PREDICTING THE TIME TO DEATH FOLLOWING SEPSIS IN UGANDA: A BIOMARKER-BASED APPROACH

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Abstract

The overall goal of this project was to accurately predict the time to death for patients with sepsis in Uganda using markers involved in the endothelial response to infection. There is substantial evidence from developed countries that endothelial markers measured at the time of hospital admission are associated with increased risk of death within 28 days, and we hypothesized that they would also discriminate between patients who die shortly after admission from those who have slower clinical progression.

We first investigated the underlying heterogeneity in sepsis. We hypothesized that patients presenting with severe sepsis represent a mixture of latent processes and subgroups of individuals that can be grouped by their "endothelial response profile". We characterized the underlying processes and subgroups using latent factor analysis (LFA) and latent profile analysis (LPA), respectively. We then identified biomarkers that accurately predict which patients will die by examining the discriminative value of the candidate predictors. Biomarkers and patient characteristics with the highest predictive accuracy were used to model the relative time to death using a generalized gamma model.

The LFA results suggested four latent processes, interpreted as "inflammation", "vessel stability", "leukocyte recruitment", and "vessel instability" based on the known biologic functions of the constituent biomarkers. Using LPA, we identified three subgroups of patients with endothelial response patterns that were homogenous within the group and distinct from the other groups. The patterns were interpreted as "quiescent", "endothelial dysfunction", and "endothelial repair". Death by 28 days was best predicted with a model consisting of endothelial dysfunction, $CD4^+$ T cell count less than 50 cells/mm^3,

Karnofsky score of 20 or less, and the 5th quintile of sFlt-1 concentration, a soluble receptor involved in vascular leak. The area under the curve (AUC) for the model for 28-day mortality was 0.73 in the derivation set and 0.77 in the validation set. The survival time for patients with endothelial dysfunction was approximately half that of patients with similar CD4^{+} T cell counts, Karnofsky scores, and sFlt-1 concentrations (relative time = 0.49, 95%CI: 0.32, 0.75). Profiling patients based on their endothelial response may provide a clinically meaningful way to categorize patients into homogenous subgroups and may identify patients at risk of imminent death.

Acknowledgments

Sepsis is referred to as the “graveyard for drug discovery”, and at the outset of this project there was some concern that it would be a graveyard for my dissertation as well. Countless discussions with my advisor, Dr. Bill Moss, were invaluable in helping me think through the underlying issues with sepsis and sepsis research. These discussions largely shaped my approach to solving the problem. Several remarkable individuals at Johns Hopkins took the time to impart their knowledge and expertise, including Drs. Alvaro Muñoz, Karen Bandeen-Roche, and David Dowdy. In every discussion I was challenged to think carefully about what the data were telling me, and whether that was what I really needed to know. The inspiration for this project came from several directions. Dr. Matt Hepburn continuously motivates me to try to save the world and perpetuates my interest in infectious diseases. My mom is the source of my strength, persistence, and patience. And none of it would have been possible without the love and support of my friends and family. For all of this, I am very thankful.

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I. Introduction

What is sepsis?

Sepsis is defined as a systemic inflammatory syndrome in response to an infection (probable or documented), and is classified as severe when accompanied by evidence of hypoperfusion or acute organ dysfunction.[1] The criteria for systemic inflammation are based on temperature, heart rate, respiratory rate, and white blood cell count, and can occur in both infectious and non-infectious conditions. Severe sepsis accompanied by hypotension that is refractory to adequate fluid resuscitation constitutes septic shock. This broad framework of definitions was proposed in the American College of Chest Physicians and Society of Critical Care Medicine Consensus Conference in 1991.[2] The definition of sepsis was modified in a Consensus Conference in 2001,[3] to include additional clinical and laboratory parameters (Appendix 1).

Sepsis is caused by a pathogenic host response to a wide range of microorganisms, including bacteria, viruses, fungi and parasites. The infecting pathogen is often not identified.[4] In developed countries the majority of infections are bacterial, with gram-negative infections (62.2%) slightly more frequent than gram-positive infections (46.8%).[5] The predominant organisms typically isolated include *Staphylococcus aureus*, *Pseudomonas* species, *Escherichia coli*, and *Candida* species. Very limited data exist on the etiology of sepsis in developing countries. A recent systematic review of community acquired bloodstream infections in Africa found *Salmonella* species (42.3%), *Brucella* species (13.2%), *Streptococcus pneumoniae* (9.5%), and *Staphylococcus aureus* (5.4%) as the predominant organisms isolated from adults.[6]

Sepsis can originate from a range of sites of infection, with the respiratory tract, genitourinary tract, and abdomen the most frequent sites.[5] The pathogen, pathogen load, site of infection, and host susceptibility contribute to the clinical presentation and course of disease. The signs and symptoms are highly variable and typically non-specific. The clinical spectrum can range from minor signs and symptoms such as fever, elevated heart rate, and altered mental status, to severe illness with organ dysfunction, shock, and death.

The broad definition of sepsis combines a wide range of pathogens and sites of infection into one complex syndrome with substantial heterogeneity. The underlying assumption is that the host response to the infectious insult is consistent across the spectrum of pathogens and affected organ systems. The failure to develop effective therapeutics and the limited success in developing diagnostic or prognostic tests is often attributed to the heterogeneity inherent in the syndrome.[7,8] There are currently no Food and Drug Administration (FDA) approved treatments for sepsis, despite several decades of clinical trials. Activated protein C was FDA approved but then removed from the market after further studies failed to demonstrate a treatment effect. Restricting the evaluation of candidate therapeutics to homogeneous subsets of patients, such as patients with a specific infectious etiology, has been proposed.[7,9] However, despite widespread recognition that the pathogenic processes leading to organ failure and death differ between microorganisms, most clinical trials continue to group together all patients with sepsis.[8] Furthermore, given the wide spectrum of pathogens causing sepsis, powering studies for a specific organism would be challenging.

The host response

Sepsis is conceptualized as a pathogenic host response to a microorganism. Researchers concluded that the host response, rather than the direct effects of the microorganism, was pathogenic because many patients with sepsis die despite clearance of the infection.[10,11] The striking clinical presentation of patients with sepsis, often consisting of high fever, shock, and respiratory failure, contributed to the theory that sepsis was due to an uncontrolled inflammatory response.[12] Attention initially focused on excessive inflammation, and many clinical trials of immunosuppressive therapies were conducted. In particular, the efficacy of corticosteroid treatment on reducing 28-day mortality was thoroughly investigated. A recent systematic review and meta-analysis found that corticosteroid treatment did not improve 28-day mortality; however, analysis of a subgroup of trials of prolonged low-dose treatment found a small reduction in mortality.[13] After more than 30 clinical trials of various immunosuppressive therapies failed, the idea of a "compensatory anti-inflammatory response syndrome" (CARS) that followed the initial pro-inflammatory response was introduced.[14] The current thinking recognizes a complex response consisting of both proinflammatory and anti-inflammatory mechanisms, as well as dysfunction of the vascular endothelium.[11]

Inflammation and immunosuppression

The initial host response to an infection consists of activation of pattern recognition receptors, particularly toll-like receptor (TLR)2 and TLR4 for bacterial infections. TLR activation initiates a signaling cascade through NF-κB, resulting in release of

proinflammatory cytokines and chemokines, such as interleukin (IL)-1α, IL-1β, IL-6, IL-8 and tumor necrosis factor –alpha (TNFα).[15] Release of these cytokines is necessary for a normal, effective immune response against a pathogen. However, excessive production is associated with sepsis[16,17] and the development of organ dysfunction and death.[18,19] Subsequent studies found concentrations of anti-inflammatory cytokines such as IL-10 were also elevated in patients with septic shock versus sepsis alone[20], and the concentrations were significantly higher in patients who died.[21,22] A recent review summarized that patients with sepsis typically followed one of three patterns of cytokine responses: 1) rapid production of both proinflammatory and anti-inflammatory cytokines, 2) predominance of anti-inflammatory cytokines, or 3) globally depressed production of cytokines.[12]

There is substantial evidence that patients who survive the initial hyper-inflammatory phase of sepsis enter an immunosuppressed phase. Patients with severe sepsis in intensive care units (ICUs) are at increased risk for nosocomial opportunistic infections, such as *Candida* spp, *Pseudomonas* spp, and *Klebsiella pneumoniae* spp.[12,23] Otto et. al found that the percent of blood cultures positive for an opportunistic bacteria was 9.1% in the first phase of sepsis (days 1-5 of hospitalization), versus 17.8% in the late phase of sepsis (beginning day 16).[23] The investigators also found 12.6% of blood cultures positive for *Candida* spp in the first phase versus 30% in the late phase. However, this study did not have a non-septic comparison group, so it is difficult to attribute the observed increase to sepsis versus the inherent risk of nosocomial infection in the ICU. A recent study conducted by Boomer et. al harvested spleens and lung tissue postmortem from patients who died from severe sepsis and critically ill controls within 30-180 minutes of death.[24]

The investigators stimulated the cells and examined the cytokine secretion (TNF, interferon [IFN]-γ, IL-6, and IL-10) and used flow cytometry to examine cell surface receptor-ligand expression profiles. Cytokine secretion in patients with severe sepsis was less than 10% that in controls, controlling for age, duration of illness, corticosteroid use, and nutritional status. In addition, they demonstrated depletion of $CD4^+$ T cells, $CD8^+$ T cells, and HLA-DR cells on splenocytes isolated from patients with severe sepsis, and increased percentages of inhibitory receptor ligands on lung epithelial cells.[24] Collectively these studies support the theory of an immunosuppressive phase in sepsis.

Microcirculatory alterations

Sepsis is characterized by microvascular leak, which manifests clinically as hypotension, tissue edema, hypoperfusion, and organ dysfunction.[25,26] The vascular leak resulting from endothelial activation is thought to contribute to the tissue hypoxia and organ dysfunction that are integral to the pathogenesis of sepsis. Compared to ICU controls, the capillaries of patients with severe sepsis have decreased or intermittent flow as well as decreased vascular density.[25] Several mechanisms have been proposed to account for the microcirculatory alterations, including endothelial dysfunction.[25]

This project evaluates several biomarkers involved in the endothelial response to sepsis, including angiopoietin-1 (Ang-1) and Ang-2, soluble Tie-2 receptor (sTie-2R), vascular endothelial growth factor (VEGF), and fms-like tyrosine kinase-1 (sFLT-1), markers of inflammation (Chi3L1, IP-10, TREM-1, and ICAM), and molecules involved in

coagulation, including von Willebrand factor (vWF) and platelet factor 4 (PF4). The role of these biomarkers in sepsis is shown in Table 1.

Biomarker	Role in Sepsis
Angiopoietin-1 (Ang1)	Inhibits endothelial activation through VEGF and NF-κB signaling pathways, enhances endothelial barrier function[26]
Angiopoietin-2 (Ang2)	Opposes Ang1, stimulates vascular leak and endothelial cytoskeleton rearrangement[26]
Tie-2 Receptor (sTie-2R)	Receptor for Ang1 and Ang2[26]
Vascular endothelial growth factor (VEGF)	Stimulates vascular leak,[8] hypoxia inducible mitogen[28]
fms-like tyrosine kinase-1 (sFLT-1)	Soluble receptor for VEGF, inhibits VEGF signaling, may lead to immune suppression[27]
Chitinase-3 like protein-1 (Chi3L1)	Elevated in inflammation or ongoing fibrosis
Interferon –inducible protein 10 (IP-10)	Regulates lymphocyte trafficking[29]
Triggering receptor expressed on myeloid cells type 1 (TREM1)	Amplifies the inflammatory response
Intracellular adhesion molecule-1 (ICAM-1)	Indicator of endothelial activation, role in initiation of inflammation[28]
von Willebrand factor (vWF)	Ultra-large vWF multimers cause platelet clot formation[28]
Platelet factor 4 (PF4)	Promotes blood coagulation, released following platelet activation, (aka chemokine CXCL4)[30]

Table 1. Role of biomarkers in sepsis

The endothelium is a monolayer of cells lining of the interior surface of blood vessels that acts as a selective barrier to control the passage of fluids, electrolytes, proteins and cells into and out of the bloodstream. During the course of an infection the endothelium becomes activated, meaning that it is more prone to clots, there is increased transmigration of leukocytes to sites of infection, and the vessels become leaky.[26] Adhesion molecules, such as intercellular adhesion molecule (ICAM)-1, are expressed by the endothelial cells and bind to ligands on leukocytes in the bloodstream to slow and eventually stop the cells so they can migrate into the underlying tissue. While essential for an effective defense against invading pathogens, extravasation of leukocytes across

the endothelium can result in tissue damage when cytotoxic mediators are released.[31] Neutrophils and monocytes express high levels of a receptor called triggering receptor expressed on myeloid cells (TREM)–1 in extracellular bacterial infections. TREM-1 triggers secretion of potent proinflammatory mediators.[32] Lymphocytes are also recruited to the site of infection through molecules such as interferon inducible protein 10 (Ip10).[29] Lymphocytes have been shown to promote systemic inflammation in septic shock, and lymphocyte activation is associated with development of multi-organ failure.[29] Immune cells such as neutrophils and macrophages also secrete factors including chitinase-3 like protein-1 (Chi3L1) to further stimulate inflammation and promote remodeling.[33] Platelet factor 4 (PF4) is secreted by activated platelets, and plays a role in coagulation.[30] PF4 has also been found to inhibit angiogenesis, the formation of new blood vessels from pre-existing blood vessels.

