190}

Furthermore, when applied to endothelial cells in culture, patient serum from the final day of IL-2 therapy resulted in endothelial gaps and increased actin stress fibres, pathophysiologic correlates of the vascular leak syndrome \textit{in vivo}. In patients receiving the anti-VEGF antibody bevacizumab, upregulation of Ang-1 as a result of VEGF inhibition limits tumour hypoxia and protects the tumour vasculature from regression, thereby potentially contributing to tumour recurrence despite ongoing VEGF blockade.\textsuperscript{191}

\section*{1.7.7 Angiopoietins in Respiratory Diseases}

The rationale for the study of the angiopoietins in obstructive airway diseases derives from clinicopathologic studies that have documented an increase in the number and permeability of blood vessels in the airway walls in chronic obstruction as recently reviewed by Zanini \textit{et al}, and from the growing need for novel, non-steroid-based therapies.\textsuperscript{192} Expression of Tie-2 is upregulated in airway epithelial cells and selected macrophages in the lungs of ovalbumin-sensitized and -challenged mice, a recognized animal model for airway hyperresponsiveness, and is correlated with the degree of airway remodeling.\textsuperscript{193} Ang-2 expression was increased, and Ang-1 expression decreased, in the same cells. In clinical studies, asthmatics who smoked had significantly higher levels of Ang-2 in induced sputa than did asthmatics who did not smoke, and these levels were positively correlated with the airway vascular permeability index.\textsuperscript{194} In patients with chronic obstructive pulmonary disease (COPD), serum Ang-2 levels were significantly higher in those with stable disease as compared to healthy controls, and higher still in those with acute exacerbations.\textsuperscript{195} Ang-2 levels in this instance were inversely correlated with PaO\textsubscript{2}.

Furthermore, both former and current smokers had higher serum levels of Ang-2 than did those patients who had never smoked. Since VEGF is also increased in obstructive airway diseases,\textsuperscript{196} and since Ang-2 is known to promote new vessel sprouting with endothelial migration and
proliferation in the presence of VEGF, the predicted impact of the net increase in Ang-2 is increased angiogenesis, thereby providing a plausible explanation and potential therapeutic target for the airway vascular proliferation characteristic of chronic obstructive lung diseases.

Chronic pulmonary vascular remodelling also occurs in pulmonary hypertension. In experimental models of pulmonary arterial hypertension (PAH), Ang-1 has been found to be protective against vascular remodelling, while Ang-2 is elevated in hypoxia-induced PAH, consistent with the ability of hypoxia to trigger Weibel-Palade body release from endothelial cells. In patients with PAH, elevated plasma Ang-2 has been found to be inversely correlated with the response to treatment, and to be an independent predictor of mortality.

1.7.8 Angiopoietins in Autoimmune Connective Tissue Diseases

Angiopoietin dysregulation has been associated with a variety of autoimmune disorders. When compared to healthy controls, patients with newly diagnosed rheumatoid arthritis (RA) had elevated levels of serum Ang-2, and these levels correlated with the severity of disease and measures of disease activity. Interestingly, patients who later developed cardiovascular disease (at a mean interval of 12.5 years after the onset of RA) had higher levels of Ang-2 at arthritis diagnosis than did those patients who never developed cardiovascular disease over the same length of follow-up. Elevated serum Ang-2 levels (and the Ang-2:Ang-1 ratio) have also been found to correlate with disease activity and proteinuria in patients with Systemic Lupus Erythematosus (SLE), and with disease activity and/or renal involvement in patients with various vasculitides. Furthermore, Ang-2 expression was upregulated in the glomeruli of patients with SLE-associated renal disease. Additional data suggesting that the angiopoietin system may contribute to the pathogenesis of autoimmune diseases is derived from patients with autoimmune thyroid disease, in whom monocyte overexpression of the Tie-2 receptor appears to enhance the chemotactic response to Ang-1 and Ang-2 overproduction by thyroid follicular cells, leading to an inflammatory cell infiltrate in the thyroid parenchyma.

Serum Ang-1 levels in patients with early RA (at the time of first clinic visit) were found to be elevated when compared with those in patients with other collagen vascular diseases, a discriminatory effect not seen with serum C-reactive protein or rheumatoid factor. Serum Ang-1 levels are likewise elevated in patients with Behçet’s disease as compared to healthy controls, suggesting that angiopoietin dysregulation is not a uniform process across
rheumatologic disorders, and therefore, that its clinical utility should be carefully evaluated with particular attention to the type, stage, and degree of complications in each autoimmune disease.

1.7.9 Angiopoietins in Miscellaneous Diseases and Syndromes

Plasma levels of Ang-2 have been shown to rise within 30 minutes of major trauma, and to correlate with the severity of injury, the degree of tissue hypoperfusion, and in-hospital mortality in trauma victims.\(^{208}\) Circulating Ang-2 is elevated in asymptomatic patients with sickle cell disease as compared to healthy controls (HbSS > HbSC > HbAA), and is further elevated in both groups during painful crises.\(^{209,210}\) Since vascular remodelling is prominent in the pathophysiology of inflammatory bowel disease, serum Ang-2 was investigated and found to be elevated in patients with Crohn’s disease and ulcerative colitis (UC).\(^{211}\) Epithelial cells from crypt abscesses in biopsy specimens from UC patients were found to overexpress Ang-1, Ang-2, and Tie-2, while the neutrophils in those abscesses overexpressed Tie-2, suggesting the ability to respond to Ang-1 or Ang-2 as chemotactic factors.\(^{212}\) These findings are strikingly similar to those of autoimmune thyroid disease (discussed above). In patients with acute pancreatitis, admission serum Ang-2 levels were found to be predictive of later organ failure that persisted for more than 48 hours.\(^{213}\) Finally, serum Ang-2 levels have been found to be increased in patients with hepatitis C-induced liver disease as compared to healthy controls, and to decline in patients with a sustained virologic response to therapy.\(^{214}\) Consistent with these findings, \textit{in vitro} experiments have shown that Ang-2 is upregulated in hepatocytes infected with either hepatitis C or hepatitis B.\(^{215}\)
1.8 Angiopoietin-based Therapies

Treatment strategies targeting the angiopoietin/Tie-2 system have been proposed for a wide variety of disorders and diseases, although most have not yet progressed beyond testing in experimental models. *In vitro* or animals models using Ang-1 mimetic or Tie-2 stimulatory therapies have been successful in the treatment of: acute lung injury (ALI), pulmonary hypertension, diabetic wounds, fractures, ischemic necrosis, and destructive bone diseases (through enhancement of osteoblast differentiation and to promote angiogenesis in *ex vivo* bone tissue engineering), cardiac, cerebral, and limb ischemia, ischemia-reperfusion-induced acute kidney injury, radiation-induced apoptosis of the microcapillary endothelial cells of the intestinal villi, and spinal cord injury, while those using Ang-2 inhibitory therapies have demonstrated positive results in the treatment of hematologic and solid tumour malignancies. Not all studies have been positive, however. Despite multiple animal and human studies documenting a link between angiopoietin dysregulation and pulmonary edema, treatment with Ang-1 did not attenuate ventilator-induced lung injury in mice. The sole clinical study of angiopoietin therapy involved AMG 386, a peptide-Fc fusion protein that blocks the Tie-2 receptor. In a Phase 1 study of 22 patients with various advanced solid tumour malignancies, AMG 386 was well-tolerated and appeared to exert some anti-tumour effect, although all patients were receiving concomitant full-dose standard chemotherapy. While the angiopoietin/Tie-2 system is an attractive therapeutic target, further studies are clearly needed to determine when, how, and in what patient population anti-angiogenic therapy is best deployed.
1.9 Biomarkers of Endothelial Dysfunction in Sepsis

The manifestations of sepsis are notoriously protean (the 2001 International Definitions Conference limned 24 criteria, some or all of which may be used to diagnose sepsis in association with documented or suspected infection), yet early diagnosis and treatment is crucial, with delay directly associated with mortality.\textsuperscript{25,231,232} The results of confirmatory diagnostic tests, and in particular microbiologic cultures, can take hours to days. Furthermore, clinical scores and traditional markers of inflammation (leukocyte count) and tissue hypoxia (lactate) are non-specific or reflect the end result of sepsis rather than the underlying pathophysiology. Therefore, the ideal biomarker for use in sepsis would derive from one of the two major dysregulated systems (either the inflammatory or endothelial/vascular system), would discriminate between septic and non-septic patients with SIRS, would allow prognostication at the time of first contact with the healthcare system, and would be reproducible across various patient populations. What follows is a discussion of the evidence for and against several proposed diagnostic and/or prognostic biomarkers, representative of either the inflammatory or vascular pathway, and their use in sepsis.

1.9.1 Procalcitonin

A prodigious literature has been generated describing the rationale for and clinical utility of procalcitonin (PCT) as a diagnostic and prognostic biomarker in a variety of infectious diseases, and has been most recently reviewed by Gilbert.\textsuperscript{233} Procalcitonin is the 12.6 kDa prohormone of calcitonin, itself produced by the medullary C cells of the thyroid gland in response to hypercalcemia. Plasma levels in healthy adults are low, but procalcitonin levels rise within 2-4 hours of bacteremia or endotoxemia, earlier than other markers of inflammation such as C-reactive protein (CRP) or the erythrocyte sedimentation rate (ESR). PCT appears to be relatively specific for infection, and does not increase in response to non-infectious inflammatory states such as autoimmune connective tissue disease or inflammatory bowel disease.\textsuperscript{234,235} Interestingly, PCT production by endothelial-adherent monocytes is transient, and has only been documented \textit{in vitro}.\textsuperscript{236} PCT in patients with infection appears to arise from monocyte-stimulated adipocytes, and has been documented to act as an \textit{in vitro} monocyte chemoattractant.\textsuperscript{237,238} Human neutrophils increase production of pro-inflammatory cytokines in response to PCT \textit{in vitro}, but demonstrate decreased migration.\textsuperscript{239} Hence, the pathophysiologic role of PCT in the \textit{in
in vivo response to infection is not clear. Further obscuring its true in vivo effect are studies demonstrating that exogenous procalcitonin produces no effect when administered to healthy animals, but increases mortality when administered during sepsis.240

Despite its questionable role in the immune response, much interest has been generated in the potential utility of PCT to differentiate bacterial from non-bacterial infection. A 2004 meta-analysis by Simon et al included 12 prospective studies that compared PCT and CRP, and calculated sensitivities of 85% and 82%, and specificities of 83% and 88%, for the use of PCT to differentiate between bacterial infection and either non-infectious inflammatory states or viral infections, respectively.241 In both cases, PCT outperformed CRP. A second meta-analysis focused on PCT in the Emergency Department or at admission for the diagnosis of bacteremia, and included 17 studies (none included in the 2004 analysis) with a total of 2008 patients.242 PCT was found to be moderately predictive of bacteremia, with a negative likelihood ratio of 0.3 helping to rule out the diagnosis. PCT levels have also been found to discriminate between Gram positive and Gram negative bacteremia in septic patients in the ICU.243 A cut-off value of 16 ng/mL yielded a positive predictive value of 83% and a negative predictive value of 74% for the diagnosis of Gram negative bacteremia in this population. Neither CRP, nor leukocyte count, nor the SOFA score were discriminative in this setting. Notably, however, mortality was also higher in those patients with Gram negative bacteremia, and the positive likelihood ratio of 4.2 indicated only moderate clinical utility. When followed routinely in the ICU, a decrease in PCT levels between days 2 and 3 is predictive of appropriate empiric antibiotic therapy,244 suggesting that PCT may have a role in monitoring therapeutic response. PCT was able to distinguish between patients with community-acquired pneumonia and those with non-infectious exacerbations of asthma or COPD, however, CRP was slightly more predictive than PCT in this setting.245 PCT at a cut-off value of 0.8 ng/mL had a sensitivity of 91% and specificity of 68% for the diagnosis of secondary bacterial infection during the 2009 H1N1 pandemic influenza season.246 It should be noted, however, that not all studies have confirmed the specificity of PCT for bacterial infection.247

PCT has been evaluated in at least two meta-analyses, one including 33 studies and the other 18, for its utility in distinguishing sepsis from non-infectious inflammatory causes of SIRS in critically ill patients. However, the two studies reported widely divergent results. The first, by Uzzan et al, evaluated 3943 patients and concluded that PCT had good predictive value and
outperformed CRP for the differentiation of sepsis versus SIRS in critically ill patients after surgery or trauma, while the second, by Tang et al, evaluated 2097 patients and concluded that PCT was insufficiently sensitive (71%) for the differentiation of sepsis versus SIRS in a mixed population (medical and surgical) of critically ill adults. The diagnostic odds ratio for PCT reported by Tang et al was less than half that reported by Uzzan et al. In at least one study reported since, of 539 consecutive adult patients presenting to the Emergency Department with suspected infection, PCT could distinguish between severe sepsis and other infectious/non-infectious presentations, but not amongst non-severe sepsis, non-septic bacterial infection, and non-infectious presentations, the clinical situations in which it would be most helpful. Based on these results, the utility of PCT seems to vary significantly according to the clinical setting and patient population, making generalization difficult and reinforcing the need for specific study in each population in which its use is intended.

Importantly, procalcitonin levels are not affected by advanced human immunodeficiency virus (HIV) disease, and can distinguish between respiratory infection with Pneumocystis jirovecii, Mycobacteria tuberculosis, and community-acquired bacteria in this population, as well as between bacteremia and disseminated viral, mycobacterial, and fungal infections, localized bacterial infections, and cerebral toxoplasmosis.

In addition to its potential role in the diagnosis of bacterial infection, procalcitonin has also been proposed as a prognostic marker in sepsis and severe bacterial infection. Table 1 presents a thorough, though not exhaustive, compilation of positive and negative studies examining procalcitonin for the prediction of mortality at Emergency Department presentation, hospital or ICU admission, onset of illness, or up to 1 week into the course of illness. Some studies have also advocated the use of the PCT trajectory over time as a means to increase predictive value. Regardless, as is illustrated by Table 1, the most appropriate use of PCT (in terms of timing, duration of sampling, and target patient population) for the prediction of mortality in sepsis remains controversial.
<table>
<thead>
<tr>
<th>Author</th>
<th>Study Population</th>
<th>Significant PCT Measure</th>
<th>Predicted Outcome</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bloos F et al&lt;sup&gt;254&lt;/sup&gt;</td>
<td>175 patients requiring mechanical ventilation for CAP, HAP, and (new) VAP</td>
<td>PCT on days 1-14, starting within 48 hours of diagnosis</td>
<td>28-day mortality</td>
<td>Daily PCT levels higher in non-survivors from days 1-14; Initial cut-off value 1.1 ng/mL, peak cut-off value 7.8 ng/mL&lt;br&gt;Predictive value equal to APACHE II</td>
</tr>
<tr>
<td>Boeck L et al&lt;sup&gt;255&lt;/sup&gt;</td>
<td>101 patients with VAP</td>
<td>Serum PCT at diagnosis</td>
<td>28-day mortality</td>
<td>PCT levels failed to decline over time in non-survivors</td>
</tr>
<tr>
<td>Boussekey N et al&lt;sup&gt;256&lt;/sup&gt;</td>
<td>110 patients requiring ICU admission for CAP</td>
<td>Serum PCT within 48 hours of ICU admission</td>
<td>ICU mortality</td>
<td>CRP not significant</td>
</tr>
<tr>
<td>Charles PE et al&lt;sup&gt;244&lt;/sup&gt;</td>
<td>180 patients with sepsis in the ICU</td>
<td>PCT on day 3</td>
<td>ICU mortality</td>
<td>PCT levels on day 1 not predictive</td>
</tr>
<tr>
<td>Clec’h C et al&lt;sup&gt;257&lt;/sup&gt;</td>
<td>75 consecutive ICU patients with shock</td>
<td>Serum PCT on day of diagnosis</td>
<td>ICU mortality</td>
<td>SN 87.5%, SP 54%, 6 ng/mL cut-off</td>
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<tr>
<td>Author</td>
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<tr>
<td>Dahaba AA et al 258</td>
<td>69 patients requiring ICU admission for severe sepsis within 24 hours of surgery</td>
<td>Plasma PCT at day 6</td>
<td>28-day mortality due to sepsis</td>
<td>No difference in PCT between survivors and non-survivors on days 1-4; SN 85%, SP 89%, 3.2 ng/mL cut-off day 6</td>
</tr>
<tr>
<td>Giamarellos-Bourboulis EJ et al 259</td>
<td>1156 hospitalized patients with sepsis (234 in the ICU)</td>
<td>PCT with 24 hours of diagnosis of sepsis</td>
<td>In-hospital mortality</td>
<td>Cut-off values 0.12 ng/mL in non-ICU patients and 0.85 ng/mL in ICU patients</td>
</tr>
<tr>
<td>Gibot S et al 260</td>
<td>63 critically ill patients with sepsis</td>
<td>None</td>
<td>ICU mortality</td>
<td>Only sTREM-1 remained predictive of mortality in a multivariate analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plasma PCT within 12 hours of ICU admission was measured</td>
</tr>
<tr>
<td>Guan J et al 261</td>
<td>37 patients with septic shock and initial PCT levels &gt; 10 ng/mL</td>
<td>Δ PCT from days 1-5</td>
<td>28-day mortality</td>
<td></td>
</tr>
<tr>
<td>Hillas G et al 262</td>
<td>45 consecutive patients with VAP</td>
<td>PCT and ΔPCT on days 1 and 7</td>
<td>28-day mortality</td>
<td>OR 7.23 for ΔPCT1-7</td>
</tr>
<tr>
<td>Author</td>
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<tr>
<td>Huang DT <em>et al</em> 263</td>
<td>1651 patients presenting to the ED with CAP</td>
<td>PCT at presentation</td>
<td>30-day mortality</td>
<td>PCT identifies low-risk patients from amongst those identified as high risk by PSI and CURB-65</td>
</tr>
<tr>
<td>Karlsson S <em>et al</em> 264</td>
<td>242 critically ill patients with sepsis</td>
<td>None</td>
<td>In-hospital mortality</td>
<td>Serum PCT at day 0 measured</td>
</tr>
<tr>
<td>Lee CC <em>et al</em> 265</td>
<td>525 consecutive patients presenting to the ED with sepsis</td>
<td>Serum PCT on admission</td>
<td>Early (≤ 5 d) and late (6-30 d) mortality</td>
<td>MEDS score &gt; PCT &gt; CRP for predicting either outcome</td>
</tr>
<tr>
<td>Menendez R <em>et al</em> 266</td>
<td>453 patients hospitalized for CAP</td>
<td>None</td>
<td>30-day mortality</td>
<td>Only CRP remained a significant predictor of mortality in a multivariate analysis</td>
</tr>
<tr>
<td>Meng FS <em>et al</em> 267</td>
<td>86 patients with severe sepsis requiring admission to the ICU</td>
<td>Serum PCT on the day of ICU admission</td>
<td>28-day mortality</td>
<td>Outperformed CRP and APACHE II for prediction of mortality</td>
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<tr>
<td>Author</td>
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<tr>
<td>Novotny A et al 268</td>
<td>160 patients with sepsis following major visceral surgery</td>
<td>Serum PCT on the day of diagnosis of sepsis</td>
<td>Sepsis-related mortality</td>
<td>SN 71%, SP 77% for PCT + APACHE II at diagnosis</td>
</tr>
<tr>
<td>Oberholzer A et al 269</td>
<td>124 patients with severe sepsis</td>
<td>None</td>
<td>28-day mortality</td>
<td>Neither baseline plasma PCT nor change over days 1-4 were predictive of mortality</td>
</tr>
<tr>
<td>Phua J et al 270</td>
<td>72 consecutive patients with septic shock within 24 hours of admission to the ICU</td>
<td>Increase in plasma lactate + serum PCT between days 1 and 2</td>
<td>28-day mortality</td>
<td>APACHE II was the only significant variable at admission; IL-1β, IL-6, IL-10, TNF not significant</td>
</tr>
<tr>
<td>Rau BM et al 271</td>
<td>82 patients with secondary peritonitis, within 96 hours of symptom onset</td>
<td>PCT ≥ 1 ng/mL after the first week</td>
<td>In-hospital mortality</td>
<td>Outperformed CRP</td>
</tr>
<tr>
<td>Ruiz-Alvarez MJ et al 272</td>
<td>103 consecutive patients requiring ICU admission for suspected sepsis</td>
<td>None</td>
<td>ICU mortality</td>
<td>Neither serum PCT nor CRP within 24 hours of admission were predictive of mortality</td>
</tr>
<tr>
<td>Author</td>
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<tr>
<td>van Langevelde P et al 273</td>
<td>464 febrile patients presenting to the Medicine ED</td>
<td>PCT at presentation</td>
<td>In-hospital mortality</td>
<td>RR 5.0 (95% CI 1.7 - 15) for PCT level ≥ 0.5 ng/mL</td>
</tr>
<tr>
<td>Viallon A et al 274</td>
<td>131 consecutive patients presenting to the ED with sepsis</td>
<td>PCT at presentation</td>
<td>30-day mortality</td>
<td>SAPS II and lactate significant; serum IL-6, IL-8, TNF, and CRP not significant</td>
</tr>
<tr>
<td>Wunder C et al 275</td>
<td>33 consecutive ICU patients with severe sepsis</td>
<td>Plasma PCT</td>
<td>28-day mortality</td>
<td>APACHE III score also significant, but not IL-6 or IL-10</td>
</tr>
</tbody>
</table>