Activation of the endothelium leads to the expression of adhesion molecules, and release of proteins including angiopoietin (ang)-2 and von Willebrand factor (vWF) into the bloodstream.[34] Ang-2 is a growth factor, which competes with ang-1 for their receptor, tyrosine kinase-2 (Tie2). Ang-2 has a destabilizing effect on the endothelium,[35] whereas ang-1 improves endothelial barrier function, inhibits vascular leak, and acts on adhesion molecules and cell junctions to exert anti-inflammatory effects.[36,37] Ang-2 primes the endothelium to respond to proinflammatory (TNF and IL-1) and angiogenic (vascular endothelial growth factor [VEGF]) stimuli, which propagates further endothelial activation and increases vascular permeability.[38] The soluble form of the receptor for VEGF, fms-like tyrosine kinase (sFLT)–1, competes with the membrane-bound form of the receptor to bind VEGF.

In summary, the host response to sepsis is a complex interplay between inflammatory, anti-inflammatory, and microcirculatory alterations. This project focuses on 11 biomarkers involved in the endothelial response to sepsis. The endothelium is a key regulator in maintaining vascular homeostasis and endothelial activation is thought to contribute to the vascular leak, tissue hypoxia, and multi-organ failure in severe sepsis. The mechanisms leading to a pathogenic endothelial response are not completely understood but likely involve prolonged, systemic endothelial activation leading to release of proinflammatory and cytotoxic mediators and growth factors, resulting in vessel instability and leakage of fluids from the vasculature.

Biomarkers for prediction of sepsis mortality

A recent systematic review by Pierrakos and Vincent identified 178 biomarkers in 3,370 clinical and experimental studies evaluated for their diagnostic or prognostic value in patients with sepsis.[39] The biomarkers encompassed a wide range of biologic pathways, including coagulation, the complement cascade, endothelial activation, inflammation, and apoptosis. The authors grouped the biomarkers as follows: 1) cytokine/chemokines (12 biomarkers); 2) cell markers (14 biomarkers); 3) receptors (17 biomarkers); 4) coagulation (8 biomarkers); 5) vascular endothelial damage (15 biomarkers); 6) vasodilation (15 biomarkers); 7) organ dysfunction (17 biomarkers); 8) acute phase proteins (9 biomarkers); and 9) other (71 biomarkers). The majority of the biomarkers were assessed for their prognostic value, and none had sufficient (defined as >90%)

sensitivity or specificity to be clinically useful. The authors concluded that combinations of biomarkers should be evaluated in future studies.

A recent systematic review by Xing et al. searched for studies evaluating 12 endothelial activation markers for their diagnostic or prognostic value, and identified 61 studies meeting their criteria.[40] Eleven studies investigated the relationship between sICAM-1 and mortality, only five of which found high concentrations of sICAM-1 associated with death.[40] Six studies investigated the association between Ang-2 concentration and mortality. Four of the six studies found that elevated concentrations of Ang-2 were significantly associated with mortality. Other studies found that Ang-1, but not Ang-2 concentrations predict 28-day mortality.[41]

The association of vWF with sepsis mortality was investigated in ten studies, six of which found a significant relationship between elevated vWF concentration and death.[40] Four studies investigated the relationship between VEGF concentration and mortality, and its soluble receptor sFlt-1 was evaluated in one study.[40] Two studies found no association between VEGF concentration and mortality,[27,42] one study reported significantly higher concentrations of VEGF in survivors,[43] and one study found significantly higher concentrations of VEGF in non-survivors.[44]

Epidemiology of sepsis

Incidence and mortality

The difficulty in clearly defining sepsis has hampered efforts to understand the epidemiology. The definitions developed in the Consensus Conferences differ from those used in the International Statistical Classification of Diseases and Related Health Problems (ICD) coding system for septicemia, which is defined as bacteria in the blood. Estimates derived from hospital discharge records from seven large states in the US in 1995 suggested a national annual incidence of 3 cases of severe sepsis per 1,000 population (751,000 cases per year).[45] The current number of cases of severe sepsis in the US is likely over 1 million per year.[4] The incidence of severe sepsis has been increasing in the US over the last 20 years, likely partially due to the growing elderly population.[46] Approximately 50% of patients with severe sepsis receive intensive care, and 25% die in the hospital.[5] With advances in intensive care and increased awareness, the case fatality is decreasing for patients with severe sepsis.[11,46]

Epidemiologic data are lacking for sepsis in developing countries; however, a recent systematic review and meta-analysis estimated that approximately 13.5% of adult patients admitted to the hospital in Africa had a bloodstream infection.[6] In developed countries, the majority of patients with severe sepsis are elderly. In the US, the incidence of severe sepsis was over 100 times higher in those over 85 years of age versus children.[45]

Although data are limited, HIV infection is thought to be an important contributor to sepsis incidence in developing countries.[47] A recent systematic review of community-

acquired bloodstream infections (BSI) in hospitalized patients compared the relative risk of BSI in HIV infected versus uninfected patients.[48] The investigators identified 16 studies of BSI in adults and found that 20% of HIV infected patients had a positive blood culture compared to 9.2% of HIV uninfected patients. The authors concluded that HIV infected patients are at increased risk of bloodstream infections. The increased risk is thought to be due to immunosuppression; however, a study conducted in the US found no difference in $CD4^+$ T cell counts between HIV infected patients with and without BSIs.[49] In contrast, other studies have reported increased incidence of BSI in HIV infected patients with lower $CD4^+$ T cell counts.[50–52]

Project Aims

The overall goal of this project was to accurately predict the time to death for sepsis patients in Uganda using markers involved in the endothelial response to infection. There is substantial evidence from developed countries that endothelial markers measured at the time of hospital admission are associated with increased risk of death within 28 days. We hypothesized that these markers would discriminate patients who die shortly after admission from those who have slower clinical progression.

We first investigated the underlying heterogeneity in sepsis. We hypothesized that patients presenting with severe sepsis represent a mixture of latent processes and subgroups of individuals that can be grouped by their "endothelial response profile". We characterized the underlying processes and subgroups using latent factor analysis and latent profile analysis, respectively (**Aim 1**). We then identified biomarkers that

accurately predict which patients will die (**Aim 2**). Biomarkers and patient characteristics with the highest predictive accuracy were used to model the relative time to death using a generalized gamma model (**Aim 3**).

Conceptual Framework

Our conceptual framework is grounded in what is known about the function of the biomarkers in sepsis pathogenesis (Figure 1). These 11 markers were selected based on their known biologic involvement in processes related to endothelial activation in patients with sepsis. In our framework, the biomarkers were grouped by their hypothesized role in the pathogenesis of sepsis (i.e. inflammation, expression of adhesion molecules, vessel instability, and platelet activation). Latent factor analysis was used to explore the correlation structure of the biomarkers and revise the conceptual framework (Chapter 2).

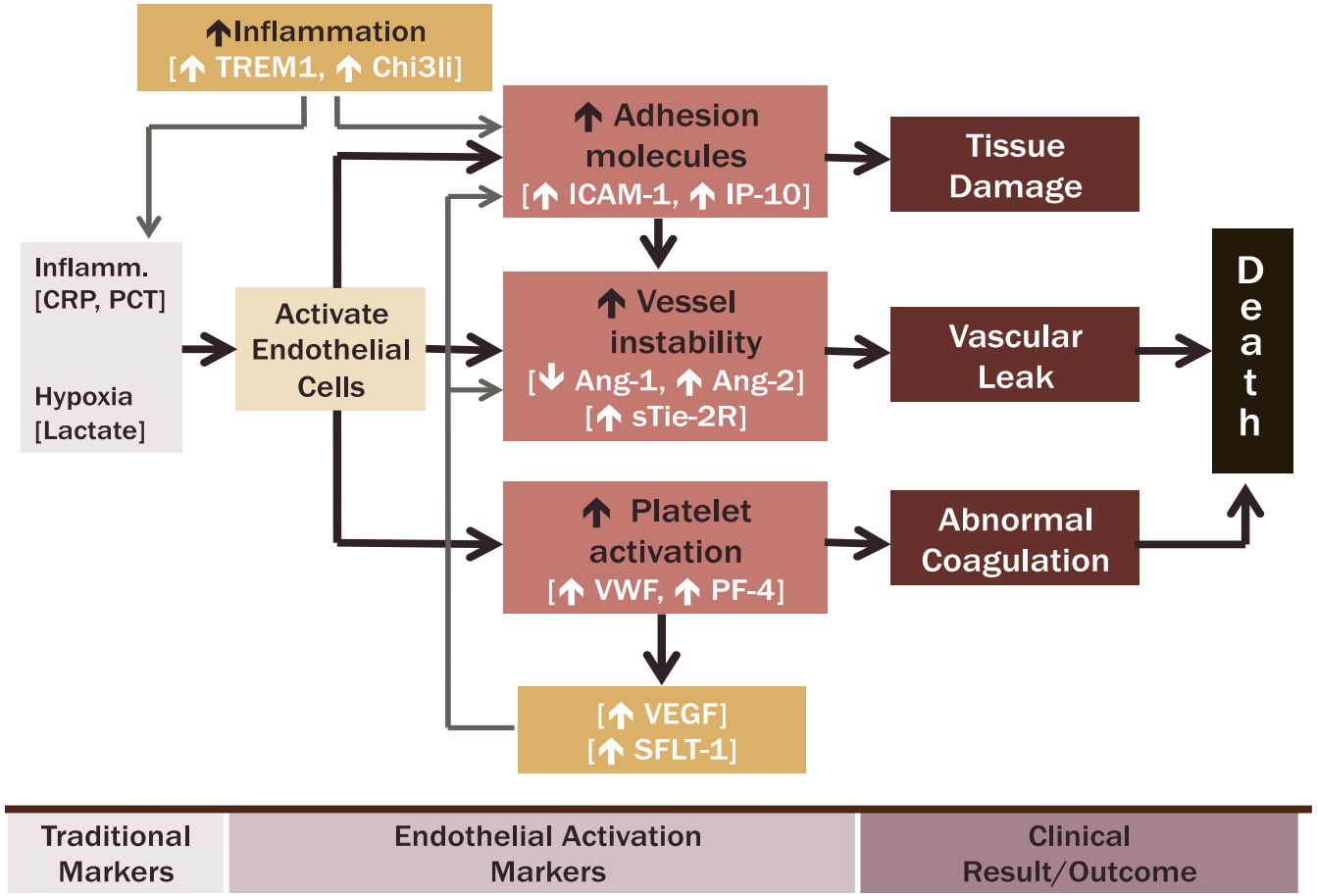

Figure 1. Conceptual framework

II. Heterogeneity in Sepsis

Background

Sepsis is defined as a systemic inflammatory syndrome in response to an infection, and is classified as severe when accompanied by evidence of hypoperfusion or acute organ dysfunction.[1] This broad definition combines a wide range of pathogens and sites of infection into one complex syndrome with substantial heterogeneity. Despite widespread recognition that the biologic processes leading to death differ, most studies continue to group together all patients with sepsis.[8] The underlying assumption is that the host response to the infectious insult is consistent across the spectrum of pathogens, affected organ systems, and patient comorbidities. While it is unlikely that there is one consistent response, it is worth evaluating the hypothesis that there are distinct patterns of host responses.

Microcirculatory alterations are thought to play a large role in the host response to sepsis. Sepsis is characterized by microvascular leak, which manifests clinically as hypotension, tissue edema, hypoperfusion, and organ dysfunction.[25,26] The endothelium is a monolayer of cells lining of the interior surface of blood vessels that acts as a selective barrier to control the passage of fluids, electrolytes, proteins and cells into and out of the bloodstream. During the course of an infection the endothelium becomes activated, meaning it is more prone to clots, there is increased transmigration of leukocytes to sites of infection, and the vessels become leaky.[26]

This study evaluates 11 biomarkers involved in the endothelial response to sepsis, including angiopoietin-1 (Ang-1) and Ang-2, soluble Tie-2 receptor (sTie-2R), vascular endothelial growth factor (VEGF), and fms-like tyrosine kinase-1 (sFLT-1), markers of inflammation (Chi3L1, IP-10, TREM-1, and ICAM), and molecules involved in coagulation, including von Willebrand factor (vWF) and platelet factor 4 (PF4). These markers were selected based on their hypothesized role in endothelial activation in patients with sepsis.

To evaluate whether the endothelial response to sepsis consists of one unified biological process, or multiple processes, latent factor analysis (LFA) was used to analyze the correlation structure of the biomarkers. LFA is a multivariate statistical method for determining the number and nature of patterns of an observed correlation structure. In this study, each factor represents an underlying biological process comprised of a set of correlated biomarkers.