**Table 1.** Selected studies evaluating the use of procalcitonin to predict mortality in adult patients with sepsis or severe bacterial infection. Sepsis defined as per ACCP/SCCM standard definitions unless otherwise indicated. Community-acquired pneumonia (CAP), hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), Procalcitonin (PCT), Acute Physiology And Chronic Health Evaluation (APACHE), Intensive Care Unit (ICU), C-reactive protein (CRP), Sensitivity (SN), Specificity (SP), soluble Triggering Receptor Expressed on Myeloid cells-1 (sTREM-1), Emergency Department (ED), Pneumonia Severity Index (PSI), CURB-65 (alternate pneumonia severity score), Mortality in Emergency Department Sepsis (MEDS) score, Odds Ratio (OR), Tumour Necrosis Factor (TNF), Relative Risk (RR), Simplified Acute Physiology Score (SAPS), Change in (Δ).
Procalcitonin has also been proposed for use as a prognostic indicator in non-septic patients. In a study of 472 consecutive patients admitted to the ICU of a tertiary care hospital in Denmark, routine daily procalcitonin measurements revealed that a maximum procalcitonin level of ≥ 1 ng/mL and a rising procalcitonin level were both independent predictors of 90-day all-cause mortality.\(^{276}\) It should be noted that leukocyte count and C-reactive protein (CRP) were also measured but were not similarly predictive. Schneider et al studied 220 unselected patients who required post-operative admission to an intensive care unit for therapy or monitoring and found that, in a multivariate analysis, admission procalcitonin values were independently predictive of in-hospital mortality (sensitivity 81% and specificity 80% for a cut-off value of 1.44 ng/mL) and were superior in this regard to the APACHE II score and to IL-6.\(^{277}\)

In children, procalcitonin levels have been associated with severity of illness in meningococcal and sepsis post-bone marrow transplant, and performed better than CRP in both populations.\(^{278, 279}\) In the latter group, procalcitonin was also associated with mortality. Similarly, in a study of 75 children with septic shock, ICU admission levels of procalcitonin, TNF, and IL-10 were all associated with mortality, however, none performed as well as a pediatric clinical risk score unless followed serially.\(^{280}\) In a recent meta-analysis, procalcitonin proved to have good discriminative capacity for the differentiation of sepsis from other critical illnesses in neonates.\(^{281}\) Carrol et al evaluated 377 Malawian children with meningitis or pneumonia, of whom 279 had a confirmed bacterial etiology.\(^{282}\) Plasma procalcitonin was higher in severe bacterial infection regardless of HIV status (as was CRP), and predicted mortality. Of note, however, the area under the ROC curve for procalcitonin in the prediction of mortality was only 0.61 (95% CI 0.50 - 0.71). Furthermore, the ability of both PCT and CRP to distinguish between viral and bacterial pneumonia in Mozambican children was abolished in the presence of detectable *Plasmodium falciparum* (malaria) parasitemia,\(^{283}\) indicating the need for caution in both the use and study of PCT in malaria-endemic areas.

In summary, PCT may be useful in the diagnosis of bacterial infections, and may be a more specific predictor of outcome than cytokines and other non-specific markers of inflammation such as CRP. However, it may need to be measured over time, and it is not clear that it is a better predictor of outcome in all cases than are standard clinical prediction scores. Furthermore, PCT
shows considerable variability in its utility in different populations, particularly amongst those with malarial co-infection, indicating the need for further study.

1.9.2 C-reactive protein

C-reactive protein (CRP) is a 21 kDa acute phase reactant protein synthesized by the liver in a process upregulated by inflammation. When evaluated for use as a biomarker, CRP has often yielded contradictory results in patients with sepsis. As illustrated in Table 1, CRP, when compared to PCT, has variously been found to be more, less, or equally predictive of mortality in sepsis. CRP levels in ALI/ARDS have been reported in at least one study to be higher in survivors than non-survivors, even when adjusted for potential confounding factors such as corticosteroid use and hepatic failure. Therefore, despite the fact that CRP assays are widely available for clinical use, the appropriate interpretation of the results in any given clinical context will require further study.

1.9.3 sTREM-1

Triggering Receptor Expressed on Myeloid cells (TREM)-1 is found on monocytes, macrophages, and neutrophils, and consistent with its distribution on cells of the innate immune system, is upregulated by TLR signalling. TREM-1 is thought to amplify the immune response to pathogens, but whether it is beneficial, detrimental, or potentially both (in a context-dependent manner) is in question. In a mouse model of pneumococcal pneumonia, TREM-1 signalling enhances cytokine production, bacterial clearance, and survival. Consistent with this protective effect, soluble TREM-1 (sTREM-1) levels on the day of ICU admission in 63 patients with sepsis were lower in non-survivors than survivors, and remained predictive of outcome in a multivariate analysis (in contrast to procalcitonin and CRP). In contrast, blockade of TREM-1 has been variously reported to increase both survival and mortality in murine models of polymicrobial sepsis induced by cecal ligation and puncture, but has also been shown to improve survival in a murine model of endotoxemia. Consistent with the latter finding, sTREM-1 has been shown to correlate with severity of illness in a study of 52 critically ill patients with sepsis, while PCT and CRP did not. sTREM-1 levels were found to be higher in the non-survivors than survivors at days 10 and 14 of admission. Other authors have also documented an association between elevated levels of sTREM-1 and severity of illness in sepsis. Further complicating the interpretation of sTREM-1 levels in sepsis are the findings from a study of 65
septic patients in a surgical ICU, in whom plasma sTREM-1 measured within 24 hours of diagnosis could not distinguish between SIRS and sepsis, nor between survivors and non-survivors (28-day mortality).\(^\text{291}\)

sTREM-1 has also been proposed as a means to distinguish infectious from non-infectious inflammatory states.\(^\text{292}\) In a small study of patients with febrile neutropenia, both sTREM-1 and PCT were associated with bloodstream infections and mortality, with levels of both markers higher in non-survivors, while in a larger study of patients with febrile neutropenia, sTREM-1 performed better for the diagnosis of microbiologically documented infection than did PCT, with sensitivities of 88% and 48%, respectively.\(^\text{293, 294}\) sTREM-1 has been reported to discriminate between infectious and non-infectious pulmonary infiltrates in hospitalized, non-critically ill patients with acute respiratory illness, yielding a sensitivity of 83% and specificity of 63%.\(^\text{295}\) Furthermore, sTREM-1 performed better than usual clinical markers in predicting bacteremia in patients with community-acquired pneumonia, with low admission sTREM-1 levels demonstrating a negative predictive value of 99%.\(^\text{296}\) However, at least one study has reported that sTREM-1 performs less well than CRP in distinguishing septic versus non-septic patients with SIRS,\(^\text{297}\) underscoring the variability found in clinical studies of sTREM-1.

1.9.4 Chi3L1 (YKL-40)

Chitinase-3-like protein 1 (Chi3L1) is a 40 kDa protein secreted by activated neutrophils and macrophages in response to IL-6. Levels of Chi3L1 peak 6 to 24 hours following IL-6 infusion.\(^\text{298}\) The function of Chi3L1 is not known, and although it is a secreted protein, no cell surface or soluble receptors have yet been identified.

In hospitalized patients with bacterial community-acquired pneumonia, serum Chi3L1 levels are elevated as compared to healthy controls, peak on day 1, and decline to baseline levels by day 3.\(^\text{299}\) Interestingly, patients with pneumococcal pneumonia demonstrated the greatest elevation in Chi3L1, while those with pneumonia due to \textit{H. influenzae} or the “atypical” organisms had Chi3L1 levels only modestly above baseline. In keeping with this finding, serum Chi3L1 levels are elevated in patients with pneumococcal bacteremia as compared to healthy controls, and relate to the severity of infection (defined as need for hemodialysis, vasopressor medications, or mechanical ventilation) and in-hospital mortality in these patients.\(^\text{300}\) In a multivariate analysis, Chi3L1 was found to be an independent predictor of mortality in the same population. Elevated
Chi3L1 levels have also been documented in association with Gram negative sepsis. In experimental human *Escherichia coli* (*E. coli*) endotoxemia, Chi3L1 levels rose significantly above baseline at 2 hours and peaked at 24 hours post-infusion. The Chi3L1 response trailed that of TNF and IL-6, whose levels peaked at 1.5 and 2 hours post-infusion, respectively. In addition, proteomic analysis identified Chi3L1 as one of three proteins uniquely upregulated in septic, as compared to non-septic, critically ill patients. Serum levels at the time of ICU admission were significantly higher in septic versus non-septic patients, and correlated with severity of illness and circulating levels of IL-6.

### 1.9.5 IP-10

Interferon-γ-inducible protein-10 kDa (IP-10 or CXCL10) is a pro-inflammatory chemokine that is involved in the chemotaxis of monocytes and T cells to sites of inflammation. IP-10 was found to correlate with severity of illness and mortality in a univariate analysis of a small study of 16 patients with sepsis, and in addition was found to have excellent sensitivity and specificity in the diagnosis of bacterial sepsis in very low birth weight neonates. IP-10 has been shown to effectively discriminate between survivors and non-survivors of severe malaria in a study conducted in Ugandan children, a finding in keeping with evidence from animal models in which IP-10 gene knockout reduced peripheral parasitemia and mortality.

IP-10 is known to be elevated in both latent tuberculosis (TB) and active TB, regardless of immunosuppression due to corticosteroids and disease-modifying anti-rheumatic drugs (DMARDs). IP-10 levels in the pleural fluid are significantly different between patients with tuberculous pleural effusions and those with pleural effusions of other etiologies. Furthermore, IP-10 has been studied and found to be effective as a novel measurable end-product of a modified Interferon-γ Release Assay (IGRA) in which patient plasma is incubated *ex vivo* with purified specific *M. tuberculosis* proteins.

### 1.9.6 PF4

Platelet Factor 4 (PF4) is a marker of platelet activation after release from platelet alpha granules, and is upregulated in septic shock. By binding to both protein C and thrombomodulin, PF4 can augment activated protein C generation *in vivo*, and improve survival in animal models of sepsis. In a small study of ten patients with sepsis, PF4 levels were
elevated in the acute phase of illness and declined significantly with effective therapy, but the utility of PF4 as a biomarker in sepsis has not been further investigated.

1.9.7 vWF

von Willebrand Factor (vWF) is stored in the Weibel-Palade bodies of endothelial cells for release upon endothelial cell activation. It acts to stabilize the adhesion of platelets at sites of vascular injury, and its release has been associated with microvascular thrombosis in animal models of experimental endotoxemia, although not in cecal ligation and puncture-induced polymicrobial sepsis. In a small study of 14 patients with sepsis, bacteremia, and disseminated intravascular coagulation (DIC), plasma levels of vWF were significantly elevated when compared to non-septic hospitalized patients, and correlated with the plasma level of fibrinogen degradation products (FDPs), a marker of the severity of DIC and therefore indirectly of sepsis. There was no correlation with lactate or platelet count. Other studies have also documented an association between vWF and severity of illness in sepsis. A study of 50 mechanically ventilated patients in the ICU, enrolled within 12 hours of the diagnosis of sepsis, found that the vWF concentration on the day of enrolment, but not thereafter, was predictive of mortality during the ICU admission. However, not all studies have confirmed the association between vWF levels and mortality in sepsis. In addition, there is conflicting literature as to whether vWF is predictive of the development of ARDS in at-risk patients, although elevated vWF levels have been associated with mortality in patients with ALI.

1.9.8 sICAM-1

ICAM-1 plays a crucial role in neutrophil recruitment to sites of inflammation and injury. Expressed constitutively at low levels on the endothelial cell surface but substantially upregulated during endothelial cell activation, ICAM-1 interacts with β2-integrin on the surface of the neutrophil to mediate firm adhesion, a necessary prerequisite to eventual neutrophil extravasation. A soluble form of ICAM-1 can be detected in plasma and increases in concert with the cell surface form during inflammation. Hence, soluble ICAM-1 (sICAM-1) has been studied as a marker of inflammation.

In a small study of 25 critically ill patients with sepsis, plasma levels of sICAM-1 at the time of diagnosis/study entry were significantly higher than those in non-septic hospitalized patients and
in eventual non-survivors versus survivors. In a multivariate analysis, sICAM-1 remained predictive of survival. Similar results were seen in other studies, including a large trial of 221 patients with sepsis, however, sICAM-1 performed less well in this setting than did other markers of endothelial dysfunction such as sFlt-1 (see below). 

1.9.9 VEGF

VEGF plays a pivotal role in angiogenesis as outlined in Chapter 1.2, and is also linked to the inflammatory pathway. In a mouse model of sepsis, circulating VEGF increases in a time-dependent manner. Furthermore, reducing VEGF signalling via a soluble form of its receptor attenuated the clinical signs of sepsis in the same model. Despite such promising results, the findings in human studies have been contradictory. While most authors agree that circulating levels of VEGF are elevated in sepsis, studies have yielded conflicting results as to whether the degree of elevation correlates with disease severity. Although VEGF has been associated with the development of ALI/ARDS in septic patients, it is less predictive of this outcome than are the angiopoietins. Also unexpected given the results of the animal model was a large study of 293 Malawian children with severe bacterial infection, in whom plasma VEGF concentrations were higher in survivors than in non-survivors. However, this difference did not remain significant in a multivariate analysis. Other studies of sepsis have also failed to detect an association between VEGF level and outcome.

1.9.10 sFlt-1

Fms-like tyrosine kinase receptor 1 (sFlt-1) is the first of two VEGF receptors (VEGFR1). Both the transmembrane and soluble form of Flt-1 may act to trap VEGF and prevent binding to Fms-like tyrosine kinase receptor (Flk-1; VEGFR2), which is capable of transducing a much stronger signal. This role is consistent with the interactions of sFlt-1 and the Dll4/Notch system. During sprouting angiogenesis, VEGFR1 is upregulated, and VEGFR2 downregulated, by Notch signalling in stalk cells, thereby limiting their responsiveness to the surrounding high concentrations of VEGF required to guide the tip cells, and promoting organized, unidirectional angiogenesis. In this way, sFlt-1 may function to fine-tune the actions of VEGF.

In 83 patients with suspected infection, sFlt-1 levels upon presentation to the Emergency Department were higher in those with than without shock, and correlated with the APACHE II
and SOFA scores.\textsuperscript{331} A similar study, this time including 221 patients with sepsis of varying severity, confirmed those findings.\textsuperscript{34} In 10 patients with febrile neutropenia, sFlt-1 measured 48 hours after fever onset was significantly higher amongst those patients who would develop septic shock than amongst those who would continue to manifest only uncomplicated sepsis.\textsuperscript{332} Nonetheless, neither the pathophysiologic role nor the source of sFlt-1 in sepsis is clear.

Of note, sFlt-1 has also been implicated in malaria, and plasma levels were predictive of mortality in a large study of 156 Ugandan children with malaria.\textsuperscript{305}

\section*{1.9.11 Miscellaneous Markers of Endothelial Dysfunction in Sepsis}

As might be expected, many other circulating factors have been explored as potential prognostic indicators in sepsis. A recent review documented 3370 references detailing studies on 178 different biomarkers.\textsuperscript{336} Few of those that pertain to endothelial dysfunction have as well-defined a pathophysiologic role and as reproducible a clinical prognostic utility as the angiopoietins. For instance, although pro-inflammatory cytokines would seem an obvious choice for prognostic indicators in sepsis, their utility has not been confirmed in large studies. In a study of 60 patients with severe sepsis, plasma levels of pro-inflammatory cytokines, including IL-1\(\beta\), IL-6, IL-8, and TNF, were higher at diagnosis in patients with shock than in those without.\textsuperscript{337} Plasma levels of IL-8 and monocyte chemoattractant protein (MCP)-1 correlated best with the SOFA score, and also predicted 48-hour and 28-day mortality, however, only MCP-1 was associated with outcome in a multivariate analysis. One of the largest studies, involving 1690 patients at enrolment in the PROWESS (recombinant human activated Protein C Worldwide Evaluation in Severe Sepsis) study, measured IL-1\(\beta\), TNF, IL-6, and IL-8, the pro-inflammatory cytokines most commonly associated with sepsis.\textsuperscript{338} While IL-8 and TNF correlated with APACHE II score, few patients had detectable IL-1\(\beta\), TNF, or IL-10 (an anti-inflammatory cytokine) levels at study entry, reflecting their early and transient release during sepsis. Furthermore, serum IL-6 levels at the time of enrolment did not predict 28-day mortality in the 840 patients randomized to the study’s placebo arm. In addition, anti-cytokine therapies have not proven useful in the treatment of septic patients.\textsuperscript{339, 340}

Many other candidate biomarkers have also been studied in sepsis. E-selectin has been associated with severity in sepsis and mortality in critically ill patients.\textsuperscript{34, 342} While some studies have
documented an association with outcome in severe infection, others have not.\textsuperscript{326, 342-344}

Endothelial progenitor cells are known to play a role in vascular repair, are elevated in sepsis, and have been found to correlate with the outcome of sepsis in at least one small study.\textsuperscript{345-347}

Circulating endothelial microparticles have also been evaluated as markers of severity in sepsis, however, controversy still exists as to whether they exert a pro- or anti-inflammatory effect.\textsuperscript{348, 349}

Finally, serum levels of various matrix metalloproteinases (MMPs) were found to be abnormal at the time of diagnosis of sepsis, although the direction of change was dependent on the particular MMP studied. MMP-10 was higher in septic than non-septic patients, while MMP-9 was lower in non-survivors than survivors of sepsis.\textsuperscript{350} Since conflicting data regarding MMPs in sepsis exist, further study is needed.\textsuperscript{351}

1.9.12 Conclusion

In summary, although multiple candidate biomarkers have been proposed for use in sepsis, none have been uniformly and consistently shown to be clinically useful as either diagnostic or prognostic indicators in sepsis. Nor have any met the prespecified criteria delineated in Chapter 1.9 for the ideal biomarker: one that would derive from one of the two major dysregulated systems, discriminate between septic and non-septic patients with SIRS, allow prognostication at the time of first contact with the healthcare system, and be reproducible across various patient populations. Thus there is the need for further study in this area, and importantly, for the direct comparison of performance characteristics of candidate molecules in a single patient population.
Chapter 2
Research Aims and Hypotheses

2.1 Research Aims

The specific aims of the project are:

1) To determine whether angiopoietin-1 and -2 are dysregulated in human diseases characterized by diffuse endothelial cell activation,

2) To define the clinical consequences (morbidity and mortality) associated with abnormal angiopoietin levels, and the prognostic or predictive value of these levels,

3) To explore the clinical utility of biomarker combinations to predict mortality in septic patients in a resource-limited setting.

2.2 Hypotheses

1) Since the angiopoietins are prominent regulators of endothelial cell function, dysregulation of the angiopoietins (decreased Ang-1 and increased Ang-2) will correlate with disease severity and outcome in infectious diseases associated with significant endothelial cell dysfunction, including STSS, HUS, and sepsis, and will be predictive of disease progression (reflecting the severity of endothelial dysfunction) when measured early in illness.

2) As outlined in Chapter 1, multiple pathways mediate reciprocal interactions between the immune system and the endothelium. Since sepsis is characterized by both immune and endothelial cell activation, combining biomarkers from the inflammatory and vascular pathways will more closely approximate the underlying pathophysiology and therefore will more accurately predict mortality in sepsis.
Chapter 3
Angiopoietin-1 and -2 in Invasive Group A Streptococcal Infection

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3.1 Introduction

Streptococcal toxic shock syndrome (STSS) remains a serious disease associated with poor clinical outcomes despite prompt recognition, timely surgical debridement, and rapid initiation of appropriate antimicrobial therapy. Much of the morbidity and mortality results from the release of toxins by invading group A streptococcal bacteria. GAS pyrogenic exotoxins function as superantigens to activate T cells in a Major Histocompatibility Complex (MHC) class II unrestricted manner, initiating a series of inflammatory reactions that result in diffuse endothelial cell activation, vascular leak, and the clinical characteristics of STSS: hypotension, major organ dysfunction, and coagulopathy. While massive pro-inflammatory cytokine release has been implicated in the pathogenesis of STSS, we hypothesized that angiopoietin-1 and angiopoietin-2, critical regulators of endothelial cell activation, might also play central roles in the development of STSS.

3.2 Methods

Beginning in 1992, prospective, population-based surveillance for invasive group A streptococcal disease has been undertaken in Ontario, Canada via mandatory laboratory reporting of S. pyogenes isolates cultured from normally sterile sites. Thirty-seven patients, enrolled between 1999 and 2009, were included in the current study. Informed consent was obtained to collect bacterial isolates and plasma samples, as well as selected clinical data from interviews with the attending physicians and patient chart review. Patients with invasive disease were considered to have streptococcal toxic shock if they met the consensus definition: hypotension in combination with at least two of coagulopathy, acute kidney injury, elevated serum aminotransferases, ARDS, rash, or necrotizing fasciitis.

Of the 37 patients included in this study, 16 had invasive streptococcal infection and toxic shock (STSS), while 21 had invasive streptococcal infection alone (no STSS). The median age was not significantly different between these groups (41.5 years with STSS versus 38 years without;
range from 10 to 74 years; \( P = \text{NS} \). The patients were predominantly female (56% in the group with STSS and 67% in the group without, \( P = \text{NS} \), and otherwise healthy, with only one-third of the patients in either group describing a pre-existing illness. Only 3 patients (2 with STSS and 1 with invasive disease alone) reported pre-existing illnesses potentially associated with angiopoietin dysregulation. The underlying source of infection was similar in each group, with the majority of patients in both groups having skin and soft tissue infections (7 patients (44%) with STSS and 12 patients (57%) with invasive streptococcal infection alone, \( P = \text{NS} \)). Amongst the patients with skin and soft tissue infections, 5 (71%) with STSS and 4 (33%) with invasive infection alone had necrotizing fasciitis, \( P = \text{NS} \). Presenting group A streptococcal infections in the remaining patients included respiratory tract infections, bacteremia without an identified source, post-partum infections, and peritonitis, and did not differ significantly between the groups. The two groups were significantly different only in the diagnostic criteria for STSS; hypotension was present in 100% of patients with STSS and 33% of patients without \( (P < 0.0001) \). Five patients with STSS died as compared to one patient with invasive infection alone (31% versus 5%, \( P = 0.06 \)).

Acute phase plasma samples were collected in heparinised tubes upon study enrolment and stored at -70°C until use. Convalescent plasma samples were collected at least one month post-enrolment and once all signs and symptoms of infection had resolved. Plasma concentrations of angiopoietin-1 and -2 were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis MN) according to the manufacturer’s instructions. Each sample was tested in duplicate and all samples were assayed simultaneously. The upper and lower limits of detection for the assays were \( 10000 \) pg/mL and \( 9.77 \) pg/mL for Ang-1 and \( 2520 \) pg/mL and \( 2.46 \) pg/mL for Ang-2, respectively. Samples were diluted in assay diluent (1:20 for Ang-1 and 1:4 for Ang-2) to fall within the range of the standard curves. Laboratory testing was performed by an investigator blinded to all clinical data.

Statistical analysis was performed using GraphPad Prism v4.03 and Instat v3.06 (GraphPad Software, San Diego, CA). To describe the study population, categorical variables were analyzed by the Fisher’s exact test and continuous variables with the Student’s t-test. Differences in median serum angiopoietin levels were assessed by the Kruskal-Wallis test followed by Dunn’s test for multiple comparisons. Matched acute and convalescent samples were compared using the Wilcoxon matched pairs test. Receiver operating characteristic (ROC) curves were generated.
using patients with STSS as “cases” and those with invasive streptococcal disease alone as “controls”, with the null hypothesis that the area under the curve (AUC) equals 0.5.

### 3.3 Results

Angiopoietin dysregulation (decreased Ang-1 and increased Ang-2) was associated with disease severity in patients with invasive group A streptococcal infection (Figure 1A). The median plasma concentration of Ang-1 was lower during the acute phase of illness in patients with invasive infection and STSS than in those with invasive streptococcal infection alone (13,915 pg/mL, [interquartile range (IQR): 5295 - 23,876 pg/mL] vs. 29,084 pg/mL [IQR: 11,855 - 54,803 pg/mL]), while the median plasma concentration of Ang-2 was higher (5752 pg/mL, [IQR: 1250 - 7695 pg/mL] vs. 1337 pg/mL [IQR: 539 - 2371 pg/mL]). As a result, the normally low Ang-2:Ang-1 ratio was significantly higher amongst patients with invasive infection and STSS as compared to those with invasive streptococcal infection alone (0.437 [IQR: 0.122 - 0.775] versus 0.048 [IQR: 0.014 - 0.327], P < 0.05).

Ang-1/2 dysregulation declined with convalescence in both groups of patients (Figure 1A). In the cohort of patients with STSS, the median plasma concentration of Ang-1 rose from 13,915 pg/mL to 21,115 pg/mL (IQR: 3517 - 41,373 pg/mL), the median plasma concentration of Ang-2 decreased from 5752 pg/mL to 378 pg/mL (IQR: 225 - 1097 pg/mL), P < 0.01, and the median Ang-2:Ang-1 ratio fell from 0.437 to 0.019 (IQR: 0.011 - 0.139), P < 0.05.

Receiver operating characteristic (ROC) curves were generated for Ang-1, Ang-2, and the Ang-2:Ang-1 ratio, and the area under the ROC curves indicated that the degree of magnitude of Ang-1/2 dysregulation accurately differentiated between those individuals with STSS and those without STSS (Figure 1B). The ROC curves for plasma Ang-2 (AUC: 0.759; 95% confidence interval (CI): 0.596 - 0.921; P = 0.009) and for the Ang-2:Ang-1 ratio (AUC: 0.791; 95% CI: 0.643 - 0.938; P = 0.003) discriminated between patients with STSS and those with invasive streptococcal infection alone (no STSS). The ROC curve for plasma Ang-1 concentration trended towards, but did not reach, statistical difference (AUC: 0.683; 95% CI: 0.506 - 0.860; P = 0.07).
Angiopoietin-1 and -2 (Ang-1 and Ang-2) in patients with invasive Group A streptococcal disease with and without streptococcal toxic shock syndrome (STSS). A. Absolute and median concentrations of Ang-1 and Ang-2, as well as Ang-2:Ang-1 expressed as log base 10, in acute and convalescent plasma from patients with or without STSS. * $P < 0.05$; ** $P < 0.01$. ● = survived; ○ = died. B. Receiver operating characteristic curves for each marker: Ang-1 (AUC: 0.683; 95% confidence interval (CI): 0.506 - 0.860; $P = 0.07$), Ang-2 (AUC: 0.759; 95% CI: 0.596 - 0.921; $P = 0.009$), and Ang-2:Ang-1 (AUC: 0.791; 95% CI: 0.643 - 0.938; $P = 0.003$).
In individual patients with STSS, the matched acute and convalescent plasma Ang-2 concentrations and the Ang-2:Ang-1 ratios also differed significantly (Figure 2). Although the same pattern was observed in the cohort of patients with invasive streptococcal disease without STSS, the changes in Ang-1/2 concentrations were more modest. The median plasma concentration of Ang-1 in this group increased only from 29 084 pg/mL to 31 743 pg/mL (IQR: 8443 - 54 645 pg/mL), while the Ang-2 concentration declined from 1337 pg/mL to 535 pg/mL (IQR: 203 - 1702 pg/mL), and the Ang-2:Ang-1 ratio decreased slightly from 0.048 to 0.027 (IQR: 0.008 - 0.091), $P = \text{NS}$ for all comparisons.
**Figure 2.** Angiopoietin-1 and -2 (Ang-1 and Ang-2) concentrations, and the ratio between the two (Ang-2:Ang-1), in matched acute and convalescent plasma samples from patients with STSS. \( P = 0.01 \) for Ang-2 in acute vs. convalescent plasma, and \( P = 0.03 \) for Ang-2:Ang-1 in acute vs. convalescent plasma by the Wilcoxon matched pairs test.
3.4 Discussion/Conclusion

This study represents the first report of an association between angiopoietin dysregulation and disease severity in invasive streptococcal infection. Ang-1/2 dysregulation, as reflected by an increased Ang-2:Ang-1 ratio, was significantly greater during the acute phase of illness in individuals with STSS than in those without (Figure 1). This finding confirms that angiopoietin dysregulation in invasive streptococcal disease is specifically related to the degree of endothelial dysfunction present, and not simply associated with the underlying infection. Further, the observation is consistent with our current understanding of angiopoietin dysregulation in other infectious disease syndromes associated with prominent endothelial cell activation. Ang-2 and/or the Ang-2:Ang-1 ratio have been found to be markers of disease severity in sepsis, ALI/ARDS, febrile neutropenia, and malaria. In our study, ROC curves suggested that both the absolute Ang-2 concentration and the Ang-2:Ang-1 ratio could effectively discriminate between patients with and without STSS (Figure 1).

The sequence of events leading to angiopoietin dysregulation during STSS is not yet known. S. pyogenes produces a variety of factors, including the M proteins, that have been shown to mediate endothelial cell function. Streptococcal superantigens may also be involved, whether through direct action on the vascular endothelium or through intermediary molecules. Streptococcal superantigens have long been known to mediate the release of pro-inflammatory cytokines, including TNF, which might function as a link to the angiopoietin system. Circulating and tissue-infiltrating mononuclear cells from patients with STSS have been shown to produce significantly higher levels of TNF and other inflammatory cytokines as compared to cells from patients with invasive streptococcal infection without shock, and TNF itself has been associated with the upregulation of Ang-2 expression in human endothelial cells in vitro, although not definitively with Ang-2 release. In turn, Ang-2 is known to sensitize endothelial cells to the pro-inflammatory effects of TNF, reflecting the type of complex interplay between the cytokine and angiopoietin systems that may occur during STSS. Ultimately, increased Ang-2 release in STSS leads to increased Ang-2 binding to the endothelial Tie-2 receptor, with subsequent induction of the activated, pro-thrombotic endothelial cell phenotype that contributes to fluid extravasation, shock, organ dysfunction, and coagulopathy. A similar mechanism has been proposed to explain angiopoietin dysregulation in sepsis. Together, our findings as well
as those of others are consistent with the possibility of a shared final common pathway of endothelial activation and diffuse vascular leak in the pathogenesis of sepsis, ALI/ARDS, and STSS. Although the current study cannot prove causation, it suggests that angiopoietin dysregulation may contribute to disease severity, and provides a rationale for future translational studies in this area.

Consistent with this hypothesis, convalescence and clinical recovery were associated with progressive normalization of the balance between Ang-1 and Ang-2, most dramatically in survivors of STSS (Figure 2). Similar results have been reported in sepsis, in which Ang-2 levels were also found to decline with convalescence. In agreement with the observation that endothelial cell function (activation versus quiescence) reflects the balance between local concentrations of both Ang-1 and Ang-2, we observed that the Ang-2:Ang-1 ratio was a consistent marker of both STSS and convalescence.