Complex diseases such as sepsis are comprised of a heterogeneous mixture of patients with a spectrum of underlying pathophysiologic processes. Latent profile analysis (LPA) is a method to ascertain subgroups of patients conforming to a particular pattern of indicators out of an otherwise heterogeneous population. In LPA, subgroups of individuals are formed such that individuals within the subgroup have common response probabilities. In turn, the fitted model can be used to classify patients with different biomarker patterns into different subgroups. LPA provides a useful means of identifying subgroups of patients with homogenous biomarker patterns, thus reducing the heterogeneity in the study population. LPA is similar to latent class analysis, but allows for continuous indicators.

Methodology

Study population

The data for this project are from the second "Promoting Resource-Limited Interventions for Sepsis Management in Uganda" (PRISM-U2) study.[53] PRISM-U2 was a prospective study of fluid resuscitation in sepsis conducted at two hospitals in Uganda from May 2008 to May 2009. Fluid resuscitation is considered standard care in sepsis but had not been evaluated in developing country settings. All patients included in this analysis were in the intervention cohort, in which the study team managed the clinical care of the patient.

Adult patients admitted to Mulago National Referral Hospital and Masaka Regional Referral Hospital with suspected infection were evaluated for inclusion. In order to be included, patients had to meet the following criteria: 1) suspected infection as determined by the admitting medical officer; 2) two or more of the following: a) axillary temperature >37.5°C or < 35.5°C, b) heart rate >90 beats/minute, c) respiratory rate >20 breaths/minute; 3) systolic blood pressure (SBP) ≤100 mmHg; and 4) whole blood lactate concentration >2.5 mmol/L or Karnofsky Performance Status (KPS) score ≤40. The KPS measures the patient's ability to conduct normal activities, ranging from no specialized care needed (100) to dead (0).[54] A score of 40 indicates that the patient requires specialized care, 20 indicates the need for active supportive treatment, and moribund patients are assigned a score of 10. Patients with acute cerebrovascular events or gastrointestinal hemorrhage, or those admitted to a non-medical ward (i.e. surgical or

maternity wards) were ineligible for the study. A total of 426 patients were enrolled. The ethics committees of the University of Virginia, Makerere University, Mulago Hospital, the Infectious Disease Institute, and the Uganda National Council of Science and Technology approved the study. Informed consent was obtained from the patient or a surrogate if the patient was unable to provide written consent.

Data collection

Data were collected on patient demographics and clinical characteristics, and blood was drawn on enrollment for laboratory testing. The patient management and data collection were provided by trained study personnel and used a standardized data collection instrument. Demographic information included patient age and sex. If the patient was unable to provide demographic information, the data were collected from a surrogate. Clinical characteristics were collected on admission, and included vital signs, use of highly active antiretroviral therapy (HAART), duration of illness prior to hospitalization, Karnofsky Performance score, and Glasgow Coma Scale. Double data entry was conducted for quality control using Epi Info.

Laboratory Testing

Malaria thick smears, HIV serology, and point-of-care lactate assays were conducted at the hospitals. Lactate was measured on whole blood using a point of care lactate assay (I-

STAT, Abbott Point of Care, Inc.). Clinical laboratories near the two hospitals conducted the complete blood counts, $CD4^+$ T cell counts, and aerobic cultures.

Mycobacterial blood cultures: Mycobacterial blood cultures were conducted at the Joint Clinical Research Center in Kampala, which participates in the World Health Organization External Quality Assurance program for microscopy, culture and drug susceptibility testing. Mycobacterium tuberculosis (MTB) bacteremia was defined as mycobacteria blood culture positive and confirmed as tuberculosis by polymerase chain reaction (PCR) or antigen test.[47] Bactec Myco/F Lytic media vials were inoculated with 3-5 mL of blood and incubated in the Bactec 9120 at 35°C. Positive vials were examined for acid-fast bacilli (AFB) and morphology consistent with MTB. AFB positive cultures were subcultured on selective media. PCR of the IS6110 target insertion sequence or detection of the MPB64 protein (Capilia TB assay,[55] Nippon Becton Dickinson Co., Ltd, Tokyo, Japan) was used to confirm MTB.

Biomarker assays: For this study, data on 11 endothelial biomarkers were analyzed: Ang-1, Ang-2, sTie2R, VEGF, sFlt-1, sICAM-1, TREM-1, Chi3L1, vWF, PF4, and IP10. All biomarker assays were conducted at the University of Toronto using blood drawn at study enrollment. The clinical samples were centrifuged at the hospital, and serum was stored at -20°C. Commercial enzyme-linked immunosorbent assays were used to measure biomarker levels (Ang-1, Ang-2, sICAM-1, TREM-1, Chi3L1, PF4, IP-10, sFlt-1, sTie2R, VEGF: R&D Systems, Minneapolis, MN; vWF: antibody from Dako, Carpinteria, CA, standard from American Diagnostica, Stamford, CT). All assays were conducted in duplicate. The biomarker results were reported on a continuous scale, measured in either picograms or nanograms per milliliter. The upper and lower limits of

detection for each assay are: Ang-1 (0.039 –20.000 ng/mL); Ang-2 (0.016–8.000 ng/mL); sICAM (0.078–4.000 ng/mL); TREM-1 (93.8-6,000 pg/mL); Chi3L1 (31.2-2,000 pg/mL); PF4 (15.6-1,000 pg/mL); IP-10 (31.2-2,000 pg/mL); sFlt-1 (125-8,000 pg/mL); sTie2R (156-10,000 pg/mL); VEGF (31.2-2,000 pg/mL); vWF (1.95–2000.00 ng/ mL).

Data analysis

The analysis set was comprised of the 426 patients enrolled in the prospective study (the "full sample"), excluding patients missing mortality data (5) or biomarker values (106, Figure 2). Ninety-three of the missing biomarker values were due to loss of a shipment of samples, suggesting the data were missing completely at random. The final analysis set included 315 patients. The biomarker variables were plotted to identify outliers and evaluate normality. The natural logarithms of the biomarker concentrations were used for all biomarkers except sTie2R and IP10, which better approximated a normal distribution with a square root transformation. There were no severe outliers (three times the interquartile range below the 25^{th} percentile or above the 75^{th} percentile) after the transformations. The transformed variables were standardized to have a mean of zero and standard deviation of one.

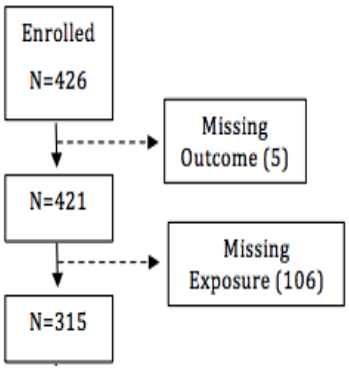

Figure 2. Patients Excluded from the Analysis

Latent factor analysis: Principal components analysis (PCA) was used to parse the biomarkers into separate indices. We determined the number of factors using several criteria, including the proportion of variance explained by the factor,[56] having an eigenvalue greater than one,[57] through the use of scree plots,[58] as well as parallel analysis[59] (PA). The eigenvalue greater than one criterion was used as an upper bound for the number of factors to retain.[56] In PA, 1000 datasets were simulated with the same number of observations and variables as the study dataset. As the generated data were random, any correlation in the indicators was due to sampling error. Factors corresponding to eigenvalues greater than the random eigenvalues obtained from the PA were retained. Factors corresponding to eigenvalues less than or equal to the random eigenvalues were considered to be due to sampling error.[56] The iterated principal factor method was used to estimate the factor loadings. Correlation in the biologic processes was expected; therefore, a promax rotation was used.[60] Factor rotations simplify the factor structure and interpretability. The rotated factor pattern matrix was used to interpret the meaning of the factors. The rotated factor loadings in this matrix were standardized regression coefficients, representing the correlation between a biomarker

and the factor holding other factors constant. The LFA was conducted using Stata (StataCorp. 2009, Stata Statistical Software: Release 11. College Station, TX).

Latent profile analysis: A series of latent profile models was evaluated to determine the number of latent subgroups. Several criteria were used to determine the best fitting model, including the Bayesian Information Criterion (BIC),[61,62] the log likelihood, the Lo-Mendel Rubin test,[63] entropy,[64] and clinical interpretability.[65] Once the optimal number of classes was determined, subjects were assigned to the most-likely class based on the posterior probability of class membership. Multinomial logistic regression was used to investigate the demographic and clinical characteristics of the latent subgroups. These models provide the odds of membership in a given latent class versus a reference latent class, with the corresponding confidence interval. The 3-step approach was used to account for the measurement error in the classification of patients into their most-likely class.[66] Age, sex, and the natural logarithm of the $CD4^+$ T cell count were included in the models as potential confounders. M-plus v.7 (Muthén and Muthén, Los Angeles, CA) was used to identify the best fitting LPA model and for multinomial logistic regression analysis.

Results

Demographic and clinical characteristics

The demographic and clinical characteristics of the full sample and the analysis sample were not substantially different (Table 2). All remaining analyses were conducted using

the analysis set (N=315). The median patient age was 35 years (IQR 27-40), with approximately equal numbers of males and females. The majority of patients had a primary (53%) or secondary (33%) school education, and earned less than 50,000 Ugandan Shillings (USH) per month (53%). This currently equates to approximately $20 US dollars per month. The median systolic blood pressure was 86 mmHg (IQR 78-90). The patients were predominantly HIV infected (85%), with a median $CD4^+$ T cell count of 40 cells/mm^3 (IQR 11-118). Twelve percent of the patients (N=39) had a positive malaria smear.

Characteristic	Full Sample N=426	Analysis Set N=315
Demographics		
Age in years [median (IQR)]	34 (27-40)	35 (27-40)
Female [n (%)]	219 (51)	163 (52)
Education [n (%)]		
None	35 (9)	26 (9)
Primary school	231 (56)	159 (53)
Secondary school	127 (31)	98 (33)
More than secondary school	17 (4)	17 (6)
Income		
<50,000 USH/mo	213 (53)	154 (53)
50,000-99,999 USH/mo	82 (21)	51 (17)
100,000-299,999 USH/mo	77 (19)	61 (21)
≥300,000 USH/mo	28 (7)	26 (9)
Clinical variables		
SBP, mmHg [median (IQR)]	85 (78-90)	86 (78-90)
HIV infected [n (%)]	368 (87)	267 (85)
$CD4^+$ T count, cells/mm^3 [median (IQR)]	63 (15-178)	40 (11-118)

Table 2. Demographic and Clinical Characteristics
IQR: interquartile range; USH: Ugandan Shillings; SBP: systolic blood pressure.

Biomarkers

Male and female patients did not have substantially different biomarker concentrations (Figures 3a and 3b), nor did the concentrations vary with age. Patients infected with HIV also had similar concentrations of biomarkers to non-infected patients. Visual inspection of scatterplots of the biomarkers (Figure 4) suggested correlation between Ang-1 and PF4, and between ICAM and IP10.

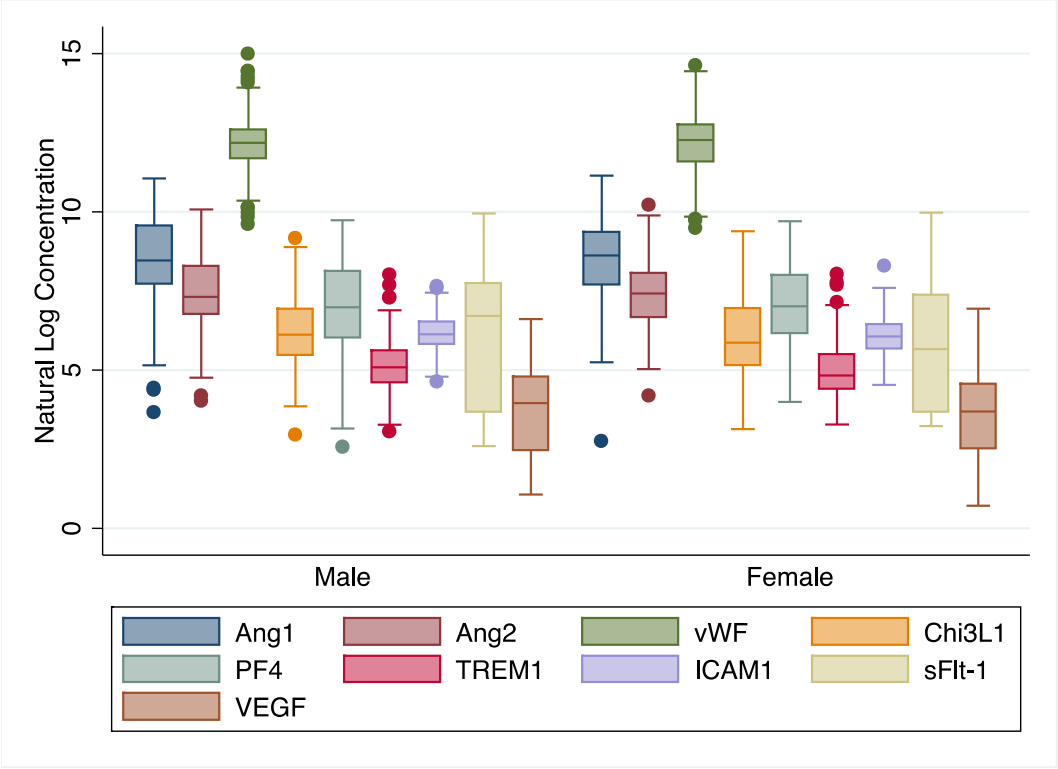

Figure 3a. Concentration of Biomarkers by Sex (Natural Logarithm Transformation)