There are some notable limitations to this study. The small sample size restricted our ability to detect potentially important differences, particularly in Ang-1 levels that displayed a trend towards statistical significance. In addition, the overall mortality rate was low and not significantly different between those with STSS and those without, which limited the potential for conclusions regarding the prognostic value of plasma concentrations of Ang-1 and/or Ang-2 for clinical outcome. Finally, since serial samples were not available during the acute phase of illness in the current study, it is not possible to comment upon the predictive value of early angiopoietin dysregulation for disease progression and the development of STSS in invasive streptococcal infection. Elevation of the Ang-2:Ang-1 ratio and serum Ang-2 concentration have recently been reported to be predictive of the development of septic shock in patients with febrile neutropenia when measured at or within 48 hours of fever onset, respectively. It is therefore possible that close monitoring of the kinetics of angiopoietin dysregulation in the plasma of patients with invasive streptococcal disease could provide a reliable prognostic biomarker for disease progression and severity, and an indicator of response to various therapeutic interventions.

Despite the above limitations, our study adds to the growing body of evidence implicating endothelial cell dysfunction and angiopoietin dysregulation in life-threatening infectious disease syndromes, and suggests that angiopoietin dysregulation may be both a contributor to, and a
clinically informative biomarker of, disease severity in invasive streptococcal infection. On this basis, further studies exploring the potential role of angiopoietin dysregulation in the pathogenesis of STSS and the predictive value of angiopoietin dysregulation for both the development of STSS and mortality in invasive streptococcal infection are warranted.
Chapter 4
Angiopoietin-1 and -2 in the Hemolytic-Uremic Syndrome
Submitted for Publication, 2012

4.1 Introduction

The haemolytic-uremic syndrome (HUS) is defined by the triad of non-immune hemolytic anemia, thrombocytopenia, and renal failure. Once thought to be a consequence of toxin-induced glomerular endothelial cell ribosomal arrest and apoptosis, recent evidence suggests a more complex pathogenesis in HUS, involving Shiga toxin-induced microvascular endothelial cell activation. Although studies have shown that Shiga toxin can induce a prothrombotic and adhesive endothelial cell phenotype, the precise mechanism for endothelial cell activation/dysfunction in HUS has yet to be definitively determined.

We hypothesized that angiopoietin dysregulation (decreased Ang-1 and increased Ang-2) might be present and contribute to the pathogenesis of HUS. The bacterial serotype most commonly implicated in typical HUS is E. coli O157:H7, and those most at risk of developing HUS as a consequence of STEC hemorrhagic colitis are children. Therefore, we studied serum angiopoietin concentrations at various time points throughout illness in children with E. coli O157:H7 infection, with and without HUS.

4.2 Methods

Study population and specimen collection

Beginning in 1997, population-based surveillance for E. coli O157:H7 infection in children less than 10 years of age was undertaken in Washington, Oregon, Idaho, and Wyoming through mandatory laboratory reporting of positive stool cultures as previously described. Children from whom a positive culture was obtained within the first seven days after the onset of diarrhea were eligible for enrolment. Seventy-eight children, enrolled between 1998 and 2005, were included in the current study. Written informed consent from the parents, and assent from the child where appropriate, was obtained at study entry for the collection of epidemiologic and clinical data and for phlebotomy both at enrolment and as clinically indicated thereafter. HUS was diagnosed in the setting of hemolytic anemia (a hematocrit < 30% with evidence of
schistocytes on peripheral blood film), thrombocytopenia (platelet count < 150,000 cells/mm$^3$), and renal insufficiency (serum creatinine above the age-adjusted upper limit of normal); participants who had not met these criteria by day 14 following the onset of diarrhea were considered to have had uncomplicated infection. In total, 84 serum samples were tested (Figure 3): 26 from patients on the day of diagnosis of HUS, 8 from patients who would subsequently be diagnosed with HUS but had not yet met diagnostic criteria (pre-HUS), and 50 from patients with uncomplicated infection. Six patients had samples taken both prior to (pre-HUS) and on the day of, HUS diagnosis.

**Figure 3.** Timeline of blood sampling and division into study groups (uncomplicated infection, pre-HUS, HUS) of participants at various stages of *E. coli* O157:H7 infection.

**Measurement of serum Ang-1 and Ang-2**

Serum samples were stored in aliquots at -80°C until use. Serum concentrations of Ang-1 and Ang-2 were measured by ELISA (R&D Systems, Minneapolis MN) as per the manufacturer’s instructions. Each sample was tested in duplicate and all samples were assayed simultaneously. The technical upper limits of detection for the assay were 10,000 pg/mL for Ang-1 and 2520
pg/mL for Ang-2, yielding effective upper limits of detection of 200 000 pg/mL and 10 080 pg/mL, respectively, for the dilutions employed in the analysis. Lower limits of detection for the assay were 9.77 pg/mL for Ang-1 and 2.46 pg/mL for Ang-2.

**Statistical analyses**

Statistical analysis was performed using GraphPad Prism v.4.03 (San Diego, CA). Median serum angiopoietin levels were compared using the Kruskal-Wallis test followed by Dunn’s test for multiple comparisons. Matched pre-HUS and HUS samples were compared using the Wilcoxon matched pairs test. Receiver operating characteristic (ROC) curves were generated using pre-HUS patients as “cases” and those with uncomplicated infection as “controls”, with the null hypothesis that the area under the curve (AUC) equals 0.5.

**4.3 Results**

In this study of 78 children with *E. coli* O157:H7 infection, angiopoietin dysregulation (decreased Ang-1 and increased Ang-2) was associated with illness severity (Figure 4A). The median serum Ang-1 concentration in those with uncomplicated infection was significantly higher than in those with HUS (77 357 pg/mL [interquartile range (IQR): 53 437 - 114 889 pg/mL] versus 10 622 pg/mL [IQR: 3464 - 43 523 pg/mL]), *P* < 0.001. Conversely, the median serum Ang-2 concentration was significantly lower in those with uncomplicated infection than in those with HUS (1140 pg/mL [IQR: 845 - 1492 pg/mL] in patients with uncomplicated infection versus 1959 pg/mL [IQR: 1057 - 2855 pg/mL] in those with HUS), *P* < 0.05. Finally, the Ang-2:Ang-1 ratio was 0.014 (IQR: 0.011 - 0.023) in patients with uncomplicated infection, and more than 10-fold higher, at 0.18 (IQR: 0.066 - 0.51) in those with HUS, *P* < 0.001.
Figure 4. Serum Angiopoietin Concentration in *E. coli* O157:H7 Infection. A. Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), and the Ang-2:Ang-1 ratio expressed as log base 10 in children with uncomplicated *E. coli* O157:H7 infection (infected), children prior to the diagnosis of HUS (pre-HUS), and children at the time of diagnosis of HUS (HUS). *P*<0.05; **P**<0.001, o = outlier (1.5 x interquartile range [IQR]); • = extreme outlier (3 x IQR). B. Receiver Operating Characteristic (ROC) curves comparing Ang-1, Ang-2, and the Ang-1:Ang-2 ratio amongst children with uncomplicated infection and those in the pre-HUS phase of illness, with the null hypothesis that the area under the curve is 0.5. *P* = 0.01 for Ang-1.
Furthermore, serum Ang-1 concentration at the time of presentation to hospital effectively discriminated between two populations of clinically indistinguishable children: 1) those with uncomplicated hemorrhagic colitis and 2) those with hemorrhagic colitis who would eventually develop HUS (Area under the ROC curve [AUC]: 0.785, 95% confidence interval (CI): 0.641 - 0.923; \( P = 0.01 \)) (Figure 4B). A serum Ang-1 cut-off value of less than 48 000 pg/mL yields a sensitivity of 75% and a specificity of 80% for the diagnosis of the pre-HUS phase of illness. The ROC curves for the serum Ang-2 concentration and the Ang-2:Ang-1 ratio trended towards, but did not reach, statistical difference (AUC: 0.600, 95% CI: 0.357 - 0.843; \( P = 0.37 \)) and AUC: 0.685, 95% CI: 0.499 - 0.872; \( P = 0.09 \), respectively).

Data from individual patients closely mirrored the aggregate data presented above. Paired samples from patients on the day of diagnosis of infection (day of positive stool culture, or pre-HUS) and on the day of diagnosis of HUS, revealed the same trend: serum concentrations of Ang-1 fell, while the Ang-2:Ang-1 ratio rose (Figure 5).
Figure 5. Serum Angiopoietin Concentration in Individual Patients with *E. coli* O157:H7 Infection before and after the diagnosis of HUS. Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), and the Ang-2:Ang-1 ratio in matched serum samples from individual patients at the time of diagnosis of *E. coli* O157:H7 infection (pre-HUS) and at the time of diagnosis of HUS (HUS).
4.4 Discussion/Conclusion

The finding of angiopoietin dysregulation in HUS is consistent with the known biologic effects of Ang-1 and Ang-2, and with their predicted impact on endothelial cell function during the course of *E. coli* O157:H7 infection. Ang-1 and Ang-2 compete for binding to the shared Tie-2 receptor. In healthy individuals, constitutive release and binding of Ang-1 results in phosphorylation of the Tie-2 receptor, inhibition of the NF-κB pathway, and endothelial cell quiescence. In patients with HUS, inflammation-induced release and binding of Ang-2 is predicted to block the action of Ang-1, release the NF-κB pathway from inhibition, and activate the endothelium. The serum Ang-1 and Ang-2 concentrations reported here for children with uncomplicated infection are comparable to those found in the serum of healthy children and adults included as control subjects in other studies, and are in keeping with the clinical observation that there is little if any endothelial dysfunction present in these patients. In contrast, the relative deficit of Ang-1 and excess of Ang-2 in children with HUS would be expected to produce significant endothelial cell activation. As a consequence, constitutive Ang-1 inhibition of the actions of TNF and VEGF, upregulated by Shiga toxin or in HUS respectively, would be diminished, leading to increased expression of tissue factor, VCAM-1, ICAM-1, and E-selectin on the endothelial cell surface. Furthermore, Ang-2 itself has been shown to sensitize endothelial cells to the effects of TNF. The endothelial phenotype that is predicted to result from an increased Ang-2:Ang-1 ratio is therefore prothrombotic and adhesive, and in keeping with the characteristic thrombotic microangiopathy of HUS. Our observations are similar to those made in other disorders of endothelial cell function, including sepsis, cerebral malaria, and streptococcal toxic shock. In each of these infectious syndromes, an association has been described between the degree of angiopoietin dysregulation and the severity of illness, and likely reflects a progressive degree of endothelial dysfunction. Taken together, these findings suggest that angiopoietin dysregulation may represent a final common pathway through which various infectious insults act to promote endothelial cell activation.

In HUS, angiopoietin dysregulation likely occurs either directly or indirectly through the action of the Shiga toxin. Angiopoietin-2 is stored in the Weibel-Palade bodies of endothelial cells for rapid release upon detection of an inflammatory stimulus. Binding of the Shiga toxin to its receptor, globotriaosylceramide (Gb3), concentrated in the renal microvasculature, may directly
stimulate Weibel-Palade body exocytosis, a hypothesis in keeping with the finding that other constituents of Weibel-Palade bodies, such as von Willebrand Factor (vWF) are released after Shiga toxin exposure in vitro. Alternatively, Shiga toxin may act indirectly through a mediator such as VEGF, known to stimulate Weibel-Palade body release.

In contrast, the decline in serum Ang-1 levels may reflect Shiga toxin-induced injury to the pericytes, or frank thrombocytopenia in the case of children with HUS. However, the Ang-1 concentration declines in the pre-HUS phase of illness, before the onset of thrombocytopenia, making the latter an unlikely mechanism.

Previous work by Chandler et al has shown that children in the pre-HUS phase of illness have normal blood cell counts, yet show evidence of early vascular injury with levels of prothrombin fragment 1+2, tissue plasminogen activator (tPA) antigen, tPA-plasminogen-activator inhibitor type 1 complex, and D-dimer, that are significantly higher than those in children with uncomplicated infection. Our work supports the concept that subclinical endothelial dysfunction precedes the development of overt HUS and can be detected before the classically recognized markers of disease.

Serum or plasma levels of Ang-1/2 have also been proposed as clinically informative biomarkers in several other important clinical syndromes, including: sepsis, cerebral malaria, severe bacterial infection in children. Although previous studies have investigated the role of markers of inflammation, such as procalcitonin and interleukin-6, in established HUS, our study is unique in that it demonstrates the potential utility of a biomarker to detect impending HUS in the setting of E. coli O157:H7 infection, a stage of illness for which no diagnostic tests currently exist. Although the degree of overlap between the groups precludes the use of Ang-1 as a definitive single predictor of HUS, one or both of the angiopoietins might be combined with other early markers of endothelial cell dysfunction, such as those described by Chandler et al, to derive useful discriminatory or predictive classification rules. Our study therefore provides a rationale for prospective validation of circulating angiopoietins as outcome-specific biomarkers for children with E. coli O157:H7 infection. If the utility of one or more biomarkers were to be confirmed clinically, it might provide the opportunity to initiate early aggressive intervention, particularly fluid resuscitation, which has been shown to limit morbidity in children with E. coli O157:H7 infection who ultimately progress to HUS.
There are some important limitations to our study. We chose to investigate *E. coli* O157:H7-associated HUS because it remains the most common serotype, and prior to the European outbreak, was associated with the most severe disease and sequelae. Although the clinical syndrome of HUS is largely the same regardless of serotype, we cannot conclusively state that our findings apply to illness caused by non-O157 serotypes. In addition, we cannot exclude the impact of renal failure itself on angiopoietin dysregulation in HUS. However, renal function was intact in children in the pre-HUS phase of illness, and therefore, this limitation should not detract from the finding that vascular injury precedes the overt clinical syndrome of HUS, nor from the utility of Ang-1 to discriminate between children with uncomplicated infection and those in the pre-HUS phase of illness. Finally, although we cannot exclude a contribution of platelet-derived Ang-1, due to *ex vivo* activation of platelets after venipuncture, to the high serum Ang-1 concentrations found in individuals with uncomplicated infection, we would expect the degree of *ex vivo* activation to be the same between the children with uncomplicated infection and those in the pre-HUS phase of illness. Therefore, it is unlikely that this particular limitation influenced the relative difference in serum Ang-1 concentration between these two groups.

In conclusion, this study is the first to demonstrate angiopoietin dysregulation in *E. coli* O157:H7 infection, in which the degree of dysregulation is associated with disease severity and predictive of disease progression upon presentation with bloody diarrhea. If these observations are confirmed in prospective clinical studies, peripheral blood levels of Ang-1/2 may be clinically useful to identify children with *E. coli* O157:H7 infection at high risk of progression to HUS.
Chapter 5
Prognostic Biomarkers for Sepsis in a Predominantly HIV-infected Population in a Resource-limited Setting

5.1 Introduction

Sepsis is a significant cause of infection-related mortality in high-income countries, and, although data are limited, an emerging and increasingly recognized issue in low- to middle-income countries. In both settings, careful and directed triage of limited resources and personnel is required, yet at present there is no well-defined, rapid, and specific test to identify those patients at highest risk of mortality, and therefore most in need of aggressive therapeutic intervention.

The clinical manifestations of sepsis occur because of an exaggerated response - both immunologic and vascular - to an infection, typically bacterial. Angiopoietin-1 and -2, sTie-2, procalcitonin, C-reactive protein, sTREM-1, Chi3L1, IP-10, PF4, vWF, ICAM-1, VEGF, and sFlt-1 have each been proposed as individual prognostic biomarkers in sepsis. However, as described in Chapter 1, no single biomarker has been demonstrated to have adequate sensitivity and specificity in all populations tested, and controversy persists in the literature surrounding each candidate molecule. There are few studies in which the prognostic utility of these markers has been explored in combination, and even fewer in resource-limited settings.

Therefore, the aim of this study is twofold: 1) to determine whether a combination of biomarkers, representing activation of both the immunologic and vascular pathways, can predict mortality from an episode of sepsis in a predominantly HIV-infected population in a resource-limited setting, and 2) to explore the utility of each of these markers, or a combination thereof, to predict the etiology of an episode of sepsis in the same setting.

5.2 Methods

Study population and enrolment criteria

Beginning in 2009, consecutive patients who presented on weekdays to the Emergency Departments of two large referral hospitals in Uganda (Mulago Hospital in Kampala and Masaka Regional Referral Hospital in Masaka) were evaluated for trial enrolment. Inclusion criteria were
defined as 2 of 4 criteria for SIRS or thermal dysregulation alone, in combination with systolic blood pressure \( \leq 100 \) mmHg, suspected infection as per the admitting physician, and an elevated plasma lactate level \( (>2.5 \) mmol/L) or low Karnofsky Performance score \( \leq 40 \). Use of the Karnofsky Performance Score, a well-described and commonly cited morbidity assessment tool, for this indication had been investigated previously and found to correlate well with mortality in Ugandan patients with sepsis.\(^{367}\) Exclusion criteria included: age < 18 years, pregnancy, gynecologic or surgical presenting illness, acute stroke, and gastrointestinal hemorrhage.

Informed consent was obtained from the patient and her/his relative(s), and demographic, clinical, biochemical, microbiologic, and outcome data were collected. Resources available in each hospital for the diagnosis and treatment of infection were previously outlined by Jacob et al.\(^{367}\)

In total, 336 patients were included in this study, of whom 170 (50.6\%) were female. The median age at enrolment was 34 years (range: 18 - 73 years). 282 patients (84\%) had HIV disease, and amongst those, the median CD\(_4\)+ T cell count was 45 cells/\( \mu \)L (range 1 - 746 cells/\( \mu \)L). Previous studies in this population indicated that only 12\% of the patients infected with HIV were actively receiving antiretroviral therapy.\(^{367}\) Fourteen percent of patients had positive malaria smears. At enrolment, the median lactate level was 3.9 mmol/L (range 1 - 16.6 mmol/L) and the median Karnofsky Performance score was 40 (range 10 - 80). The median white blood cell count was 4.8 x 10\(^9\) cells/L (range 0.1 - 82 x 10\(^9\) cells/L).

In-hospital mortality data was available for 332 of 336 patients, of whom 89 of 332 (27\%) died. Twenty-eight day mortality data was available for 306 of 336 patients, of whom 119 (39\%) died. In a univariate analysis of positive microbiologic results (any mycobacterial culture, aerobic blood culture, or malaria smear), only a positive mycobacterial culture was significantly associated with a risk of death (data not shown).

The candidate was not involved in this aspect of the study.

**Biomarker Assays**

Plasma was obtained at study enrolment, divided into aliquots, and stored at -70°C until use. Care was taken to maintain the frozen samples during prolonged transportation and shipping. Plasma concentrations of all biomarkers were measured by ELISA (R&D Systems, Minneapolis
MN except where indicated) as per the manufacturer’s instructions. The technical upper limits of
detection for the assays were 250 ng/mL for vWF, 20 000 pg/mL for Ang-1, sTie-2, and sFlt-1,
7000 pg/mL for Ang-2, 5000 pg/mL for PF4, and 4000 pg/mL for C-reactive protein, sTREM-1,
Chi3L1, IP-10, ICAM-1, and VEGF, yielding effective upper limits of detection of 500 μg/mL
for vWF, 80 000 pg/mL for Ang-1, 400 000 pg/mL for sTie-2, 40 000 pg/mL for sFlt-1, 28 000
pg/mL for Ang-2, 50 ng/mL for PF4, 400 μg/mL for CRP, 4000 pg/mL for sTREM-1, 2 ng/mL
for Chi3L1, 8000 pg/mL for IP-10, 4 ng/mL for ICAM-1, and 8000 pg/mL for VEGF, for the
dilutions employed in the analysis. Lower limits of detection were as follows: 244 pg/mL for
vWF, 19.5 pg/mL for Ang-1, sTie-2, and sFlt-1, 6.84 pg/mL for Ang-2, 4.88 pg/mL for PF4, and
3.91 pg/mL for CRP, sTREM-1, Chi3L1, IP-10, ICAM-1, and VEGF. Plasma concentrations of
procalcitonin were measured by ELISA (RayBiotech Inc, Norcross GA) as per the
manufacturer’s instructions, with a technical upper limit of detection of 60 000 pg/mL, an
effective upper limit of detection of 300 000 pg/mL, and a lower limit of detection of 58.6
pg/mL.

All assays were performed by an investigator blinded to clinical and microbiologic data, as well
as outcome, and each sample was tested in duplicate.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism v.4.03 (San Diego CA) and SPSS
v.16.0 (IBM, Chicago IL). Median plasma levels of all biomarkers upon enrolment in survivors
and non-survivors were compared using the Mann-Whitney test. Receiver operating
characteristic (ROC) curves were generated for each biomarker for the prediction of in-hospital
and 28-day mortality, as well as positive mycobacterial or aerobic bacterial cultures, with the
null hypothesis that the area under the curve (AUC) equals 0.5. Classification and Regression
Tree (CaRT) analysis was performed to predict the outcome (survival versus death) of an episode
of sepsis or the likelihood of a laboratory-confirmed microbiologic diagnosis by a combination
of clinical factors (Karnofsky Performance score) and host biomarkers measured upon first
presentation to hospital. CaRT analysis has been used extensively in biomarker studies in cancer,
cardiology, and infectious diseases, among other disciplines, and uses recursive partitioning to
derive cut-points for the independent variables that are then combined to create an algorithm that
can be used to classify patients according to the dependent variable. All biomarkers that
discriminated between survivors and non-survivors for each of the primary (in-hospital) and secondary (28-day mortality, positive aerobic culture, and positive mycobacterial culture) endpoints on univariate analysis were entered into each combinatorial analysis. The cost of misclassifying an eventual death or positive culture was considered to be 5 times that of misclassifying survival or a negative culture. CaRT analysis was chosen for this study because it yields a clinically relevant and easily followed algorithm, and because it makes no assumptions about the distribution or completeness of the data. Because over-fitting, the creation of an excessively complex tree that does not generalize well, is a potential limitation of CaRT analysis, the following criteria for tree construction were specified: minimum 10 cases for a parent node and 5 for a child node, customized prior probabilities based on a prospective observational study undertaken at the same sites, maximum depth of 3 nodes, and pruning to reduce overfitting. The cut-points as selected by the analysis for each biomarker are indicated between parent and child nodes, while the predicted classification of patients in each terminal node (no further branching) is highlighted.

5.3 Results

In this large cohort of Ugandan patients presenting to the Emergency Department with sepsis, multiple biomarkers were significantly associated with in-hospital mortality (Figure 6). In particular, angiopoietin dysregulation (an increase in Ang-2 and the Ang-2:Ang-1 ratio) was more severe in those patients who later died during hospital admission. The median plasma Ang-2 level was 1927 pg/mL (IQR: 1073 - 5494 pg/mL) in non-survivors and significantly lower at 1366 pg/mL (IQR: 748 - 3122 pg/mL) in survivors, \( P < 0.01 \), while the median Ang-2:Ang-1 ratio was 0.40 (IQR: 0.17 - 1.9) in non-survivors and only 0.23 (IQR: 0.074 - 0.84) in survivors, \( P < 0.01 \). In addition, biomarkers representing both the inflammatory (procalcitonin, sTREM-1, Chi3L1, and IP-10) and vascular (vWF, ICAM-1, and sFlt-1) pathways were each significantly different in those patients who survived to hospital discharge versus those who did not. The median plasma procalcitonin level was significantly elevated in non-survivors (11 104 pg/mL, IQR: 2666 - 34 475 pg/mL) as compared to survivors (6425 pg/mL, IQR: 815 - 23 411 pg/mL), \( P < 0.05 \), as were the median concentrations of sTREM-1 (247 pg/mL, [IQR: 107 - 446 pg/mL] versus 124 pg/mL, [IQR: 83 - 223 pg/mL], \( P < 0.001 \)), Chi3L1 (813 ng/mL, [IQR: 253 - 1589 ng/mL] versus 359 ng/mL, [IQR: 178 - 827 ng/mL], \( P < 0.001 \), and IP-10 (771 pg/mL, [IQR: 309 - 1273 pg/mL] versus 540 pg/mL, [IQR: 256 - 942 pg/mL], \( P < 0.01 \), all in non-survivors.
versus survivors, respectively. Amongst markers of endothelial or vascular activation, the median plasma concentration of vWF was significantly higher in patients who died prior to hospital discharge than in those who survived (265 ng/mL, [IQR: 188 - 394 ng/mL] versus 186 ng/mL, [IQR: 98 - 297 ng/mL], P < 0.001), as was also true of the median plasma concentrations of ICAM-1 (564 ng/mL, [IQR: 357 - 929 ng/mL] versus 439 ng/mL, [IQR: 303 - 608 ng/mL], P < 0.001), and sFlt-1 (1133 pg/mL, [IQR: 39 - 3051 pg/mL] versus 319 pg/mL, [IQR: 39 - 1596 pg/mL], P < 0.01).

ROC curves constructed for each of these biomarkers indicated that all nine indices were predictive of in-hospital morality, albeit to differing degrees: Ang-2 (AUC: 0.597, 95% CI: 0.529 - 0.665; P < 0.01), the Ang-2:Ang-1 ratio (AUC: 0.611, 95% CI: 0.544 - 0.677; P < 0.01), procalcitonin (AUC: 0.571, 95% CI: 0.503 - 0.639, P < 0.05), sTREM-1 (AUC: 0.668, 95% CI: 0.599 - 0.738, P < 0.001), Chi3L1 (AUC: 0.634, 95% CI: 0.564 - 0.705, P < 0.001), IP-10 (AUC: 0.610, 95% CI: 0.541 - 0.680, P < 0.01), vWF (AUC: 0.645, 95% CI: 0.580 - 0.710, P < 0.001), sICAM-1 (AUC: 0.634, 95% CI: 0.564 - 0.705, P < 0.001), and sFlt-1 (AUC: 0.600, 95% CI: 0.529 - 0.671, P < 0.01) (data not shown). ROC curves for the other biomarkers tested did not meet statistical significance (data not shown). Although the ROC curve for sTREM-1 demonstrated the greatest AUC, a plasma sTREM-1 cut-off value of 164.7 pg/mL yielded only 62% sensitivity and specificity for the prediction of in-hospital mortality at the onset of an episode of sepsis in this population.

Both plasma lactate and the clinical Karnofsky Performance score were significantly different between those patients who died prior to hospital discharge and those who survived: 4.4 mmol/L (IQR: 3.3 - 5.7 mmol/L) versus 3.8 mmol/L (IQR: 3.0 - 4.6 mmol/L), P < 0.001, and 30 (IQR: 30 - 40) versus 40 (IQR: 30 - 50), P < 0.001, respectively (data not shown). ROC curves for both were significantly predictive of in-hospital mortality, however, plasma lactate performed slightly worse (AUC: 0.609, 95% CI: 0.543 - 0.676, P < 0.001) than many of the newer biomarkers discussed above, and Karnofsky Performance score (AUC: 0.668, 95% CI: 0.609 - 0.728, P < 0.001) approximately equally (data not shown).
**Figure 6.** Plasma Biomarkers Associated with In-hospital Mortality in Ugandan patients with sepsis. Absolute and median concentrations of Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), Ang-2:Ang-1 ratio, soluble Tie-2 receptor (sTie-2), C-reactive protein (CRP), Procalcitonin (PCT), soluble Triggering Receptor Expressed on Myeloid cells-1 (sTREM-1), Chitinase-3-like protein 1 (Chi3L1), Interferon-γ-inducible Protein-10 kDa (IP-10), Platelet Factor 4 (PF4), von Willebrand Factor (vWF), soluble Intercellular Adhesion Molecule-1 (sICAM-1), Vascular Endothelial Growth Factor (VEGF), and soluble Fms-like tyrosine kinase-1 (sFlt-1) at the time of hospital admission in patients with sepsis, divided according to in-hospital mortality. *P < 0.05; **P < 0.01; ***P < 0.001.

Multiple biomarkers were also significantly associated with 28-day mortality (Figure 7). With one exception, these markers were identical to the subset that predicted in-hospital mortality. Again, angiopoietin dysregulation at hospital admission was significantly greater in those patients who died in the subsequent 28 days than in those who survived. The median plasma concentration of Ang-2 was 1927 pg/mL (IQR: 871 - 5427 pg/mL) in non-survivors and significantly lower at 1381 pg/mL (IQR: 758 - 3077 pg/mL) in survivors, *P < 0.05, while the median Ang-2:Ang-1 ratio was 0.49 (IQR: 0.17 - 1.8) in non-survivors and 0.21 (IQR: 0.072 - 0.68) in survivors, **P < 0.001. Again mirroring the results found with in-hospital mortality, biomarkers representing both the inflammatory (sTREM-1, Chi3L1, and IP-10) and vascular (PF4, vWF, sICAM-1, and sFlt-1) pathways were each significantly different in patients who survived for at least 28 days after hospital admission versus those who did not. The median plasma concentrations of sTREM-1 (178 pg/mL, [IQR: 98 - 385 pg/mL] versus 127 pg/mL, [IQR: 85 - 225 pg/mL], *P < 0.01), Chi3L1 (749 ng/mL, [IQR: 228 - 1309 ng/mL] versus 352 ng/mL, [IQR: 154 - 723 ng/mL], **P < 0.001), and IP-10 (771 pg/mL, [IQR: 300 - 1319 pg/mL] versus 504 pg/mL, [IQR: 259 - 870 pg/mL], ***P < 0.001), all differed significantly amongst non-survivors versus survivors at 28 days, respectively. Similarly, the median plasma concentrations of vWF (260 ng/mL, [IQR: 175 - 387 ng/mL] versus 172 ng/mL, [IQR: 89 - 281 ng/mL], *P < 0.001), ICAM-1 (536 ng/mL, [IQR: 357 - 880 ng/mL] versus 425 ng/mL, [IQR: 305 - 601 ng/mL], **P < 0.001), and sFlt-1 (1159 pg/mL, [IQR: 39 - 3051 pg/mL] versus 125 pg/mL, [IQR: 39 - 1224 pg/mL], ***P < 0.001) were all significantly higher at hospital admission in those patients...
who died within 28 days than in those who survived (non-survivor versus survivor, respectively, for each biomarker). In contrast, the median plasma concentration of PF4 at hospital admission demonstrated the opposite pattern and was significantly lower in non-survivors (944 ng/mL, IQR: 342 - 2150 ng/mL) than survivors (1244 ng/mL, IQR: 942 - 3535 ng/mL), \( P < 0.05 \). Notably, the median plasma procalcitonin level upon presentation to hospital did not differ significantly amongst survivors and non-survivors at 28 days as it did amongst survivors and non-survivors at hospital discharge, while the opposite was true of the median plasma PF4 concentration.

Each of these nine biomarkers discriminated between survivors and non-survivors at 28 days according to the area under the ROC curve: Ang-2 (AUC: 0.579, 95% CI: 0.513 - 0.644; \( P < 0.05 \)), the Ang-2:Ang-1 ratio (AUC: 0.619, 95% CI: 0.554 - 0.683; \( P < 0.001 \)), sTREM-1 (AUC: 0.607, 95% CI: 0.540 - 0.675, \( P < 0.01 \)), Chi3L1 (AUC: 0.639, 95% CI: 0.574 - 0.703, \( P < 0.001 \)), IP-10 (AUC: 0.619, 95% CI: 0.554 - 0.685, \( P < 0.001 \)), PF4 (AUC: 0.587, 95% CI: 0.521 - 0.652, \( P < 0.05 \)), vWF (AUC: 0.663, 95% CI: 0.602 - 0.724, \( P < 0.001 \)), sICAM-1 (AUC: 0.627, 95% CI: 0.562 - 0.693, \( P < 0.001 \)), and sFlt-1 (AUC: 0.631, 95% CI: 0.564 - 0.698, \( P < 0.001 \)) (data not shown). However, while statistically significant, none of these indices were sufficiently sensitive and specific to be clinically useful as the definitive and sole prognostic indicator in patients presenting to the Emergency Department with sepsis.

Again, both lactate (4.1 mmol/L [IQR: 3.3 - 5.6 mmol/L] versus 3.8 mmol/L [IQR: 3.0 - 4.6 mmol/L], \( P < 0.01 \) in non-survivors and survivors, respectively) and Karnofsky Performance score (30 [IQR: 30 - 40] versus 40 [IQR: 30 - 50], \( P < 0.001 \) in non-survivors and survivors, respectively) differed according to 28-day mortality. ROC curves indicated that both were predictive of 28-day mortality - lactate AUC: 0.578, 95% CI: 0.518 - 0.639, \( P < 0.01 \), and Karnofsky Performance score AUC: 0.656, 95% CI: 0.601 - 0.712, \( P < 0.001 \) - but neither was sufficiently predictive for use as the sole predictive marker in a clinical setting.
Figure 7. Plasma Biomarkers Associated with 28-day Mortality in Ugandan patients with sepsis. Absolute and median concentrations of Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), Ang-2:Ang-1 ratio, soluble Tie-2 receptor (sTie-2), C-reactive protein (CRP), Procalcitonin (PCT), soluble Triggering Receptor Expressed on Myeloid cells-1 (sTREM-1), Chitinase-3-like protein 1 (Chi3L1), Interferon-γ-inducible Protein-10 kDa (IP-10), Platelet Factor 4 (PF4), von Willebrand Factor (vWF), soluble Intercellular Adhesion Molecule-1 (sICAM-1), Vascular Endothelial Growth Factor (VEGF), and soluble Fms-like tyrosine kinase-1 (sFlt-1) at the time of hospital admission in patients with sepsis, divided according to 28-day mortality. * \( P < 0.05; \) ** \( P < 0.01; \) *** \( P < 0.001. \)

CaRT analysis was undertaken to determine whether a combination of biomarkers might be more clinically useful for the prediction of mortality at the time of presentation to the Emergency Department. With in-hospital mortality defined as the dependent variable and those biomarkers that had effectively discriminated between survivors and non-survivors in univariate analyses defined as independent variables, CaRT analysis identified a model incorporating sTREM-1 (cut-off value > 246 pg/mL), the Karnofsky Performance score (cut-off value ≤ 30), and Ang-2 (cut-off value > 1404 pg/mL) that predicted in-hospital mortality with a sensitivity of 97% (Figure 8A). Sensitivity was not appreciably increased when the model was expanded to incorporate additional biomarkers, nor when either of the standard predictive measures (Karnofsky Performance score or lactate) were specified \textit{a priori} as the first variable in the model (data not shown). Furthermore, a model incorporating only the Karnofsky Performance score and lactate yielded a higher misclassification rate (data not shown). For the prediction of 28-day mortality, CaRT analysis yielded a model that incorporated vWF (cut-off value 194 ng/mL), the Ang-2:Ang-1 ratio (cut-off value > 0.23), and Chi3L1 (cut-off value > 84 ng/mL), and identified non-survivors with 93% sensitivity (Figure 8B).
Figure 8. Classification and Regression Tree (CaRT) analysis of biomarker combinations to predict mortality following an episode of sepsis in a Ugandan population. **A.** sTREM-1, Ang-2, and the Karnofsky Performance score predict in-hospital mortality: sensitivity 97%, specificity 38%, misclassification rate 26%, standard error 2.4%. **B.** vWF, Ang-2:Ang-1, and Chi3L1 predict 28-day mortality: sensitivity 93%, specificity 38%, misclassification rate 20%, standard error 2.3%.
In addition to prognostic indicators, we sought to identify those biomarkers that might be associated with specific microbial etiologies. Of 226 patients who had blood cultures drawn, 37 (16%) were positive. This is in keeping with the incidence of positive cultures in previous studies in this population. The median plasma concentrations for 5 of 12 biomarkers tested were significantly different at hospital admission in those patients with positive aerobic blood cultures than in those patients whose blood cultures remained negative: CRP (178 μg/mL, [IQR: 98 - 385 μg/mL] versus 127 μg/mL, [IQR: 85 - 225 μg/mL], \( P < 0.01 \)), PCT (749 pg/mL, [IQR: 228 - 1309 pg/mL] versus 352 pg/mL, [IQR: 154 - 723 pg/mL], \( P < 0.001 \)), sTREM-1 (771 pg/mL, [IQR: 300 - 1319 pg/mL] versus 504 pg/mL, [IQR: 259 - 870 pg/mL], \( P < 0.001 \)), Chi3L1 (851 ng/mL, [IQR: 441 - 1992 ng/mL] versus 295 ng/mL, [IQR: 151 - 827 ng/mL], \( P < 0.001 \)), and sICAM-1 (500 ng/mL, [IQR: 386 ng/mL - 695 ng/mL], versus 407 ng/mL, [IQR: 287 - 603 ng/mL], \( P < 0.05 \)), in patients with positive and negative aerobic blood cultures, respectively (Figure 9A).