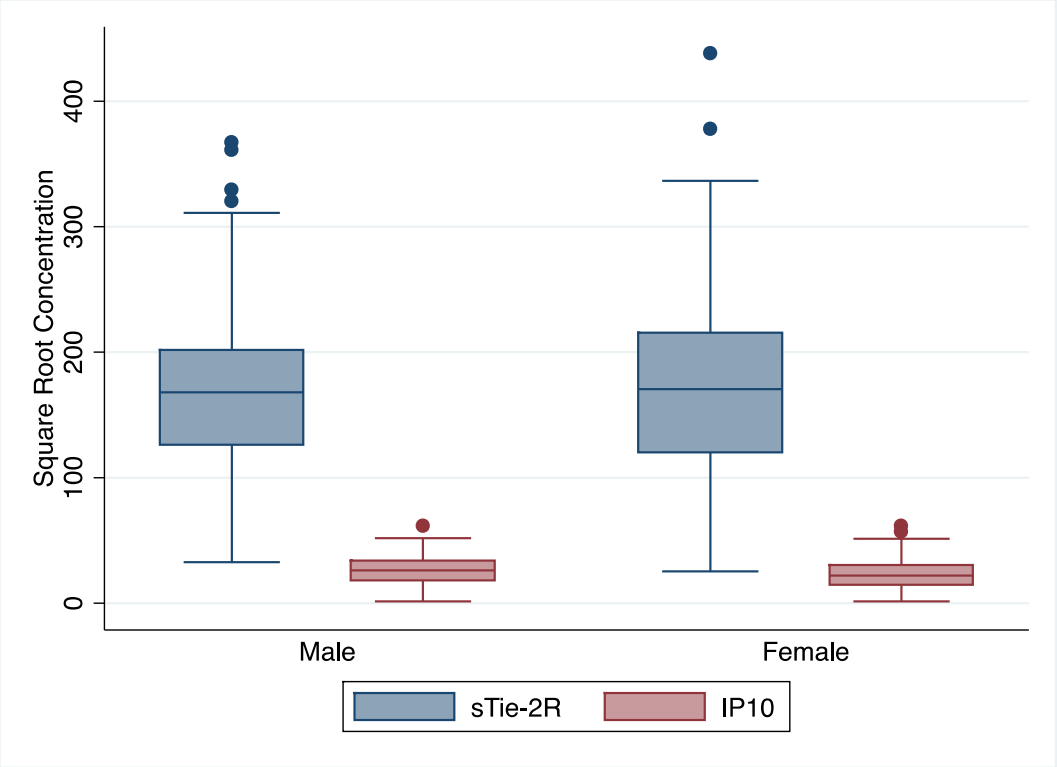

Figure 3b. Concentration of Biomarkers by Sex (Square Root Transformation)

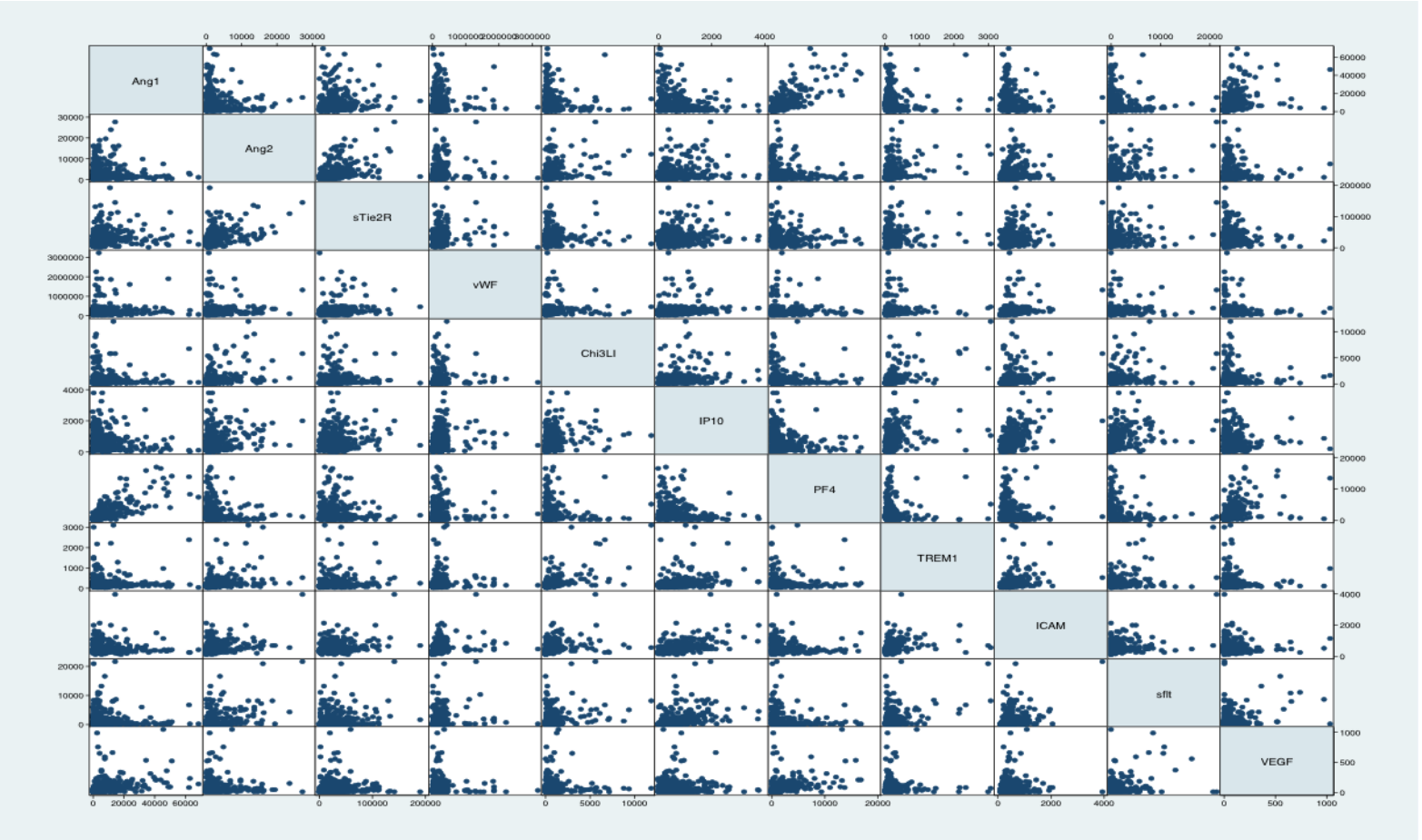

Figure 4. Scatterplot matrix of endothelial biomarkers
Visual inspection suggests correlation between Ang-1 and PF4, and between ICAM and IP10.

Latent Factor Analysis

PCA parsed the data into 11 components, 4 of which had eigenvalues greater than 1 (Table 3) suggesting that a maximum of 4 latent factors should be retained. The 4th component explained 10% of the variance in the data, which points towards retention of 4 factors. The 4 components together explained 70% of the variance in the data. The results of the scree plot and parallel analysis (Figure 5) provide additional evidence for a 4-factor model, although the 4th component is only marginally above what was observed in the randomly generated datasets. Based on these findings, a four-factor model was selected.

Component	Eigenvalue	Difference	Proportion	Cumulative
Comp1	3.2805	1.17539	0.2982	0.2982
Comp2	2.10511	.863161	0.1914	0.4896
Comp3	1.24195	.132821	0.1129	0.6025
Comp4	1.10913	.291832	0.1008	0.7033
Comp5	.817297	.246273	0.0743	0.7776
Comp6	.571024	.109918	0.0519	0.8295
Comp7	.461106	.0145727	0.0419	0.8715
Comp8	.446534	.0473304	0.0406	0.9121
Comp9	.399203	.046483	0.0363	0.9484
Comp10	.35272	.137294	0.0321	0.9804
Comp11	.215426	.	0.0196	1.0000

Table 3. Principal components analysis

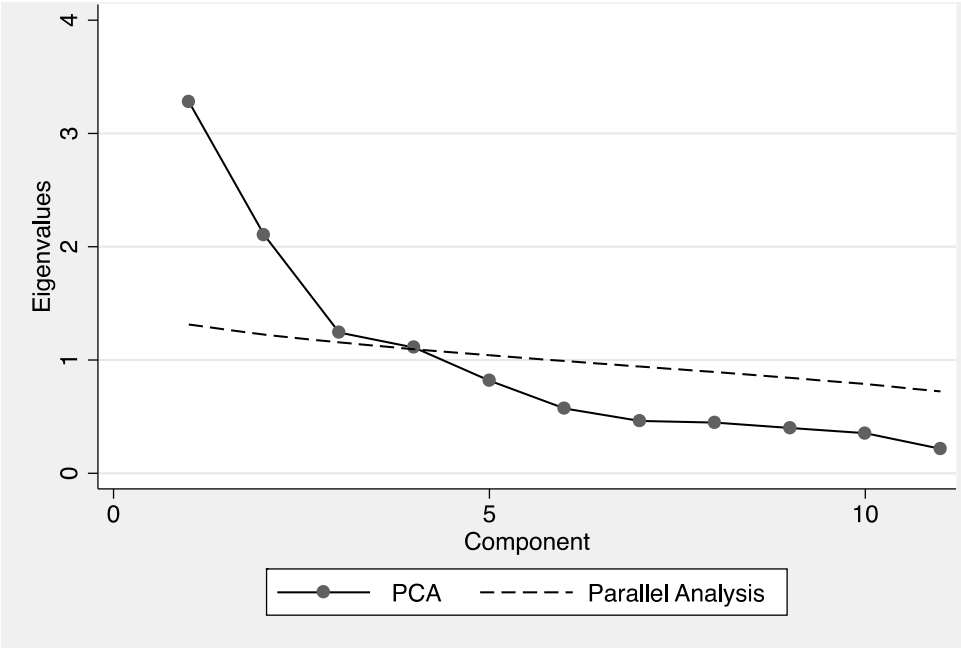

Figure 5. Scree Plot and Parallel Analysis

Factor 1 was characterized by high factor loadings for Chi3L1 (0.68), TREM1 (0.61), and sFlt-1 (0.50), and was interpreted as an inflammatory process (Table 4). Ang1 (0.81), PF4 (0.93), and VEGF (0.63) loaded on factor 2, which was interpreted as vessel stabilization. Factor 3 was characterized by high loadings of IP-10 (0.48), vWF (0.62), and ICAM1 (0.66), and was interpreted as leukocyte recruitment. Lastly, high loadings of Ang2 (0.51) and sTie-2R (0.81) characterized factor 4, interpreted as endothelial vessel instability. Factors 1 (inflammation) and 3 (leukocyte recruitment) were correlated (0.38). These results were used to modify the conceptual framework (Figure 6).