Each of these five biomarkers discriminated between those patients with positive blood cultures and those without as per the area under the ROC curves for each (Figure 9B): CRP (AUC: 0.614, 95% CI: 0.514 - 0.714, \( P < 0.05 \)), PCT (AUC: 0.757, 95% CI: 0.667 - 0.847, \( P < 0.001 \)), sTREM-1 (AUC: 0.623, 95% CI: 0.523 - 0.723, \( P < 0.05 \)), Chi3L1 (AUC: 0.730, 95% CI: 0.650 - 0.809, \( P < 0.001 \)), and sICAM-1 (AUC: 0.609, 95% CI: 0.518 - 0.700, \( P < 0.05 \)). PCT and Chi3L1 were the best predictors of positive aerobic blood cultures from amongst the five significant biomarkers, and this was confirmed in a CaRT analysis, in which these two markers, with the addition of sTREM-1, predicted positive blood cultures with a specificity of 86% (Figure 9C).

Neither lactate nor Karnofsky performance score differed amongst patients with and without positive aerobic blood cultures (data not shown).
A similar analysis was performed to examine biomarkers predictive of mycobacterial infection. Results were positive in 71 of 243 patients (29%) from whom mycobacterial cultures were taken, consistent with the findings of previous studies in the same population. In patients presenting to the Emergency Department with sepsis, all biomarkers tested were significantly different between those with and without mycobacterial infection except for sTie-2, sFlt-1, and VEGF (Table 2). Furthermore, ROC curves confirmed that each of these markers were predictive of positive mycobacterial culture results, albeit to varying degrees. sTREM-1 and IP-10 had the highest and second highest AUCs by ROC curve analysis, respectively, and CaRT analysis included both in a model that predicted positive mycobacterial culture results with 79% sensitivity and 76% specificity (Figure 10). Although plasma lactate was significantly different between those with and without mycobacterial infection, the associated AUC from the ROC curve analysis was lower than that of most of the other biomarkers tested (data not shown). Karnofsky performance score was not significantly different between patients with and without mycobacterial infection (data not shown).

Interestingly, the most commonly used indicator of infection of any type, leukocyte count, was not significantly different at hospital admission in those patients with and without either bacteremia or mycobacterial infection (data not shown). Furthermore, although the white blood cell count was slightly lower in non-survivors than survivors, and although this difference did reach statistical significance ($P < 0.05$), admission white blood cell count was very poorly
discriminative of both in-hospital and 28-day mortality, and was outperformed by all other statistically significant biomarkers (data not shown).
**Table 2. Significant predictors of mycobacterial infection in Ugandan adults with sepsis.** Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), Ang-2:Ang-1 ratio, C-reactive protein (CRP), Procalcitonin (PCT), soluble Triggering Receptor Expressed on Myeloid cells-1 (sTREM-1), Chitinase-3-like protein 1 (Chi3L1), Interferon-γ-inducible Protein-10 kDa (IP-10), Platelet Factor 4 (PF4), von Willebrand Factor (vWF), and soluble Intercellular Adhesion Molecule-1 (sICAM-1).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Positive</th>
<th>Negative</th>
<th>( P )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mycobacterial culture</td>
<td>Mycobacterial culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang-1 (pg/mL)</td>
<td>3835 (1425 - 11032)</td>
<td>5598 (2529 - 13549)</td>
<td>&lt; 0.05</td>
<td>0.588 (0.522 - 0.665)</td>
</tr>
<tr>
<td>Ang-2 (pg/mL)</td>
<td>2043 (1099 - 5078)</td>
<td>1382 (778 - 3113)</td>
<td>&lt; 0.01</td>
<td>0.606 (0.533 - 0.680)</td>
</tr>
<tr>
<td>Ang-2:Ang-1</td>
<td>0.660 (0.152 - 1.92)</td>
<td>0.225 (0.078 - 0.770)</td>
<td>&lt; 0.001</td>
<td>0.641 (0.569 - 0.713)</td>
</tr>
<tr>
<td>CRP (μg/mL)</td>
<td>134 (78.3 - 221)</td>
<td>84.9 (35.2 - 169)</td>
<td>&lt; 0.001</td>
<td>0.631 (0.561 - 0.701)</td>
</tr>
<tr>
<td>PCT (pg/mL)</td>
<td>13 709 (4858 - 44738)</td>
<td>5605 (440 - 20800)</td>
<td>&lt; 0.001</td>
<td>0.635 (0.563 - 0.707)</td>
</tr>
<tr>
<td>sTREM-1 (pg/mL)</td>
<td>294 (160 - 458)</td>
<td>117 (82 - 223)</td>
<td>&lt; 0.001</td>
<td>0.777 (0.720 - 0.834)</td>
</tr>
<tr>
<td>Chi3L1 (ng/mL)</td>
<td>726 (343 - 1309)</td>
<td>348 (172 - 932)</td>
<td>&lt; 0.001</td>
<td>0.657 (0.588 - 0.725)</td>
</tr>
<tr>
<td>IP-10 (pg/mL)</td>
<td>901 (670 - 1342)</td>
<td>444 (216 - 884)</td>
<td>&lt; 0.001</td>
<td>0.754 (0.697 - 0.811)</td>
</tr>
<tr>
<td>PF4 (ng/mL)</td>
<td>794 (317 - 2139)</td>
<td>1180 (509 - 3271)</td>
<td>&lt; 0.05</td>
<td>0.587 (0.512 - 0.662)</td>
</tr>
<tr>
<td>vWF (μg/mL)</td>
<td>249 (177 - 367)</td>
<td>192 (91 - 302)</td>
<td>&lt; 0.01</td>
<td>0.625 (0.559 - 0.691)</td>
</tr>
<tr>
<td>sICAM-1 (ng/mL)</td>
<td>592 (386 - 815)</td>
<td>432 (294 - 631)</td>
<td>&lt; 0.001</td>
<td>0.647 (0.577 - 0.716)</td>
</tr>
</tbody>
</table>

**Median Plasma Concentration (IQR)**

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Median Plasma Concentration (IQR)</th>
<th>Area under the ROC curve (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1 (pg/mL)</td>
<td>3835 (1425 - 11032)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Ang-2 (pg/mL)</td>
<td>2043 (1099 - 5078)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Ang-2:Ang-1</td>
<td>0.660 (0.152 - 1.92)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CRP (μg/mL)</td>
<td>134 (78.3 - 221)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PCT (pg/mL)</td>
<td>13 709 (4858 - 44738)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>sTREM-1 (pg/mL)</td>
<td>294 (160 - 458)</td>
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</tr>
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<td>Chi3L1 (ng/mL)</td>
<td>726 (343 - 1309)</td>
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<tr>
<td>IP-10 (pg/mL)</td>
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</tr>
<tr>
<td>sICAM-1 (ng/mL)</td>
<td>592 (386 - 815)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Figure 10. Classification and Regression Tree (CaRT) analysis for the prediction of positive mycobacterial cultures in Ugandan adults with sepsis. Interferon-γ-inducible protein-10 kDa (IP-10), soluble Triggering Receptor Expressed on Myeloid cells-1 (sTREM-1). Sensitivity 79%, specificity 76%.
5.4 Discussion/Conclusion

The current study of 336 Ugandan patients with sepsis is, to our knowledge, the largest study published to date that has investigated the role of prognostic biomarkers in a predominantly HIV-infected population in a resource-limited setting. As a result, a number of unique conclusions can be drawn.

First, angiopoietin dysregulation is a prominent feature of sepsis in this population, and both the plasma Ang-2 concentration and the Ang-2:Ang-1 ratio at the time of hospital admission predict in-hospital mortality and mortality at 28 days. Angiopoietin dysregulation, manifested primarily by increased Ang-2 resulting in an increased Ang-2:Ang-1 ratio, has been found in septic patients in high-income countries, and in these settings has been found to correlate with severity of illness, pulmonary edema, ALI/ARDS, multi-organ dysfunction, and mortality.\textsuperscript{9, 120-123, 127} The increase in Ang-2 in sepsis may reflect Weibel-Palade body exocytosis in response to reduced endothelial nitric oxide bioavailability, increased sphingosine-1-phosphate, or perturbation of another as yet unidentified factor.\textsuperscript{5, 53, 123}

Since many proposed biomarkers of infection have shown wide variation in performance characteristics when studied in different patient populations, our study is significant in that it confirms that the association between angiopoietin dysregulation and mortality in sepsis is present in a predominantly HIV-infected, significantly immunosuppressed population in which the median CD\textsubscript{4+} T cell count was 45 cells/\textmu L. Mankhambo \textit{et al} conducted the only other investigation of angiopoietin dysregulation in a similar population: a study of 293 Malawian children with meningitis or pneumonia of whom 53\% were HIV-infected and in whom a low Ang-1 concentration was associated with mortality in a multivariate analysis.\textsuperscript{129}

Our study is also unique in that, in addition to Ang-2 and the Ang-2:Ang-1 ratio, sTREM-1, Chi3L1, IP-10, vWF, sICAM-1, and sFlt-1 were all predictive both of in-hospital and 28-day mortality in this septic population, while PCT and PF4 were predictive of in-hospital and 28-day mortality, respectively. This is the first study to document an association between mortality in sepsis and Chi3L1 or PF4, and the second to document the same association with IP-10. Chi3L1 has only been examined once before in sepsis, in a small study of 45 patients in which it was associated with illness severity but not mortality,\textsuperscript{302} although elevated Chi3L1 has been associated with mortality following pneumococcal bacteremia.\textsuperscript{300} PF4 had previously been
studied in 10 patients with sepsis, but no attempt was made to correlate increased circulating levels of PF4 with mortality.\textsuperscript{314} Finally, IP-10 had previously been studied only in 16 patients with sepsis, but despite the small sample size elevated levels were nonetheless found to correlate with mortality.\textsuperscript{303} Interestingly, all of the biomarkers studied here were higher in non-survivors than survivors, with the exception of PF4, which demonstrated the opposite pattern. PF4 is known to facilitate generation of the anticoagulant molecule activated protein C, and, in keeping with our findings, increased levels of PF4 have been shown to correlate with survival in animal models of sepsis.\textsuperscript{312, 313}

While the literature is generally in agreement that sTREM-1 can predict prognosis in sepsis, there is some controversy regarding the direction of change. Our results coincide with those of Zhang \textit{et al} and Dimopoulou \textit{et al}, both of whom described higher levels of sTREM-1 in non-survivors, but conflict with those of Gibot \textit{et al}, who describe lower sTREM-1 in non-survivors.\textsuperscript{260, 289, 290} It is possible that the discrepancy reflects the inherent paradox of sepsis, namely that the inflammatory response can be both helpful and harmful. sTREM-1 amplifies the innate immune response induced by pathogens detected by pattern recognition factors such as TLR4.\textsuperscript{371} If this response is controlled, carefully regulated, and directed, the end result is pathogen clearance and minimal host morbidity. If, however, this response is exaggerated, excessive, and indiscriminate, the end result is sepsis and potentially significant morbidity. Therefore, the discrepancy may simply reflect the fact that an intermediate level of sTREM-1 is required for an appropriate response, and levels that are either too high or too low are detrimental.

PCT, vWF, sICAM-1, and sFlt-1 have all been associated with mortality and/or disease severity in other studies of septic patients, and in this regard our results confirm previous findings.\textsuperscript{34, 120, 254-263, 267, 268, 273-275, 318, 326, 331}

Of note, we did not detect an association between Ang-1, sTie-2, VEGF, or CRP and mortality in this population of adult patients with sepsis. Ang-1 has not been identified as a significant predictor of mortality in sepsis as consistently as have Ang-2 or the Ang-2:Ang-1 ratio.\textsuperscript{127, 128} Furthermore, Ang-1 has been shown to fluctuate widely in individual patients over a 24-hour period,\textsuperscript{118} and therefore may have limited clinical reproducibility and utility. With respect to the other candidate biomarkers, our results are in keeping with the published literature in which
neither sTie-2, nor VEGF, nor CRP have been consistently and reliably associated with mortality in sepsis.\textsuperscript{128, 131, 266, 272, 334}

Despite the number of biomarkers that were positively associated with mortality in this cohort, none demonstrated sufficient sensitivity or specificity to be employed clinically as the sole indicator of prognosis. Therefore, we exploited Classification and Regression Tree (CaRT) analysis to determine whether combinations of biomarkers were more predictive of mortality in this population than any single marker alone. Combining biomarkers has previously been shown to enhance their prognostic utility in both sepsis and malaria,\textsuperscript{127, 305} and likewise the combinations illustrated in Figures 6 and 7 predict death with better sensitivity than any individual marker alone. The predictive models for both in-hospital and 28-day mortality are representative of the underlying pathophysiology of sepsis and incorporate markers from both the inflammatory and vascular pathways. To maximize clinical utility and feasibility in our patient population and resource-limited setting, this analysis was performed with the prespecified goals of prioritizing sensitivity over specificity and limiting the number of markers included in each model to three. By identifying patients at highest risk of mortality, this approach may be useful for triage and directed allocation of limited resources. A recent pan-African survey of anesthesiologists found that < 2% of African hospitals regularly had access to the resources, including medications, disposable and permanent equipment, and monitoring and diagnostic tools, required to implement the Surviving Sepsis Campaign guidelines, now considered the standard of care for patients with sepsis.\textsuperscript{372, 373} A convenient, inexpensive, point-of-care test that could be used at the time of hospital admission to identify those patients at highest risk of mortality and therefore most likely to benefit from intensive therapy would be of value, and should be studied prospectively. Rapid tests are currently available for the diagnosis of HIV, syphilis, and malaria, and new microfluidics-based technology allows all of the steps of a traditional ELISA to be mimicked on a point-of-care chip, making implementation of biomarker analysis feasible even in resource-limited settings.\textsuperscript{374, 375}

Rapid identification of the specific microbial etiology responsible for an episode of sepsis would likewise be a key component of any algorithm designed to maximize appropriate resource allocation. To that end, we confirmed that procalcitonin has reasonable specificity for bacterial infection despite the high prevalence of co-infection with HIV, malaria, and mycobacteria in this population. Interestingly, Chi3L1 had equivalent specificity for bacterial infection in this
population, and has not previously been studied for its discriminative ability in this regard. Although the known association of Chi3L1 with the specific granules of activated neutrophils is in keeping with the finding of elevated Chi3L1 in bacterial infection, elevated levels of Chi3L1 have also been reported in association with HIV encephalitis and hepatitis C-induced liver fibrosis.\textsuperscript{299, 376, 377} Therefore, further study of Chi3L1 is warranted to confirm its specificity for bacterial infection in septic patients.

In contrast, IP-10 has been well-studied in association with tuberculosis and was also most predictive of mycobacterial infection in our cohort of Ugandan patients with sepsis. IP-10 acts to amplify the adaptive immune response: it can be produced by monocytes, macrophages, and vascular endothelial cells and act as a specific chemotactic signal to draw activated Th1 cells that express its cognate receptor, CXCR3, to a site of infection, and in turn, expression of IP-10 is further upregulated by interferon-γ produced by the infiltrating Th1 cells.\textsuperscript{378} Consistent with our findings and its role in the Th1 inflammatory response, elevated levels of IP-10 have been demonstrated in areas of granulomatous inflammation and pneumonitis in the lungs of patients with tuberculosis.\textsuperscript{379} However, it should be noted that elevated IP-10 levels are not pathognomonic of mycobacterial infection. IP-10 has also been shown to be elevated in, and crucial to host control of, \textit{Toxoplasma gondii} and influenza infection.\textsuperscript{380, 381}

If combined in a point-of-care test panel, these biomarkers might facilitate the initiation of rapid, appropriate, empiric antimicrobial therapy in the Emergency Department, without the time delay required for standard diagnostic methods. A recent study compared blood culture, bacterial polymerase chain reaction (PCR), and procalcitonin for the diagnosis of bacteremia, and found that the average time from testing to the receipt of results was 33 hours for blood cultures, 10 hours for PCR, and 45 minutes for procalcitonin, a significant difference that is not inconsequential given the well-documented relationship between delay of appropriate antibiotics and mortality in sepsis.\textsuperscript{231, 382} Procalcitonin is not a substitute for blood culture since the latter has a clear advantage in terms of antimicrobial identification and susceptibility testing. However, procalcitonin and similar markers may be particularly useful in resource-limited settings to prioritize those patients most likely to benefit from broad-spectrum antibiotics, or, in the case of IP-10, to identify and appropriately treat those patients likely to have a non-bacterial cause of sepsis.
In summary, our study has demonstrated that a variety of biomarkers from the inflammatory and vascular pathways are predictive of both in-hospital and 28-day mortality when measured at the time of hospital presentation in predominantly HIV-infected Ugandan patients with sepsis. Biomarker combinations warrant further study with the goal of developing rapid, point-of-care tests to assist Emergency Department physicians in the appropriate allocation of limited resources, particularly as sepsis becomes increasingly recognized as an important contributor to morbidity and mortality in low- and middle-income countries.
Chapter 6
Discussion

Angiopoietin dysregulation (decreased Ang-1 and increased Ang-2) is present in streptococcal toxic shock syndrome, *E. coli* O157:H7-induced hemolytic-uremic syndrome, and sepsis. This work is the first to identify angiopoietin dysregulation in both invasive group A streptococcal infection and *E. coli* O157:H7 infection, and adds both syndromes to the published literature on infectious diseases, including sepsis, severe bacterial infection, and malaria, in which angiopoietin dysregulation has been described.\(^{119, 128, 133}\) No association between angiopoietin dysregulation and exotoxin-mediated diseases has previously been reported, and therefore, these findings may provide the rationale for future experimental work in this area, particularly as regards the direct effects of exotoxins on the endothelium in general and on Weibel-Palade body exocytosis in particular. The impact of streptococcal exotoxins on the release of WPBs or their contents has not been studied *in vitro*, and while Shiga toxins have been linked indirectly to WPB exocytosis (via rapid toxin-mediated stimulation of vWF release), the mechanism for decreased Ang-1, as found in our study, is not known.\(^{383}\)

In patients with sepsis, our findings of angiopoietin dysregulation, and in particular of increased plasma Ang-2 and an elevated Ang-2:Ang-1 ratio, were in accordance with those reported in other cohorts of septic patients.\(^{9, 120-123, 127}\) Furthermore, angiopoietin dysregulation performed better than lactate, a standard marker of tissue hypoperfusion, for the prediction of mortality in sepsis, confirming results reported by others.\(^{124}\) Our study, however, was unique in that it was conducted in a resource-limited setting amongst adult patients with sepsis, of whom 84% were HIV-infected and 29% had mycobacteremia, and as such, is the only study of its kind to document the association between angiopoietins and sepsis in this patient population. The study of biomarkers for sepsis in resource-limited settings is crucial prior to clinical implementation, as patient populations in these settings differ markedly from those enrolled in usual clinical trials. Mortality rates are often higher than in settings in which protocolized sepsis bundles are considered standard of care.\(^{367}\) As demonstrated in the cohort under study here, patients in resource-limited areas of Africa who present with sepsis have a high rate of co-infections, including HIV, malaria, and mycobacteremia, not seen in the usual sepsis studies conducted in North America, Australia, and Europe. Co-infections that influence basal levels of circulating
inflammatory and vascular mediators are particularly significant as they may accentuate or mask between-group differences in the biomarker under study, and as a consequence, render those biomarkers either more or less useful than they appeared in studies conducted in different target populations. For example, healthy patients from malaria endemic areas are known to exhibit a different basal cytokine expression profile than that found in patients from resource-limited but malaria non-endemic areas, and active malaria infection has been shown to abrogate the clinical utility of both procalcitonin and C-reactive protein to discriminate between bacterial and viral pneumonia. Similarly, HIV infection is known to induce the production of pro-inflammatory cytokines that with treatment decline towards levels found in healthy individuals. Our study is therefore significant in that it demonstrates the utility of angiopoietin dysregulation for the prediction of mortality in a patient population with a high rate of untreated, severe HIV disease in which the median CD4+ T cell count was 45 cells/μL.

In addition, angiopoietin dysregulation was present despite a high rate of mycobacterial infection in this Ugandan population. Although plasma levels of the angiopoietins have not been studied previously in patients with mycobacterial disease, pleural fluid Ang-2 levels were found to be lower in tuberculous pleural effusions than in other types of exudative effusions. Furthermore, angiopoietin dysregulation has not been found in animal models of pulmonary tuberculosis. In fact, caseating granuloma formation in these animals has been attributed in part to impaired neovascularization and subsequent hypoxia as a result of relatively low angiopoietin levels. Given these findings, it was important that angiopoietin dysregulation be confirmed specifically in septic patients with a high rate of mycobacterial disease prior to clinical use or further study in this population.

If added to the initial panel of diagnostic tests in patients with suspected severe bacterial infection, the angiopoietins might be useful adjuncts to the clinical assessment of prognosis. The presence of angiopoietin dysregulation can differentiate between those patients with invasive streptococcal infection and those with streptococcal toxic shock, between those patients with *E. coli* O157:H7 infection who will have an uncomplicated course and those who will develop the hemolytic-uremic syndrome, and between those who will and will not survive after an episode of sepsis at the time of presentation to the Emergency Department. By providing information about the functional status of the endothelium, angiopoietin dysregulation acts as a marker of the extent of the pathophysiologic abnormalities induced by the disease process, and, at least in the case of
E. coli O157:H7 infection, is a more sensitive indicator of impending complications than are traditional laboratory investigations alone. In patients who present to the Emergency Department with sepsis, angiopoietin dysregulation is associated with an increased risk of both early and late mortality. The identification of those patients with the greatest degree of angiopoietin dysregulation, and presumably endothelial dysfunction, may be an important signal for the need for more aggressive therapeutic intervention. Early, appropriate, supportive therapy is known to improve outcome in both HUS and sepsis, and the ability to accurately triage or prioritize patients for the receipt of these interventions would enhance any Emergency Department critical illness protocol and would be especially crucial in resource-limited settings.

The clinical utility of the angiopoietins in multiple different infectious syndromes is a major advantage over biomarkers with limited or specific indications and argues for their use in the undifferentiated patient with suspected infection.

In sepsis, the prognostic utility of the angiopoietins, representative of vascular and endothelial activation, can be enhanced by their use together with other biomarkers representative of inflammation and immunologic activation. For the prediction of in-hospital mortality, Ang-2 can be combined with the Karnofsky Performance score (representing clinical assessment) and sTREM-1 (representing the inflammatory pathway), while for the prediction of 28-day mortality, the Ang-2:Ang-1 ratio can be combined with vWF and Chi3L1 (the former representing the vascular pathway and the latter the inflammatory pathway), to create in both cases clinical prediction pathways capable of identifying those patients at greatest risk of mortality with sensitivities in excess of 90%. The resulting algorithms are composed of biomarkers from the two primary pathways perturbed during sepsis and therefore may more accurately represent the underlying pathophysiology of the sepsis syndrome than might angiopoietin dysregulation alone. Biomarker combinations have likewise been proposed for use in risk assessment and outcome prediction during acute coronary syndromes (ACS). As reviewed by Morrow and Braunwald, ACS results from the maladaptive activation of multiple systems (in a manner strikingly similar to sepsis), including the inflammatory, thrombotic, and vascular systems, and therefore, prognostication is best done using an algorithm that incorporates information from each of the potentially abnormal pathways.

Testing this theory in the Emergency Department, Birkhahn et al found that a point-of-care test (POCT) incorporating multiple cardiac biomarkers was equally accurate for the diagnosis of non-ST elevation myocardial infarction as standard laboratory-
based single marker testing, but was significantly faster.\textsuperscript{390} With regards to infectious syndromes, biomarker combinations have been proposed for the prediction of mortality amongst Ugandan children with severe malaria and amongst patients with sepsis in a high-income country,\textsuperscript{127, 305} and represent the most likely form in which these prognostic biomarkers will be introduced into clinical settings.

The finding of angiopoietin dysregulation in three seemingly disparate infectious disease syndromes also suggests the possibility of a shared underlying final common pathway of endothelial injury or activation. As such, the angiopoietin system becomes an attractive target for therapeutic intervention, particularly in patients who present with non-specific hypotension and major organ (such as renal) dysfunction of unclear etiology. In such cases, empiric antibiotic therapy requires time to take effect, may be initially inappropriate for the causative organism (particularly in the era of increasingly common multi-drug resistant organisms), or may be ineffective in the case of toxin-mediated disease. This is perhaps best illustrated by recent outcome studies in streptococcal toxic shock, in which even with prompt antimicrobial therapy and surgical debridement where indicated for necrotizing fasciitis, mortality rates remain in excess of 40\%.\textsuperscript{14} Furthermore, even with protocolized sepsis management guidelines mandating early goal-directed therapy in the Emergency Department under optimal clinical trial conditions, in-hospital mortality for patients presenting with sepsis was 30\%.\textsuperscript{391} Amongst those who did not receive early goal-directed therapy, mortality was 46.5\%. Ten years after the advent of early goal-directed therapy and the subsequent widespread implementation of severe sepsis resuscitation bundles and the Surviving Sepsis Campaign recommendations, mortality rates from sepsis remain at 30\% and efforts to further improve outcomes have stalled.\textsuperscript{370, 392, 393}

Angiopoietin-based therapies might be useful adjuncts to the current best-practice interventions to support such patients until the etiology of the presentation is determined and therapy appropriately targeted.

There are limitations to the body of work presented here. These studies document an association between angiopoietin dysregulation and each of the syndromes under investigation, and therefore suggest, but do not prove, that the angiopoietins are involved in the underlying pathogenesis. Furthermore, the impact of angiopoietin testing on physician decision-making and patient outcome should be explored in a prospective manner before firm conclusions can be drawn regarding their true clinical utility. Regardless, the three studies presented here add to the
growing literature on angiopoietin dysregulation by a) identifying two syndromes (STSS and HUS) not previously known to be associated with angiopoietin dysregulation and demonstrating that dysregulation correlates with disease severity in each and is predictive of clinical outcome in *E. coli* O157:H7 infection, and b) confirming that angiopoietin dysregulation is associated with mortality in sepsis in a predominantly HIV-infected patient population and illustrating that a combination of biomarkers, including the angiopoietins as markers of vascular dysfunction and either sTREM-1 or Chi3L1 as markers of inflammation, predicts mortality better than any individual marker alone.
Chapter 7
Conclusion

Angiopoietin dysregulation is present in invasive group A streptococcal infection, *E. coli* O157:H7 infection, and sepsis. It is associated with disease severity in streptococcal toxic shock and is a prognostic biomarker in undifferentiated *E. coli* O157:H7 infection and sepsis, suggesting that measurement of the angiopoietins upon patient presentation with a suspected severe bacterial infection may offer clinically useful information beyond that which can be obtained from clinical judgement and traditional diagnostic tests.

However, in recent years many biomarkers have been proposed, studied, and ultimately associated with a variety of different diseases and syndromes, yet have failed to make the transition to clinical practice. This has prompted some authors to raise concerns regarding the true clinical utility of biomarkers identified in association studies such as those presented here. Vasan has elegantly outlined the ideal characteristics for biomarkers, referring specifically to biomarkers of cardiovascular disease but with principles equally applicable to biomarkers of infectious diseases. To be useful in clinical practice, biomarkers should be specific, reproducible, representative in a single measure but correlated with disease over time, applicable to and studied in multiple different populations, able to provide information not available from clinical assessment, and amenable to accurate laboratory testing that can be automated to achieve high throughput and short turnaround time. Based on these criteria, the angiopoietins appear well-positioned to evolve from experimental to clinical use, and will likely do so first in sepsis, in which they have been most often studied.

When compared to other biomarkers recently proposed as prognostic indicators in patients with sepsis, the angiopoietins have several advantages. First, unlike procalcitonin and C-reactive protein, the angiopoietins have been consistently associated with disease severity in sepsis in multiple different studies and wide-ranging patient populations, from undifferentiated critically ill patients in the ICU to immunocompromised patients with febrile neutropenia to predominantly HIV-infected patients as studied here. Our study, in particular, is one of the largest undertaken, with an enrolment of 336 patients, and confirms the results found in earlier, smaller studies, a major criterion for clinical use not always fulfilled by other proposed biomarkers. While a meta-analysis of studies examining the association between angiopoietin
dysregulation and disease severity in sepsis might be useful to obtain a more accurate estimate of effect size, such an analysis is unlikely to overturn the basic finding of the association itself given the almost uniformly positive results in studies published to date.

Second, in their use as prognostic biomarkers in sepsis, the angiopoietins have the added benefit of directly reflecting a component of the underlying pathophysiology, namely endothelial cell activation. Unlike procalcitonin or Chi3L1, whose precise roles in sepsis and inflammation are still unclear, angiopoietin dysregulation has a very well-defined and well-studied effect on the vascular endothelium, and therefore makes physiologic sense as a marker of both endothelial dysfunction and the resultant mortality in sepsis.

Third, the magnitude of angiopoietin dysregulation approximates disease severity both early and late in sepsis. Angiopoietin dysregulation has been shown to correlate with the evolution of endothelial activation and its clinical manifestations, including hypotension and ARDS, over time, such that angiopoietin concentrations remain abnormal during ongoing illness and normalize with convalescence. The persistence of angiopoietin dysregulation during prolonged illness is a distinct advantage over other proposed biomarkers of sepsis, such as the pro-inflammatory cytokines IL-6 and TNF, which may be elevated only transiently and therefore missed during one-time sampling and simply not useful for serial assessments over the course of a patient’s hospital or ICU admission. Although angiopoietin dysregulation can herald ongoing endothelial cell activation in prolonged critical illness, it has been studied most often for its prognostic value upon patient presentation, and has been found to be an equally effective marker of disease severity in this setting. Ang-2 concentrations rise within two hours of experimental human endotoxemia, indicating that angiopoietin dysregulation occurs, and presumably can be detected, in the earliest stages of severe systemic bacterial infection. The phenomenon of early angiopoietin dysregulation is supported by our findings in E. coli O157:H7 infection, in which angiopoietin dysregulation preceded development of the overt clinical syndrome of HUS, and could distinguish between two clinically indistinguishable patient populations: those who would experience an uncomplicated course of illness, and those who would develop HUS.

Finally, angiopoietin dysregulation is widely applicable to a variety of infectious diseases and syndromes associated with endothelial cell activation or dysfunction. In contrast to a biomarker
such as IP-10, which is relatively specific for mycobacterial infection, angiopoietin dysregulation is sufficiently generalizable to be of potential use in the estimation of disease severity in undifferentiated patients with suspected severe bacterial infection in the Emergency Department. At the same time, angiopoietin dysregulation is specific enough to distinguish septic patients from those with non-infectious critical illness.\textsuperscript{121,122}

Nonetheless, there are some aspects of the use of angiopoietin dysregulation as a prognostic biomarker in sepsis that need to be clarified or resolved prior to implementation in clinical practice. First, the ideal cut-off value for Ang-1, Ang-2, or the Ang-2:Ang-1 ratio needs to be established. Since this may differ according to whether disease severity or mortality is being predicted, both values will have to be made available in a format easily interpretable for clinicians. Furthermore, any biomarker proposed for clinical use must be sufficiently sensitive and specific to correctly classify patients. To achieve these performance characteristics, it is likely that angiopoietin dysregulation would need to be combined with other biomarkers in a prognostic algorithm or clinical decision pathway, and once again, the format of such a pathway would need to facilitate ease of clinical use. Second, a rapid test, whether laboratory-based or point-of-care, will need to be established and validated, as the ELISA method used in the studies presented above will not yield a sufficiently rapid turnaround time to enable use of angiopoietin dysregulation in clinical decision-making. This test will also need to be relatively inexpensive, or be associated with a demonstrable net cost savings, in order to justify its use in preference to the standard (lactate) and free (Karnofsky Performance score) means of prognostication traditionally used in sepsis. Finally, the ability of angiopoietin monitoring to influence physician management and patient outcome will need to be proven in prospective studies prior to widespread clinical implementation. Although there are clearly aspects on which additional research is required, angiopoietin dysregulation as a prognostic indicator in sepsis fulfills many of Vasan’s criteria for the ideal biomarker, and therefore, deserves further study.