The uniqueness of most biomarkers was low, indicating that the variance in the biomarkers was well explained by the four factors (Table 4). In particular, the high factor loadings of all three biomarkers comprising factor 2 (vessel stabilization) suggests that this factor was a strong predictor of Ang-1, PF4, and VEGF. Two of the biomarkers had

uniqueness values greater than 0.6, sFlt-1 and vWF, suggesting that there was residual variability in these biomarkers. Factor 4 (vessel instability) was identified by only two biomarkers, and is therefore at risk for misinterpretation. In other words, "vessel instability" may not be the correct interpretation of this factor

Biomarker	Factor 1	Factor 2	Factor 3	Factor 4	Uniqueness
Ang1	-0.0257	**0.8061**	-0.0120	0.1859	0.3070
Ang2	0.4548	-0.0958	-0.1203	**0.5088**	0.4739
sTie2r	-0.0488	0.0326	0.0950	**0.8099**	0.3252
Chi3L1	**0.6819**	0.0603	0.1063	-0.0285	0.4862
Ip10	0.3127	-0.1107	**0.4830**	-0.0653	0.5215
vWF	-0.0931	0.1015	**0.6180**	0.1059	0.6427
Pf4	-0.0985	**0.9275**	0.0772	-0.0925	0.1365
Trem1	**0.6129**	-0.0303	0.1176	0.0227	0.5415
Icam	0.2247	0.0425	**0.6611**	0.0411	0.3993
sFlt	**0.4979**	-0.1312	0.1539	-0.0703	0.6408
VEGF	0.3332	**0.6262**	-0.0964	-0.0950	0.5299

Table 4. Rotated Factor Pattern (Promax Rotation)

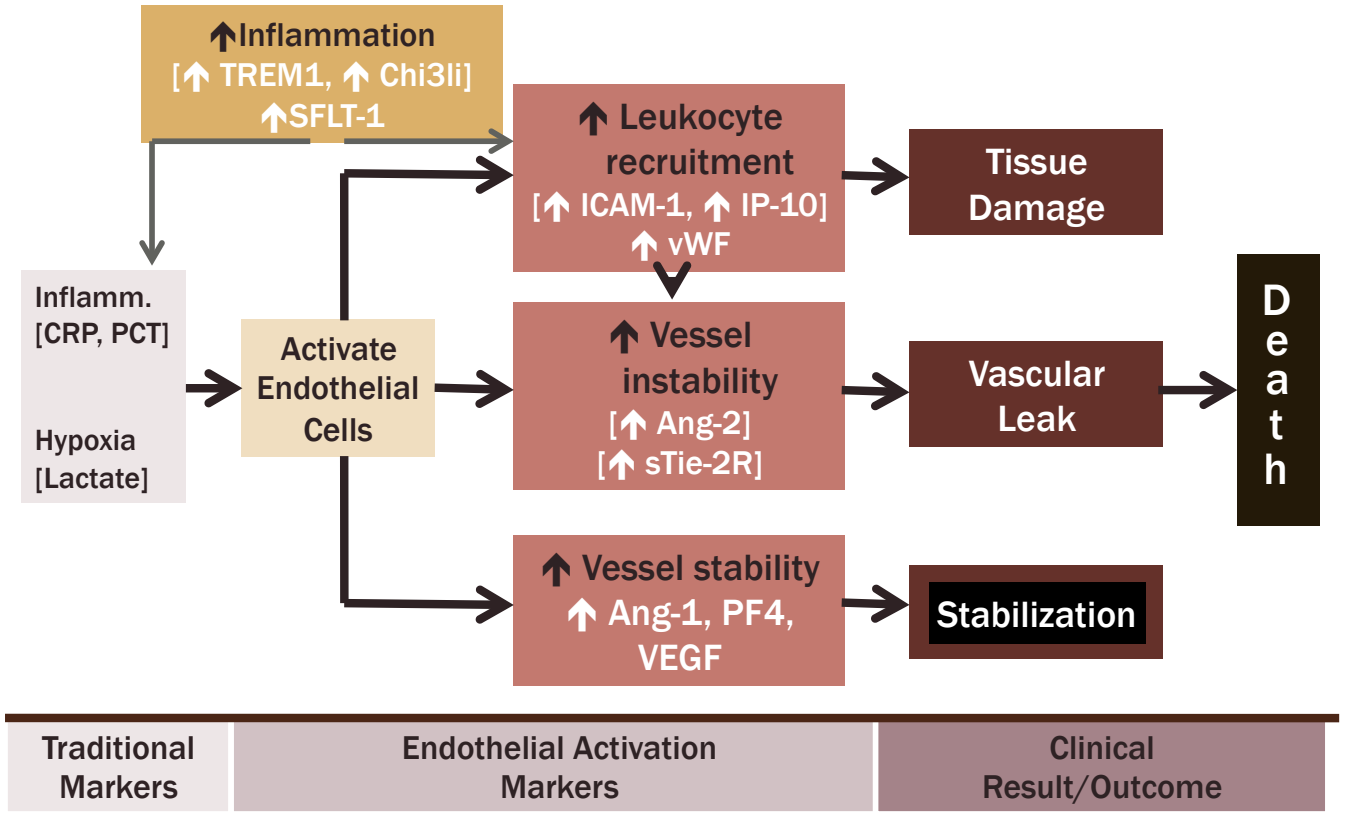

Figure 6. Revised Conceptual Framework

Latent Profile Analysis

Several fit-statistics were evaluated to determine the number of classes (Table 5). The four class model had slightly lower log likelihoods, AIC, and BIC statistics. However, the Lo-Mendel Rubin test indicated there was no improvement in fit for a four-class versus a three-class model. Furthermore, the entropy of the three-class model was higher, suggesting higher classification certainty. Based on these results, a three-class model was selected.

	Log Likelihood	**AIC**	**BIC**	**Lo-Mendel Rubin**	**Entropy**
1	-4911.1	9866.2	9948.8	-	-
2	-4675.5	9440.9	9609.8	<0.001	0.778
3	**-4540.7**	**9217.5**	**9472.7**	**0.0148**	**0.859**
4	-4473.7	9129.4	9470.9	0.5827	0.827

Table 5. Fit Statistics for Latent Profile Models with 1-4 Classes

The three endothelial response profiles were interpreted as *quiescent, endothelial dysfunction*, and *endothelial repair*. Patients in the quiescent group had biomarker concentrations that were below average for all 11 biomarkers (Table 6 and Figure 7). The biomarkers identified through the factor analysis as belonging to the vessel stabilization process (Ang1, PF4, and VEGF) were particularly low in the quiescent group. The group of patients with endothelial dysfunction was characterized by elevated concentrations of all biomarkers except for those involved in the vessel stabilization process. Conversely, the endothelial repair profile consisted of elevated concentrations of vessel stabilization markers and low concentrations of the other biomarkers. The quiescent group was the

most prevalent, comprising 39% of the patients (N=124). Thirty-four percent of the patients had a biomarker profile consistent with endothelial repair (N=107), and 27% with endothelial dysfunction (N=84).

Biomarker	Quiescent	Endothelial Dysfunction	Endothelial Repair
Ang 1	**-0.576**	-0.249	**0.867**
Ang 2	-0.230	**0.594**	-0.199
sTie2r	-0.199	0.396	-0.080
Chi3L1	-0.339	**0.755**	-0.199
ip10	-0.210	**0.944**	-0.499
vWF	-0.083	0.427	-0.239
Pf4	**-0.488**	**-0.529**	**0.985**
Trem1	-0.291	**0.772**	-0.268
ICAM	-0.243	**0.753**	-0.310
sFlt	-0.338	**1.085**	-0.460
VEGF	**-0.650**	0.020	**0.741**

Table 6. Standardized Mean Concentrations by Latent Profile

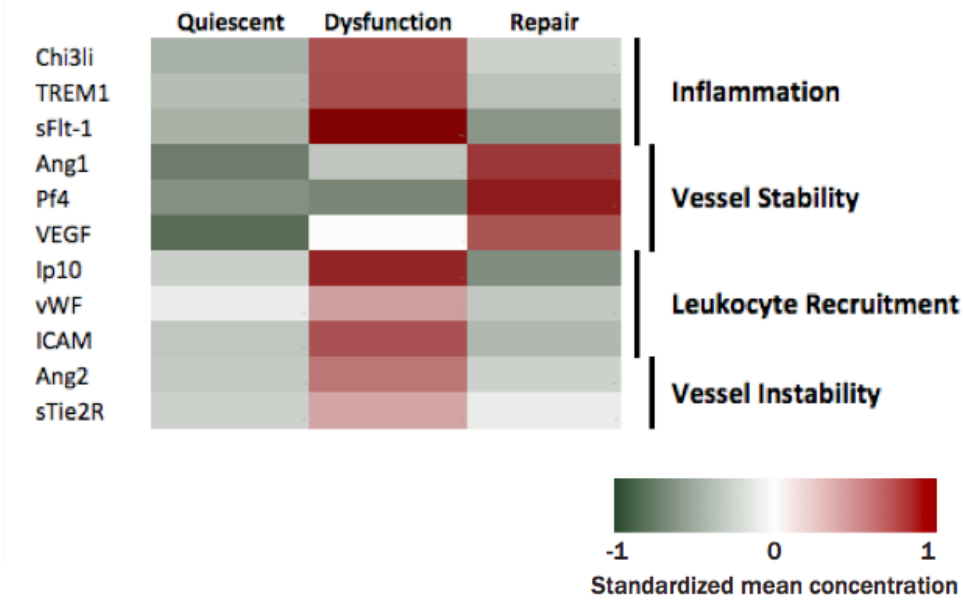

Figure 7. Heat map of Standardized Mean Biomarker Concentrations

The three groups were similar in their demographic characteristics (Table 7). The endothelial dysfunction group had a slightly lower percentage of females, but the difference was not statistically significant. The percentage of patients infected with HIV was slightly higher in the endothelial dysfunction group (91%) than in the quiescent (82%) and endothelial repair (83%) groups.

Characteristic	Quiescent (N=122)	Endothelial Dysfunction (N=86)	Endothelial Repair (N=107)
Age in years [median (IQR)]	35 (27-40)	32 (27-38)	34 (29-43)
Female [n (%)]	71 (58)	38 (44)	54 (50)
Education			
None	6 (5)	7 (8)	13 (13)
Primary school	68 (60)	45 (54)	46 (45)
Secondary school	38 (33)	25 (30)	35 (34)
More than secondary school	2 (2)	7 (8)	8 (8)
Income			
<50,000 USH/mo	53 (48)	45 (56)	56 (55)
50,000-99,999 USH/mo	22 (20)	16 (20)	13 (13)
100,000-299,999 USH/mo	26 (24)	14 (17)	21 (21)
≥300,000 USH/mo	9 (8)	6 (8)	11 (11)
Clinical variables			
SBP, mmHg [median (IQR)]	86 (80-90)	84 (76-92)	88 (80-90)
HIV infected [n (%)]	100 (82)	78 (91)	89 (83)
$CD4^+$ T count, cells/mm^3 [median (IQR)]	52 (11-192)	44 (8-119)	93 (16-241)

Table 7. Demographic Characteristics by Latent Profile
IQR: interquartile range; USH: Ugandan Shillings; SBP: systolic blood pressure.

The endothelial response profiles corresponded to differences in other frequently used clinical laboratory measures of patient status, including $CD4^+$ T cell counts, white blood cell (WBC) counts, platelet counts, and hemoglobin concentration (Table 8). The risk of endothelial dysfunction compared to quiescence nearly doubled with every 1 unit increase in log transformed WBC count (RR=1.83, 95%CI: 1.03, 3.24), and decreased

with increasing CD4+ T cell counts (RR=0.76, 95%CI: 0.59, 0.96), hemoglobin (RR=0.87, 95%CI: 0.75, 1.02), and platelet counts (RR=0.99, 95%CI: 0.99, 0.99), controlling for age and sex. All three groups differed from each other in their hemoglobin concentrations and platelet counts, with decreased risk of endothelial dysfunction with increasing hemoglobin and platelet counts.

Characteristic	Endothelial Dysfunction (vs. Quiescent)	Endothelial Repair (vs. Quiescent)	Endothelial Dysfunction (vs. Repair)
Age in years	0.99 (0.94, 1.04)	1.00 (0.96, 1.03)	0.99 (0.93, 1.05)
Female	0.49 (0.23, 1.04)	0.68 (0.32, 1.26)	0.77 (0.31, 1.89)
Ln CD4+ T cells	0.76 (0.59, 0.96)*	0.90 (0.72, 1.11)	0.84 (0.67, 1.06)
Ln WBC	1.83 (1.03, 3.24)*	1.38 (0.83, 2.30)	1.32 (0.63, 2.80)
Hemoglobin	0.87 (0.75, 1.02)*	1.19 (1.02, 1.38)*	0.73 (0.59, 0.91)*
Sqrt Platelets	0.99 (0.99, 0.99)*	1.01 (1.00, 1.01)*	0.99 (0.98, 0.99)*

Table 8. Odds Ratios for Class Membership

Mycobacterium tuberculosis infection was significantly associated with the endothelial dysfunction subgroup. The odds of endothelial dysfunction versus endothelial repair was 2.7 times higher for patients with *Mycobacterium tuberculosis* bacteremia (95% CI=1.16, 6.33), controlling for age, sex and CD4+ T cell count. Similarly, the odds of endothelial dysfunction versus quiescence was 2.5 times higher (95% CI=1.16, 5.39), controlling for age, sex and CD4+ T cell count.

Every 1 unit increase in log-transformed PCT increased the odds of endothelial dysfunction versus endothelial repair by 2.8 times (95% CI=1.98, 4.03), controlling for age, sex and CD4+ T cell count. Every 1 unit increase in log-transformed PCT increased

the odds of endothelial dysfunction versus quiescence by 3 times (95% CI=2.07, 4.50), controlling for age, sex and $CD4^+$ T cell count.

Discussion

Sepsis is widely recognized as a complex, heterogeneous syndrome.[8] This study aimed to rigorously evaluate a panel of biomarkers involved in the endothelial response to sepsis in order to better characterize the nature of the underlying heterogeneity. Analysis of the correlation structure of the biomarkers identified patterns suggesting that the biomarkers are involved in four distinct processes, interpreted as "inflammation", "vessel stabilization", "leukocyte recruitment", and "vessel instability".