Even if the angiopoietins are never employed clinically as biomarkers of infection, the studies presented here have still identified the angiopoietins as potential mediators of the endothelial dysfunction present in streptococcal toxic shock syndrome, \textit{E. coli} O157:H7-induced hemolytic-uremic syndrome, and sepsis. In that regard, these studies have provided a rationale for further investigation into the pathophysiology of each syndrome, with a focus on the regulation of angiopoietin production, release, and impact on the endothelium in each case. Furthermore, these
studies provide the basis for pre-clinical and clinical studies to explore the angiopoietins as potential therapeutic targets in undifferentiated patients with severe bacterial infections. In this way, studies such as these that document an association between angiopoietin dysregulation and various infectious diseases characterized by prominent endothelial cell dysfunction can serve to advance research in the field and potentially impact clinical outcome by means other than simply through the identification of potential prognostic biomarkers.
Chapter 8
Future Directions

The three studies presented above provide the necessary background for conceptualizing future research on the angiopoietin system. By suggesting that the angiopoietins play a role in the pathogenesis of toxin-mediated diseases such as streptococcal toxic shock syndrome and the hemolytic-uremic syndrome, these studies provide a rationale for in vitro experimental work investigating the interaction between exotoxins and the endothelium. In STSS, we reported elevated levels of Ang-2 in proportion to disease severity, however, our association study was not able to determine whether the increased circulating Ang-2 was due to a direct effect of the streptococcal exotoxin on the endothelium, or whether an intermediary molecule was required. To determine whether streptococcal exotoxins could induce WPB exocytosis, cultures of human umbilical vein endothelial cells and human dermal microvascular endothelial cells could be exposed to streptococcal exotoxins prior to live-cell imaging for the detection of WPB exocytosis and subsequent measurement of the angiopoietin-2 concentration in the cell culture supernatant by ELISA. Abrogation of the effect of the streptococcal exotoxin in an experiment repeated after treatment of the cultured cells with a specific inhibitor of WPB exocytosis would confirm the mechanism of Ang-2 release. In contrast, in E. coli O157:H7 infection, we reported decreased circulating Ang-1 levels as the predominant contributor to angiopoietin dysregulation. Although Ang-1 is produced in part by platelets, decreased Ang-1 levels were present in children in the pre-HUS phase of illness, prior to the onset of thrombocytopenia, and therefore cannot be attributed to a decline in platelet numbers. Since pericytes are the primary source of Ang-1, a co-culture of pericytes and endothelial cells could be exposed to Shiga toxin, with measurement of Ang-1 levels in the cell culture supernatant both before and after toxin treatment. Cell cultures of pericytes alone could also be observed and assayed for apoptosis and necrosis after direct exposure to Shiga toxin. In this way, in vitro studies could explain the observed clinical results.

By suggesting that the angiopoietins may be useful prognostic indicators in E. coli O157:H7 infection and sepsis, these studies provide the rationale for the prospective exploration of the impact of an angiopoietin-based algorithm on clinical decision-making in the management of patients with suspected severe bacterial infection. However, as outlined in Chapter 7, appropriate
cut-off values need to be defined, and a rapid and perhaps point-of-care test developed before such studies could be planned or implemented.

Finally, as a continuation of the studies presented above, angiopoietin dysregulation could be investigated in a variety of clinical conditions with known endothelial dysfunction in which a demonstrated association may shed light on the underlying, and heretofore unknown, pathogenesis. Additional work could explore the potential utility of the angiopoietins and other biomarkers to guide clinical decision-making in areas in which standard diagnostic tools are either absent or lacking sufficient sensitivity and/or specificity.

### 8.1 Effect of Ventilatory Strategy on Angiopoietin levels in ARDS

ARDS is characterized by the diffuse leak of intravascular fluid into the alveolar space, resulting from disruption of the alveolar-capillary membrane barrier. Pulmonary microvascular endothelial cell dysfunction is a necessary pre-requisite. Consistent with the known effects of the angiopoietins - endothelial cell quiescence induced by Ang-1 and endothelial cell activation induced by Ang-2 - a relative excess of Ang-2 has been shown to play a role in the pathogenesis of ARDS. In vitro, Ang-2 sensitizes human pulmonary microvascular endothelial cells to thrombin-induced contractility and inter-cellular gap formation. In a murine model of LPS-induced acute lung injury, an increase in alveolar Ang-2 relative to Ang-1 was associated with an increase in the neutrophil and protein content of bronchoalveolar lavage fluid, suggestive of pulmonary capillary leak. In the same model, overexpression or administration of exogenous Ang-1 produced improvements in all indices of endothelial function, lung injury, and inflammation. In humans, polymorphisms of the Ang-2 gene have been associated with increased susceptibility to ALI and ARDS. Circulating plasma Ang-2 levels can differentiate between critically ill patients with ALI and those without. Serum from critically ill patients has been shown to contain high levels of Ang-2 in proportion to the degree of hypoxemia, and to be capable of disrupting normal barrier function when added to human microvascular endothelial cells in vitro. This effect was reversed by Ang-1 and resolved as circulating Ang-2 levels declined with convalescence. High Ang2 levels also predicted mortality in these patients, as has since been confirmed in several other studies of ALI/ARDS in critically ill patient populations. Taken together, these data indicate that angiopoietin dysregulation is present in, and likely contributes to, the pathogenesis of ALI/ARDS.
Mechanical ventilation is the mainstay of supportive therapy for patients with ARDS, however, ventilator-associated lung injury (also known as ‘ventilator-induced lung injury’ [VILI]) remains a major contributor to morbidity and mortality. High frequency oscillation (HFO) is a means of delivering small tidal volume ventilation that may limit VILI and the resulting inflammatory response. When compared to lung-protective conventional ventilation providing similar mean airway pressures, HFO has been shown to reduce inflammatory cell infiltrate and pro-inflammatory cytokine production in the lungs in large animal models. Human data come primarily from studies in neonates, which have demonstrated a reduction in the circulating levels of some, but not all, pro-inflammatory cytokines with the use of HFO. Given these findings, we hypothesize that angiopoietin dysregulation will be present in patients with ARDS receiving conventional low tidal volume ventilation and will be attenuated in those patients transitioned to HFO. The only currently existing data on the effect of ventilatory strategy on levels of circulating angiopoietins are contradictory and derive from two murine models. In the first, hyperoxia-induced downregulation of Ang-1 was reversed in the setting of permissive hypercapnia, a model of low tidal volume ventilation. In the second, the administration of exogenous Ang-1 failed to prevent VILI in the setting of high tidal volume ventilation. There are no studies exploring the effect of HFO on circulating levels of the angiopoietins.

There, we propose to measure serum or plasma Ang-1 and Ang-2 levels at study enrolment and serially thereafter in patients with ALI/ARDS receiving either conventional, lung-protective, low tidal volume ventilation or high frequency oscillatory ventilation, to test the following hypotheses:

1. Circulating levels of Ang-1 and Ang-2 will correlate with the severity of illness and will predict clinical outcome in ventilated patients with ARDS

2. Lower levels of circulating Ang-2 and/or higher levels of circulating Ang-1 (and/or more rapid correction of angiopoietin dysregulation) will be found in patients receiving HFO versus those receiving conventional mechanical ventilation

Ultimately, the angiopoietins may be useful biomarkers to guide clinical management decisions concerning the choice of ventilatory strategies in patients with ARDS.
8.2 Angiopoietins in HIV

Untreated HIV disease is associated with endothelial activation and dysfunction, attenuated in part by the initiation of antiretroviral therapy (ART). IL-6, sICAM-1, sVCAM-1, P-selectin, and vWF are all elevated in patients with HIV disease as compared to healthy controls. In patients receiving ART, both CRP and IL-6 were found to be predictive of the development of an opportunistic disease. Taken together, these results indicate that there is a baseline level of inflammation and endothelial dysfunction that occurs in chronic HIV infection and which subsequently diminishes (but does not normalize) with treatment and increases again in the setting of intercurrent illness or disease.

Although the advent of truly effective ART has been associated with clear survival benefits in HIV-infected individuals, it has also exposed patients to the consequences and adverse effects of longterm ART use. Both endothelial activation and an increase in carotid intima-media thickness (a marker of subclinical atherosclerosis) are well-documented amongst treated patients with HIV infection. Furthermore, certain antiretroviral medications appear to be associated with a particular increased risk of cardiovascular disease, namely abacavir (ABC) and certain protease inhibitors (indinavir or lopinavir-ritonavir). This risk is not fully accounted for by changes in lipid profile or other metabolic disorders. Data conflict on the degree of endothelial activation specifically associated with ABC. VEGF, vWF, IL-6, CRP, and other markers of thrombosis, inflammation, and endothelial activation have been studied but not found to be different amongst patients receiving ABC and those receiving non-ABC-containing regimens. In contrast, in vitro studies demonstrated ICAM-1 upregulation and a resultant increase in rolling and adhesion of neutrophils and peripheral blood mononuclear cells along an endothelial monolayer in the presence of ABC. At this time, the pathogenesis of ABC-induced cardiovascular risk remains unknown.

Given the known association between angiopoietin dysregulation, cardiovascular disease, and the traditional risk factors for cardiovascular disease (reviewed in Chapter 1.7.3) in the general population, I hypothesize that angiopoietin dysregulation might also play a role in ABC-induced cardiovascular disease in HIV. This hypothesis is further supported by in vitro data that describe the attenuation of ABC-induced endothelial dysfunction by specific inhibitors of the Erk
signalling pathway, one of the major downstream regulators of Tie-2 activation and associated with endothelial cell proliferation.\textsuperscript{415}

Although the majority of patients in the sepsis study reported here (Chapter 5) were HIV-infected, most were not receiving ART, and all had angiopoietin levels measured in the setting of acute illness. To my knowledge, there are no studies that explore the angiopoietins in treated HIV disease, nor specifically in association with ABC-induced cardiovascular disease. Therefore, I propose to investigate Ang-1 and Ang-2 levels in a cohort of patients receiving ABC, and to compare these levels to that of a control group of patients (matched for age, duration and stage of HIV infection, and cardiovascular risk factors and disease) receiving non-ABC-containing ARV regimens. I hypothesize that angiopoietin dysregulation (decreased Ang-1 and/or increased Ang-2) will be greater in those patients receiving ABC-containing ARV regimens than in the control group. I further hypothesize that amongst those patients receiving non-ABC-containing regimens, angiopoietin dysregulation will be an independent predictor of cardiovascular disease.

\section*{8.3 Angiopoietins in Obstructive Sleep Apnea}

Obstructive sleep apnea (OSA) is defined by frequent and recurrent complete or partial collapse of the upper airway during sleep, resulting in apneas or hypopneas, intermittent asphyxia, and periodic awakenings leading to daytime somnolence.\textsuperscript{416} OSA is common, and depending on the precise disease definition used, has been documented in as many as 24\% of middle-aged men.\textsuperscript{417} OSA is also serious; it is an independent predictor of future drug-resistant hypertension, myocardial infarction, stroke, and death from cardiovascular disease.\textsuperscript{416} As recently reviewed by Drager \textit{et al}, multiple mechanisms have been proposed to link atherosclerosis and OSA, including dyslipidemia, the generation of reactive oxygen species and subsequent lipid peroxidation, and inflammation.\textsuperscript{416} Each of these risk factors for atherosclerosis can be induced in a mouse model by intermittent hypoxia, a defining characteristic of OSA in humans.\textsuperscript{416}

As the site of atherosclerosis, the endothelium is clearly implicated as a mediator of many of the adverse consequences of OSA. However, impaired endothelial function occurs even in the absence of overt cardiovascular disease, as evidenced by diminished endothelium-dependent vasodilatation, enhanced expression of cell-surface adhesion molecules, and increased circulating levels of tissue factor and vWF.\textsuperscript{418-422}
Since hypoxia is a known regulator of Weibel-Palade body exocytosis, and since a vasoconstrictive, adhesive, prothrombotic endothelial cell phenotype such as that seen in OSA is the predicted result of a net increase in circulating Ang-2, I hypothesize that angiopoietin dysregulation might occur in obstructive sleep apnea and might provide an additional link between intermittent hypoxia and the endothelium. Although angiopoietin-like protein 4 has been associated with chronic intermittent hypoxia through its role in lipid metabolism (specifically as an inhibitor of the enzyme lipoprotein lipase needed to clear triglyceride-rich lipoproteins from the circulation), Angiopoietin-1 and -2 have not been studied in OSA.

To investigate this hypothesis, I propose a study comparing plasma levels of Ang-1 and Ang-2 in three groups of patients: a) untreated patients with newly diagnosed OSA, b) patients with treated OSA adherent to the prescribed regimen of nasal continuous positive airway pressure (CPAP), and c) untreated healthy controls without OSA. Based on the above data, I expect to find angiopoietin dysregulation, independent of other cardiovascular disease risk factors, present in untreated patients with OSA. Furthermore, treated patients with OSA who are adherent to CPAP should manifest an angiopoietin profile akin to that of healthy controls without sleep-disordered breathing, provided that the prescribed CPAP therapy has been documented with a titration sleep study to reduce or eliminate apneic/hypopneic episodes. As a substudy, I propose to follow the newly diagnosed patients with serial measurements of plasma Ang-1 and Ang-2 both before and after the initiation of CPAP with the intent of documenting reduced angiopoietin dysregulation in individual patients in whom CPAP is successful in eliminating or reducing intermittent nocturnal hypoxia.

8.4 Procalcitonin in patients with Hematologic Malignancy

The duration of appropriate broad-spectrum antibiotic therapy in patients with prolonged febrile neutropenia without obvious source is a common and contentious issue. Early discontinuation in the setting of unrecognized infection could lead to morbidity or mortality, while delayed discontinuation in the absence of infection exposes patients to unnecessary antibiotics and the inherent risk therein, and may increase antimicrobial resistance. Since microbiologic cultures and other diagnostic tests are often negative in this patient population, an alternative marker of infection with sufficient negative predictive value to guide the safe discontinuation of antibiotics (although not necessarily antifungals) would be of benefit.
Procalcitonin has been proposed as a marker to guide antibiotic therapy, and in this role was the subject of a recent systematic review by Schuetz et al.\textsuperscript{425} Fourteen randomized controlled trials, enrolling a total of 4467 patients with respiratory tract infections or sepsis in primary care, emergency department or ICU settings, were included in the analysis. Use of a procalcitonin cut-off value of $< 0.25$ ng/mL in primary care or the ED, or $< 0.25 - 1$ ng/mL in the ICU to determine initiation or duration of antibiotics resulted in a significant decrease in antibiotic use and duration, without a change in mortality. A second systematic review by Agarwal and Schwartz focused on sepsis in the ICU and confirmed that procalcitonin-based algorithms for antibiotic discontinuation lead to reduced antibiotic exposure without compromising survival.\textsuperscript{426} Importantly, this finding was sufficiently robust to withstand relatively high rates of physician non-adherence to the algorithm (failure to discontinue antibiotics despite a low or declining procalcitonin level). A third systematic review focused on only those studies exploring PCT-guided antibiotic therapy in patients with respiratory tract infections, and found results identical to those of Schuetz and Agarwal and Schwartz.\textsuperscript{427}

However, since the publication of these meta-analyses, a large study of 1200 critically ill patients randomized to either standard or PCT-guided antibiotic therapy found that PCT-guided therapy did not improve survival, and in fact prolonged ICU stay and increased use of broad-spectrum antimicrobial agents.\textsuperscript{428} In this study, a PCT measurement of $\geq 1$ ng/mL triggered escalation and expansion of the spectrum of the patient’s current antimicrobial regimen. Nonetheless, PCT may still be useful to guide antimicrobial \textit{discontinuation}, as opposed to \textit{escalation}.

Before such a study is undertaken, the sensitivity and specificity of PCT for the diagnosis of bacterial infection in this population must be clarified. In a mixed population of immunocompromised patients (HIV or hematologic/solid tumour malignancies), procalcitonin levels greater than $0.5$ ng/mL displayed excellent sensitivity for the diagnosis of bacterial infection, and remained an independent predictor of such in a multivariate analysis.\textsuperscript{429} However, a separate study found that while all patients with bacteremia in the setting of febrile neutropenia ultimately manifested a procalcitonin level above $0.5$ ng/mL (the lower limit of detection for first generation assays), a substantial portion of patients with febrile neutropenia without source also manifested PCT values above this limit.\textsuperscript{430} While some studies have found that PCT can effectively identify those patients with bacteremia from amongst the total population of patients with febrile neutropenia, others have found reduced sensitivity and specificity of PCT in febrile
neutropenic as compared to febrile non-neutropenic patients for the diagnosis of bacterial infection. In a study of 90 patients undergoing chemotherapy for a hematologic malignancy and who presented with febrile neutropenia, admission plasma procalcitonin levels, using a sufficiently sensitive assay, were unable to discriminate between those with microbiologically or clinically documented infections and those with fever without source. However, if the peak procalcitonin level occurred more than three days after the onset of febrile neutropenia, it was predictive of an invasive fungal infection, perhaps indicating that elevated procalcitonin levels have a different significance or should be interpreted differently in immunocompromised patients, and certainly indicating that the utility of PCT in patients with febrile neutropenia requires further exploration.

Consequently, I would propose two sequential studies:

1. Cohort study of the performance characteristics of PCT to discriminate microbiologically-confirmed bacterial from non-bacterial infection in non-neutropenic patients with hematologic malignancy and in neutropenic patients with fever of short duration (< 5 days).

2. Randomized controlled trial of PCT-guided antibiotic discontinuation in prolonged febrile neutropenia (> 14 days) without source, provided the findings from study 1 confirm a sufficient negative predictive value for PCT in this setting.

8.5 Systematic review of procalcitonin for the prediction of mortality in sepsis and severe bacterial infection

As can be seen from the literature review in Chapter 1.9.1 and from Table 1, there are a substantial number of publications, many with conflicting results, pertaining to the use of PCT to predict outcome in sepsis or severe bacterial infection. Distilling and organizing this information in a systematic fashion may yield a better understanding of the potential benefit, or lack thereof, to the use of PCT for this indication. To my knowledge, no such review is ongoing, and searches of both the Cochrane Database of Systematic Reviews and the newly launched Prospero International Registry of Systematic Reviews yielded no registered reviews.
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