Biological processes

The inflammation factor consisted of the biomarkers Chi3L1, TREM1, and sFlt-1. All three are involved in the monocyte response to infection. TREM1 and sFlt-1 are receptors expressed on monocytes, which lead to secretion of proinflammatory mediators when activated. TREM1 amplifies the inflammatory response in extracellular bacterial and fungal infections.[67] Once activated, the cellular receptors are shed from the cell surface. Activated macrophages and neutrophils secrete Chi3L1, which has a proinflammatory effect. The biologic activity of Chi3L1 is not completely understood, but it is associated with inflammatory conditions such as rheumatoid arthritis and has been shown to upregulate VEGF expression and promote angiogenesis.[68]

Ang-1, PF4, and VEGF loaded on factor 2, which was interpreted as vessel stabilization. All three molecules are involved in angiogenesis, the formation of blood vessels from pre-existing blood vessels. Gavard et al. investigated the biologic relationship between Ang-1 and VEGF, and found that Ang-1 prevents VEGF from disrupting endothelial cell to cell contacts, thus stabilizing blood vessels and preventing vascular leak.[69] PF4 has several biological functions, and inhibits the angiogenic effects of VEGF.[70] Furthermore, a murine model of sepsis-induced acute lung injury demonstrated that disruption of PF4 prevented lung edema and tissue damage.[71] Given the biologic functions of the constituent biomarkers, this factor likely plays a protective role in sepsis pathogenesis.

Factor 3 was characterized by high loadings of IP-10 (0.48), vWF (0.62), and ICAM1 (0.66), and was interpreted as leukocyte recruitment. Transendothelial migration of leukocytes to sites of inflammation occurs in a multistep process involving rolling across the endothelium, integrin activation to stop leukocyte motility, and adhesion and transmigration of the cell across the endothelium. The processes of leukocyte rolling and leukocyte adhesion have both been shown to be dependent on the presence of vWF in inflamed veins.[72] IP-10 is a chemokine that is produced at high levels by activated endothelial cells, and is involved in leukocyte transmigration.[73] The firm adhesion of leukocytes to the endothelial cell is mediated by ICAM-1, an adhesion molecule expressed on endothelial cells.

Lastly, high loadings of Ang2 (0.51) and sTie-2R (0.81) characterized factor 4, interpreted as endothelial vessel instability. Ang-2 competes with Ang-1 for their receptor, sTie-2R. When bound to sTie-2R, Ang-2 primes the endothelium to respond to proinflammatory and angiogenic (VEGF) stimuli, propagating further endothelial

activation and destabilizing the endothelial vasculature.[38] Endothelial barrier integrity is tightly regulated and is altered during sepsis. Ang-2 and Tie-2R signaling plays a critical role in disrupting the endothelial barrier resulting in net extravasation of fluid from the vascular space into the tissues.[35]

The four-factor model explained the variability in most of the biomarkers. Of the 11 biomarkers, 2 had uniqueness values greater than 0.6, sFlt-1 and vWF, suggesting that there was residual variability in these biomarkers not explained by the 4 factors. It is possible that these biomarkers are involved in other relevant processes not captured in this study. Factor 4 (vessel instability) was identified by only two biomarkers, and is therefore at risk for misinterpretation. However, there has been extensive study on the relationship between Ang-2 and its receptor, sTie-2R, supporting the interpretation of this factor as vessel instability.

Latent subgroups

Three subgroups of patients with severe sepsis were identified with distinct host endothelial response profiles, interpreted as: *endothelial dysfunction* (27%), *endothelial repair* (34%), and *quiescent* (39%). The group with endothelial dysfunction was characterized by elevated concentrations of all biomarkers except for those involved in the angiogenic process (Ang1, PF4, and VEGF). Conversely, the endothelial repair profile consisted of elevated concentrations of angiogenic markers and low concentrations of the other biomarkers. Patients in the quiescent group had biomarker concentrations that were below average for all 11 biomarkers. The cytokine responses for

patients with severe sepsis were summarized in a recent review to typically follow one of three patterns: 1) rapid production of both proinflammatory and anti-inflammatory cytokines, 2) predominance of anti-inflammatory cytokines, or 3) globally depressed production of cytokines.[12] It would be interesting to investigate whether the three cytokine patterns described in the review correspond to the endothelial response profiles identified in this study.

The patients in the three subgroups were similar in their demographic characteristics, yet there were significant differences in their clinical laboratory values. This finding suggests that there is clinical relevance to the three endothelial response profiles. An endothelial dysfunction profile was associated with low $CD4^+$ T cell counts, low platelet counts, low hemoglobin concentrations, and elevated WBC counts, controlling for age and sex. The risk of being in the endothelial repair group increased with increasing hemoglobin concentrations and platelet counts.

Patients infected with MTB were at approximately 2.5 times higher risk of endothelial dysfunction compared to patients without MTB bacteremia, controlling for age, sex, and $CD4^+$ T cell count. This finding supports the theory that different pathogens may elicit different endothelial responses. However, only 38% of patients with endothelial dysfunction were infected with MTB, suggesting that the endothelial response is not completely pathogen specific. Few studies have specifically investigated endothelial activation in patients with MTB. Ragno et al. examined changes in gene expression in macrophages infected with MTB and found upregulation of genes encoding VEGF and its receptor sFlt-1, among other genes thought to be involved in immunoregulation.[74] VEGF [75,76] and ICAM-1[77] concentrations were also found to be higher in patients with

active pulmonary tuberculosis than in patients with inactive pulmonary tuberculosis. Patients with active pulmonary tuberculosis had significantly higher concentrations of PF4 than control patients,[78,79] and one study found PF4 levels correlated with the extent of pulmonary lesions on chest radiography.[79] In a study of patients with pleural effusions, vWF levels were significantly higher in patients with tuberculosis than the other etiologies.[80] Among patients with pulmonary MTB, TREM-1 concentration ≥128 pg/mL was associated with 6-month mortality and the presence of disseminated tuberculosis,[81] but does not differentiate tuberculosis from pneumonia caused by extracellular bacteria.[82] Although the evidence for the contribution of endothelial dysfunction to MTB pathogenesis is limited, further investigation may be warranted, particularly as most studies focused on pulmonary tuberculosis.

The decisions regarding both the number of factors as well as the number of subgroups were guided by several well-established criteria but are ultimately somewhat arbitrary. In the LFA, we chose a four-factor model, but an argument could also be made for a three-factor solution. While selecting too few or too many factors both have consequences for the interpretation of the data, specifying too many factors would likely lead to inclusion of minor factors, and was considered more acceptable than potentially losing important information by specifying too few factors. Furthermore, the fourth factor was comprised of Ang-2 and its receptor sTie-2R, which is conceptually consistent with the known biology of the two molecules. In the LPA, the log-likelihood and BIC were marginally better for the four-class solution. However, simulation studies suggest that the Lo-Mendel Rubin test is more accurate for selecting the correct number of classes. In addition, the entropy statistic suggested that the three-class solution formed more distinct groups.

Sepsis encompasses a wide range of pathogens and sites of infection within a single complex syndrome with substantial heterogeneity. We reasoned that one consistent host response is unlikely and sought to evaluate the hypothesis that there are distinct patterns of host responses. We evaluated a panel of biomarkers involved in the endothelial response to sepsis to better characterize the nature of the underlying heterogeneity. Analysis of the correlation structure of the biomarkers identified patterns suggesting that the biomarkers are involved in four distinct processes, interpreted as "inflammation", "vessel stabilization", "leukocyte recruitment", and "vessel instability". These processes may represent therapeutic targets. Three subgroups of patients with severe sepsis were identified with distinct host endothelial response profiles, interpreted as: *endothelial dysfunction*, *endothelial repair*, and *quiescent*. The patients in the three subgroups were similar in their demographic characteristics yet had significant differences in their clinical laboratory values. The presence of three endothelial response profiles supports the hypothesis that there are distinct patterns of endothelial responses, which may have important implications for patient care. Further research is needed to establish the clinical relevance of the endothelial response profiles and to determine whether similar subgroups are found in populations with different pathogens, host genetics, and patient comorbidities.

III. Predicting who dies

Background

Sepsis is a complex syndrome resulting in disturbances in a myriad of biologic systems. A recent systematic review of sepsis identified 178 biomarkers in 3,370 clinical and experimental studies.[39] The biomarkers encompassed a wide range of biologic pathways, including coagulation, the complement cascade, endothelial activation, inflammation, and apoptosis. The authors concluded that none of the biomarkers with published accuracy measures had adequate sensitivity or specificity for use in clinical practice, but that combinations of biomarkers should be evaluated in future studies. Lactate, procalcitonin (PCT) and C-reactive protein (CRP) have been studied most extensively in sepsis prognosis, and have been employed in some routine clinical practices.[39,83,84] The Surviving Sepsis treatment guidelines strongly recommend that patients with hypotension or serum lactate levels >4 mmol/L receive immediate fluid resuscitation.[85] However, accurate lactate testing is challenging, as levels continue to rise in the collected sample, resulting in falsely elevated levels unless testing can be conducted immediately.[86] Point of care lactate tests have been developed but the area under the curve was 0.72 for predicting mortality in a recent study.[86] High levels of procalcitonin were found to identify septic patients at risk of developing severe sepsis, but were not sensitive enough for clinical decision making.[87]

A recent systematic review by Xing et al. focused specifically on biomarkers of endothelial dysfunction for the diagnosis, prognosis, or risk-stratification of patients with sepsis.[40] The authors searched MEDLINE for the keyword ‘sepsis’ together with names

of relevant biomarkers (Ang-1 and -2, sTie2R, sVEGF, sFlt-1, sICAM-1, sVCAM-1, sE-selectin, endothelin-1, endocan, vWF, and ADAMTS13), and identified 1,243 studies, 61 of which met pre-specified criteria. Six studies investigated the association between Ang-2 concentration and mortality, four of which found that Ang-2 concentration was significantly associated with mortality. However, few of these studies provided discrimination or calibration metrics. Riccuito et al. enrolled 70 patients upon admission to the ICU and assessed the predictive accuracy of Ang-1 and Ang-2 concentrations.[41] Ang-1, but not Ang-2 concentrations were found to predict 28-day mortality, adjusting for age and multi-organ dysfunction score. The authors developed a score incorporating Ang-1, Ang-2, ICAM-1, vWF, and E-selectin that accurately discriminated survivors from those who died by day 28 with an area under the receiver operating characteristic (ROC) curve (AUC) of 0.80 (95%CI: 0.69-0.90). However, the score used empirically derived cut-offs for the biomarkers and the accuracy metrics were calculated based on the same data used to generate the score. Thus, the predictive accuracy of the score in other populations will likely be lower than described by the authors.

The systematic review by Xing et al. identified 11 studies investigating the relationship between sICAM-1 and mortality. Only five of the studies found that high concentrations of sICAM-1 was significantly associated with death.[40] Two of the studies reported discrimination metrics, although one study had only fourteen patients.[88] The remaining study reported that sICAM-1 predicted mortality in the emergency department with an AUC of 0.72 (95%CI: 0.57 to 0.87).[89] Ten studies evaluated vWF for association with sepsis mortality, six of which found a significant relationship between vWF concentration and death.[40]

VEGF was evaluated for its prognostic ability in four studies, and its soluble receptor sFlt-1 was evaluated in one study.[40] The results were conflicting for VEGF; two studies found no association between VEGF concentration and mortality,[27,42] one study reported significantly higher concentrations of VEGF in survivors,[43] and one study found significantly higher concentrations of VEGF in non-survivors.[44] Yang et. al investigated both VEGF and sFlt-1 in patients with pneumonia-related septic shock and found that the patients who died of septic shock had significantly higher concentrations of sFlt-1 (659 pg/ml) than patients with septic shock who survived (221 pg/ml).[27] The concentration of VEGF was not associated with mortality.

This study builds on the results obtained in Aim 1, to determine whether an endothelial dysfunction profile predicts 28-day mortality, and to evaluate other candidate predictors.

Methods

Study population

This aim utilizes the same study population as Aim 1. Briefly, data for this project are from a prospective study of fluid resuscitation in patients with severe sepsis conducted at two hospitals in Uganda from May 2008 to May 2009. Adult patients had to meet the following criteria: 1) suspected infection as determined by the admitting medical officer; 2) two or more of the following: a) axillary temperature >37.5°C or < 35.5°C, b) heart rate >90 beats/minute, c) respiratory rate >20 breaths/minute; 3) systolic blood pressure (SBP) ≤100 mmHg; and 4) whole blood lactate concentration >2.5 mmol/L or Karnofsky Performance Status (KPS) score ≤40. Patients were excluded for acute cerebrovascular

events or gastrointestinal hemorrhage, or for admission to a non-medical ward (i.e. surgical or maternity wards). A total of 426 patients were enrolled and 315 patients were included in the analysis after excluding those with missing outcome measures or biomarker measurements. The ethics committees of the University of Virginia, Makerere University, Mulago Hospital, the Infectious Disease Institute, and the Uganda National Council of Science and Technology approved the study. Informed consent was obtained from the patient or a surrogate if the patient was unable to provide written consent.

Data collection

Data were collected on patient demographics and clinical characteristics, and blood was drawn on enrollment for laboratory testing. Demographic information included patient age in years, sex, education and income. Clinical characteristics were collected on admission, and included vital signs and Karnofsky Performance score.

Laboratory Testing

As described in Aim 1, HIV serology, and point-of-care lactate assays were conducted at the hospitals. Lactate was measured on whole blood using a point of care lactate assay (I-STAT, Abbott Point of Care, Inc.). Clinical laboratories near the two hospitals conducted the $CD4^+$ T cell counts and aerobic cultures. The endothelial biomarkers described in Aim 1 were used for Aim 2: Ang-1, Ang-2, sTie2R, VEGF, sFlt-1, sICAM-1, TREM-1,

Chi3L1, vWF, PF4, and IP10. All biomarker assays were conducted at the University of Toronto using blood drawn at study enrollment.

Data analysis

Two outcomes were considered, 28-day mortality and 3-day mortality. The 3-day mortality outcome was used to predict which patients were at risk of imminent death. Time was measured in days from hospital admission.

Candidate markers included the following: endothelial biomarkers (Ang-1, Ang-2, sTie2R, VEGF, sFlt-1, sICAM-1, TREM-1, Chi3L1, vWF, PF4, and IP10), $CD4^+$ T cell count, lactate, procalcitonin, C-reactive protein (CRP), WBC count, platelet count, and hemoglobin concentration. The patient's endothelial response profile (from Aim 1) and Karnofsky score were also considered.

Statistical Methods

The data were divided into a derivation set and a validation set, with the derivation set comprised of a random sample of two thirds of the data. The candidate predictors were dichotomized for ease of interpretation in the predictive models. The endothelial biomarkers, WBC count, platelet count, and hemoglobin levels were divided into quintiles and dichotomized into above or below the fifth quintile. A low $CD4^+$ T cell count was defined as below 50 cells/mm^3. The Karnofsky score was defined as 20 or less.

The discriminative value of the candidate predictors for predicting which patients would die was assessed using logistic regression. The predictors were removed from the full model using backwards selection based on the univariate AUCs. If the AUC of the model decreased by more than 0.025 when a candidate predictor was removed, the predictor was put back in the model.

The ability of the model to discriminate those who died from those who survived was quantified by calculating the AUC. The calibration of the model was assessed using the Hosmer-Lemeshow χ^2 statistic. Calibration metrics measure how closely the predicted outcomes agree with the actual outcomes. The predicted versus actual event rates were compared for each decile of predicted risk in both the derivation and validation sets.

Results

The demographic characteristics of patients in the derivation and validation sets were similar, except that the validation set had significantly fewer females (p=0.04). The percentage of patients infected with HIV was similar in the derivation (84%) and validation (86%) sets. In both the derivation and validation sets, approximately 38% of the patients died. The demographic characteristics of patients who died were similar to the patients who survived in both the derivation and validation sets (Table 9).

Characteristic	Derivation Set		Validation Set	
	Died N = 75	Survived N = 120	Died N = 37	Survived N = 60
Demographics				
Age in years [median (IQR)]	34 (26-40)	34 (28-42)	35 (31-40)	34 (27-40)
Female [n (%)]	40 (53)	70 (58)	13 (35)	26 (43)
Education [n (%)]				
None	5 (7)	14 (12)	2 (5)	3 (5)
Primary school	38 (51)	57 (50)	19 (54)	33 (59)
Secondary school	27 (36)	37 (32)	10 (29)	18 (32)
More than secondary school	4 (5)	6 (5)	4 (11)	2 (4)
Income				
<50,000 USH/mo	38 (55)	55 (50)	15 (45)	32 (56)
50,000-99,999 USH/mo	10 (14)	24 (22)	5 (16)	10 (18)
100,000-299,999 USH/mo	17 (25)	22 (20)	7 (21)	9 (16)
≥300,000 USH/mo	4 (6)	10 (9)	6 (18)	6 (11)
Clinical variables				
SBP, mmHg [median (IQR)]	80 (70-90)	90 (80-90)	80 (70-90)	86 (80-90)
HIV infected [n (%)]	69 (92)	98 (82)	37 (100)	46 (77)
$CD4^+$ T count, cells/mm^3 [median (IQR)]	24 (7-101)	86 (24-205)	21 (6-100)	82 (12-206)

Table 9. Demographic characteristics by 28-day mortality in the derivation and validation sets
IQR: interquartile range; USH: Ugandan Shillings; SBP: systolic blood pressure.

Mortality prediction (derivation set)

The univariate AUC and odds ratio for predicting 28-day mortality are shown in Table 10. Selection of predictors with the highest discriminative value resulted in a reduced model consisting of the endothelial dysfunction profile, $CD4^+$ T cell count less than 50, and Karnofsky score less than or equal to 20. The biomarker sFlt-1 did not contribute to the prediction of which patients would die, however, it predicted the time of death (described in Aim 3) and was therefore retained in the final model.

Predictor	AUC	OR	p-value
Endothelial dysfunction	0.61	2.83 (1.50, 5.34)	0.001
Ang 1	0.48	0.72 (0.34, 1.54)	0.404
Ang 2	0.53	1.47 (0.71, 3.02)	0.300
sTie2r	0.50	0.95 (0.46, 1.95)	0.889
Chi3L1	0.56	2.06 (1.01, 4.22)	0.048
ip10	0.60	3.29 (1.59, 6.81)	0.001
vWF	0.57	2.35 (1.16, 4.76)	0.018
Pf4	0.48	0.76 (0.35, 1.69)	0.507
Trem1	0.55	2.05 (0.97, 4.33)	0.059
ICAM	0.59	2.88 (1.40, 5.90)	0.004
sFlt-1	0.53	1.50 (0.73, 3.02)	0.271
VEGF	0.46	0.56 (0.25, 1.23)	0.148
CD4<50	0.64	3.07 (1.69, 5.60)	<0.001
Lactate	0.54	1.68 (0.82, 3.45)	0.160
Hemoglobin	0.56	2.20 (1.07, 4.55)	0.033
Karnofsky score	0.57	8.18 (2.25, 29.78)	0.001
CRP	0.47	0.65 (0.29, 1.45)	0.293
PCT	0.49	0.90 (0.44, 1.84)	0.781
WBC	0.46	0.56 (0.25, 1.23)	0.148
Platelet	0.54	1.53 (0.77, 3.04)	0.221

Table 10. Univariate discriminative values for 28-day mortality

The odds of death within 28 days were approximately 3 times higher for patients with an endothelial dysfunction profile (95%CI: 1.36, 7.52), as well as for patients with a $CD4^+$ T cell count less than 50 cells/mm^3 (95%CI: 1.51, 5.38, Table 11). Patients with a Karnofsky Score of 20 or less had 6 times the odds of death (95%CI: 1.58, 23.01).

Predictor	OR	p-value
Endothelial dysfunction	3.2 (1.39, 7.52)	0.008
CD4<50	2.85 (1.51, 5.38)	0.001
Karnofsky score	6.04 (1.58, 23.01)	0.008
sFlt-1	0.66 (0.25, 1.73)	0.396

Table 11. Final model for predicting 28-day mortality

Model accuracy

The AUC of the final model for predicting 28-day mortality was 0.73 in the derivation set and 0.77 in the validation set (Figure 8). The model discrimination was improved when predicting 3-day mortality (AUC= 0.79 in the derivation set, and 0.74 in the validation set). The Hosmer-Lemeshow χ^2 statistic was 1.45 (p=0.92) in the validation set, indicating that the model fit the data well.

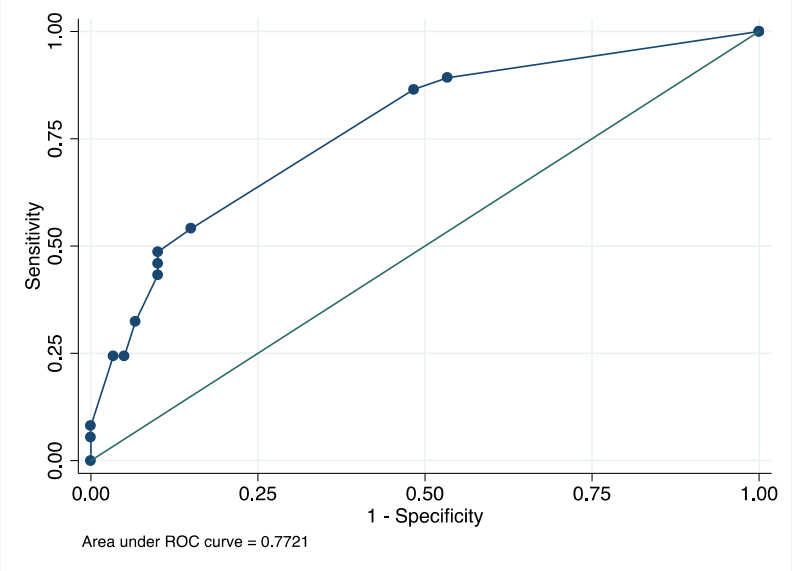

Figure 8. ROC curve for 28-day mortality, validation set

Discussion

Sepsis remains a leading cause of death in both developing and developed countries. Although accurate estimates are lacking, the burden of sepsis may be greatest in developing countries.[90] A recent systematic review and meta-analysis estimated that approximately 13.5% of adult patients admitted to the hospital in Africa had a bloodstream infection.[6] Methods to accurately predict prognosis are needed to guide clinical decision-making and avoid delays in treatment.

Nearly 200 biomarkers have been evaluated for their diagnostic or prognostic value, yet no individual or combination of biomarkers has proven to be sufficiently accurate for clinical use.[39] In this study, endothelial dysfunction, $CD4^+$ T cell count less than 50 cells/mm^3, and Karnofsky score of 20 or less predicted 28-day mortality. Endothelial dysfunction was associated with 28-day mortality, but did not accurately discriminate patients who would die within 28 days on its own. There are several likely reasons for the poor discriminative value. First, 28 days may not be an appropriate end point for endothelial biomarkers measured at hospital admission. This approach groups together patients who died on day 2 together with those who died on day 25, for example, and assumes that the biological processes leading to death are similar and measurable at baseline. This assumption is likely false, especially considering that events occurring over the course of the hospitalization, such as nosocomial infections, may have a dramatic impact on the clinical outcome. Shortening the timeframe for the prediction or repeat measurement of the biomarkers would likely improve the discriminative value. Thus, we also evaluated the discriminative value of the model for predicting a three-day

mortality outcome and found that the ability of the biomarkers to accurately predict mortality was improved.

The discriminative value of the individual endothelial biomarkers for predicting 28-day mortality in this study was similar to results obtained in developed countries. Riccuito et al. enrolled 70 patients with severe sepsis upon admission to the ICU in 3 hospitals in Canada and found that Ang-1 concentrations ≤5.5 ng/mL predicted 28-day mortality with an AUC of 0.62,[41] compared to the AUC of 0.48 observed in this study. The investigators also measured Ang-2, vWF, and ICAM-1, but did not report the individual discriminative values of these biomarkers. Shapiro et al. investigated sFlt-1 and ICAM-1 for their ability to predict in-hospital mortality among 221 patients presenting to the emergency department of a US academic medical center with clinical suspicion of infection.[89] The study population was comprised of patients with sepsis (32%), severe sepsis (30%), septic shock (32%), and non-infected controls (6%). In this population, sFlt-1 predicted in-hospital mortality with an AUC of 0.91, and ICAM-1 predicted mortality with an AUC of 0.72. Valid comparisons across studies are difficult as the biomarker cut-off values and inclusion criteria are vastly different. Furthermore, the biomarker cut-offs are often not reported or are empirically derived. Nonetheless, the similarity of the results found in this study to those from developed countries suggests that the HIV infection status of the patients may not impact the discriminative value of the endothelial biomarkers. Further research is needed to determine the effect, if any, of immunosuppressive conditions such as HIV infection on the predictive value of the biomarkers.

Our study had several strengths. We derived the predictive model in a randomly assigned derivation set and evaluated the accuracy of the model in a separate set of patients (i.e. the validation set). This approach gives more realistic measures of predictive accuracy as the data used to develop the model were not used to evaluate the model. Secondly, the discriminative values were used to select the predictors in the final model, as opposed to selecting predictors based on the p-value for their association with the outcome. Predictors may have a significant p-value yet have low discriminative value. Furthermore, these findings provide information previously lacking for patients in low-resource settings.

Our study was limited in that the biomarkers were measured only at hospital admission. While ideally a prognostic test would require only one measurement, it is possible that changes in biomarker concentration over time would be more predictive of outcome. This may partially be due to the variation in the stage at which patients present to the hospital. Some patients may seek care earlier in the course of illness than others and some infections may have a fulminant clinical course. Measuring the change in a biomarker over time may account for these differences. However, an ideal prognostic test would be informative regardless of when in the course of illness the patient presents to the hospital.

We investigated the accuracy of a novel method of profiling patients with severe sepsis based on their endothelial response patterns for predicting 28-day mortality. We identified endothelial dysfunction, $CD4^+$ T cell count less than 50 cells/mm^3, and Karnofsky score of 20 or less predicted 28-day mortality with an AUC of 0.77 in the validation set. Further research is needed to externally validate these findings in patient populations with different infectious etiologies.

IV. Predicting time of death

Background

The current treatment strategy for sepsis relies on early goal-directed therapy, including fluid resuscitation within the first 6 hours of recognition of sepsis and administration of broad-spectrum antibiotics within 1 hour of diagnosis of septic shock.[85] Early recognition and treatment is considered paramount, as several studies demonstrated an increased mortality risk in patients receiving delayed treatment.[91,92] In particular, studies have shown decreased survival with every hour delay in the time to administration of appropriate antibiotics.[93,94] Aggressive hemodynamic optimization is also most effective when administered before the development of global tissue hypoxia.[95] However, early recognition of sepsis is difficult as physiologic derangements such as hypotension may be absent early in the course of illness.[96] A prognostic test that could identify which patients are at highest risk of clinical progression, and among those at high risk, discriminate patients who will die shortly after admission from those who have slower disease progression would provide important information for clinical decision-making. Patients at high risk of imminent death may be candidates for more aggressive therapy, which might not be justified for patients with a less fulminant clinical course. Furthermore, analysis of biomarkers associated with the time of death may provide insight into sepsis pathogenesis and may facilitate identification of risk factors for rapid decline. This study builds on the results obtained in Aim 2 by incorporating information on the time of death.

Methods

Study population

This aim uses the same study population as Aim 1 and 2. Briefly, adult patients meeting the following criteria were enrolled: 1) suspected infection as determined by the admitting medical officer; 2) two or more of the following: a) axillary temperature >37.5°C or < 35.5°C, b) heart rate >90 beats/minute, c) respiratory rate >20 breaths/minute; 3) systolic blood pressure (SBP) ≤100 mmHg; and 4) whole blood lactate concentration >2.5 mmol/L or Karnofsky Performance Status (KPS) score ≤40. Patients were excluded for acute cerebrovascular events or gastrointestinal hemorrhage, or for admission to a non-medical ward (i.e. surgical or maternity wards).

Data were collected on patient demographics and clinical characteristics, and blood was drawn on enrollment for laboratory testing. Demographic information included patient age in years, sex, education and income. Clinical characteristics were collected on admission, and included vital signs and Karnofsky Performance score. The time of death was measured in days after admission to the hospital.

Laboratory Testing

As described in Aim 1, HIV serology, and point-of-care lactate assays were conducted at the hospitals. Lactate was measured on whole blood using a point of care lactate assay (I-STAT, Abbott Point of Care, Inc.). Clinical laboratories near the two hospitals conducted the $CD4^+$ T cell counts, and aerobic cultures. The endothelial biomarkers described in

Aim 1 and 2 were used for Aim 3: Ang-1, Ang-2, sTie2R, VEGF, sFlt-1, sICAM-1, TREM-1, Chi3L1, vWF, PF4, and IP10.

Data analysis

A generalized gamma model was used to model the time of death among those who died. The generalized gamma is a parametric accelerated failure time model. The exponentiated model coefficients provide the time ratio for a unit change in the predictor. Therefore, time ratios less than one indicate a decrease in survival time and time ratios greater than one indicate a prolonged survival time. The predictors selected in the logistic regression (as described in Aim 2) were retained in the generalized gamma model, and the candidate predictors were evaluated for their additional contribution to the time of death prediction. The model fit was assessed by plotting the Cox-Snell residuals against the cumulative hazard function.[97] A straight line with a slope of one indicates a good fit. The candidate predictors were evaluated in the derivation set.

The positive and negative predictive values for the model predicting the time of death were assessed using a modified version of recently developed statistical methods. These methods model the probability of death as a function of time, given a positive (or negative) biomarker result. The PPV and NPV for a binary biomarker X at time t were defined as follows[98]:

$$PPV\ x(t) = P(T \leq t \mid X=1) \qquad NPV\ x(t) = P(T > t \mid X=0)$$

The published statistical methods were modified to specifically model the time to death. The model specification was defined as follows:

$$PPV\ x(t) = \pi^* F_1^*(t) \qquad NPV\ x(t) = \pi(1-F_1(t)) + (1- \pi)$$

Where π is the proportion of patients who die, $F_1(t)$ is the distribution of times of death, and * indicates that the patients were positive for the biomarker. Therefore, the PPV for biomarker X at time t is the proportion of patients who die before time t among those who tested positive for biomarker X. The NPV is interpreted as among those who tested negative for the biomarker, the proportion of those who do died but did so after time t, plus the proportion discharged from the hospital alive. The PPV and NPV of the final model were plotted over time.

Results

The median time to death in the derivation set was 6 days for patients with a quiescent endothelial response profile, 4 days for patients with an endothelial repair profile, and 2 days for patients with endothelial dysfunction. The survival curves for the endothelial response profiles show that patients with endothelial dysfunction died significantly faster than patients in the other groups (Log-rank test $p<0.0001$, Figure 9). The predicted time to death and actual times of death were plotted for patients with and without endothelial dysfunction (Figure 10). The predicted times of death closely match the observed times of death, among those who died.

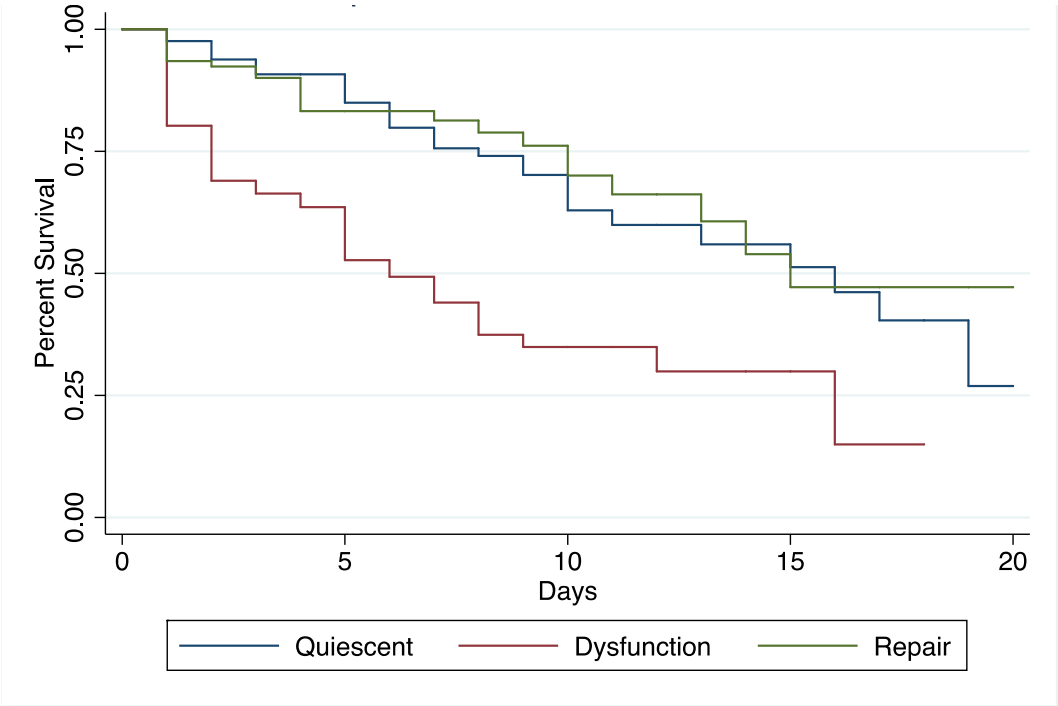

Figure 9. Kaplan-Meier survival curves by endothelial response profile

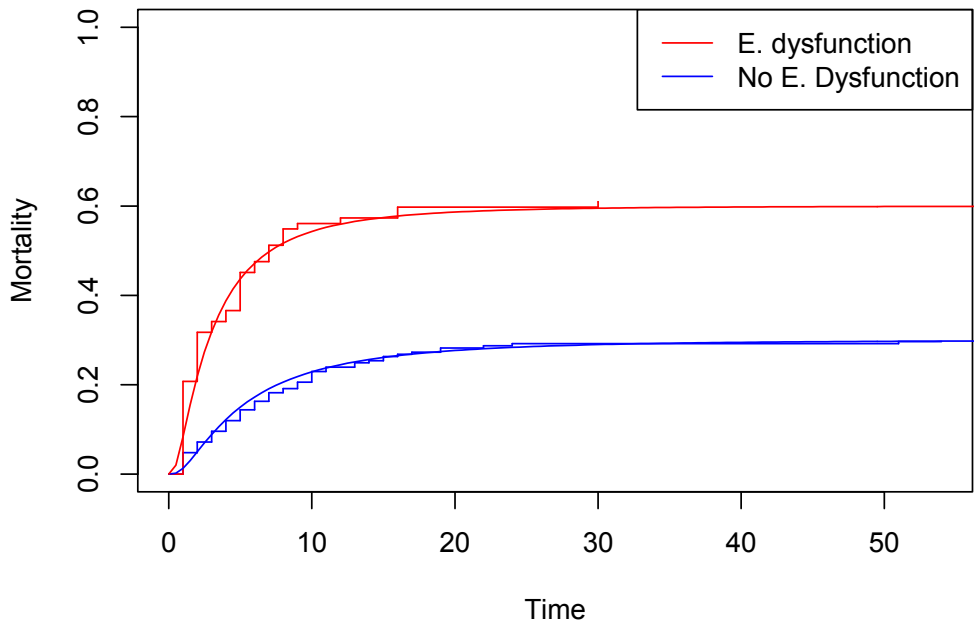

Figure 10. Predicted Time of Death versus Actual Time of Death

Time of death prediction (derivation set)

The time to death was significantly shorter for patients with an endothelial dysfunction profile versus the other endothelial response profiles (p=0.001, Table 12). The survival time for patients with endothelial dysfunction was approximately half that of patients with similar $CD4^+$ T cell counts, Karnofsky scores, and sFlt-1 concentrations (relative time = 0.49, 95%CI: 0.32, 0.75). Interestingly, neither a $CD4^+$ T cell count less than 50 nor a Karnofsky Score of 20 or less were informative for the time of death among those who died. The survival time for patients with high concentrations of sFlt-1 was reduced by 43% compared to patients with lower concentrations (relative time = 0.57, 95%CI: 0.35, 0.93). Patients with an endothelial dysfunction profile and high concentrations of sFlt-1 had a 72% reduction in their survival times. Thirty-three patients in the derivation set (16%) had both indicators of rapid decline.

Predictor	Estimate (95%CI)	p-value
Endothelial dysfunction	-0.723 (-1.14, -0.31)	0.001
CD4<50	0.022 (-0.36, 0.41)	0.912
Karnofsky score	0.022 (-0.45, 0.50)	0.928
sFlt-1	-0.557 (-1.04, -0.07)	0.024
Constant	1.74 (1.35, 2.13)	<0.001
Sigma	0.777	0.003
Kappa	-0.226	0.501

Table 12. Time of death

The model fit the data well, as the hazard function closely follows the 45-degree line with some variation at later times (Figure 11).

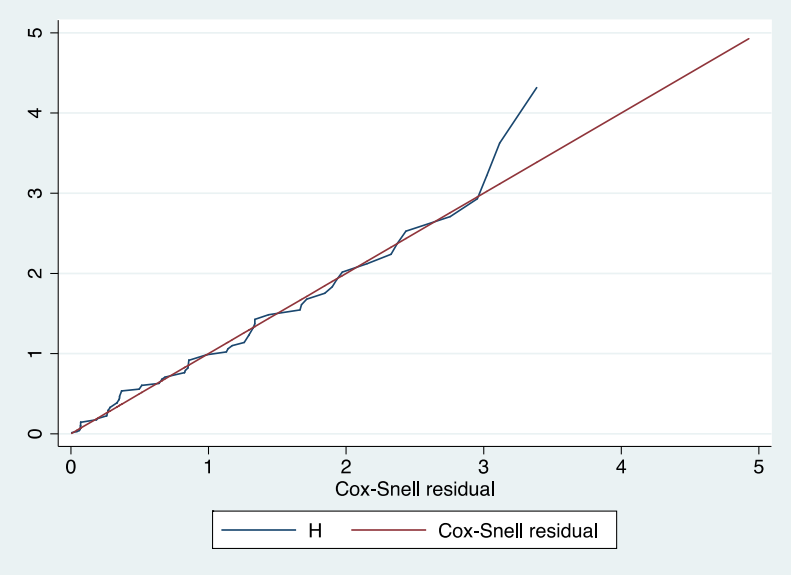

Figure 11. Cox-Snell residual plot

Positive predictive value

The positive predictive value for patients with endothelial dysfunction, a $CD4^+$ T cell count less than 50 cells/mm3, a Karnofsky score of 20 or less, and in the 5^{th} quintile of sFlt-1 concentration compared to patients without any of the above indicators of poor prognosis was plotted over time (Figure 12a). The relative positive predictive values (PPV) are shown in Figure 12b. These graphs show that the prediction of time of death is much more accurate for deaths occurring within five days of hospital admission.

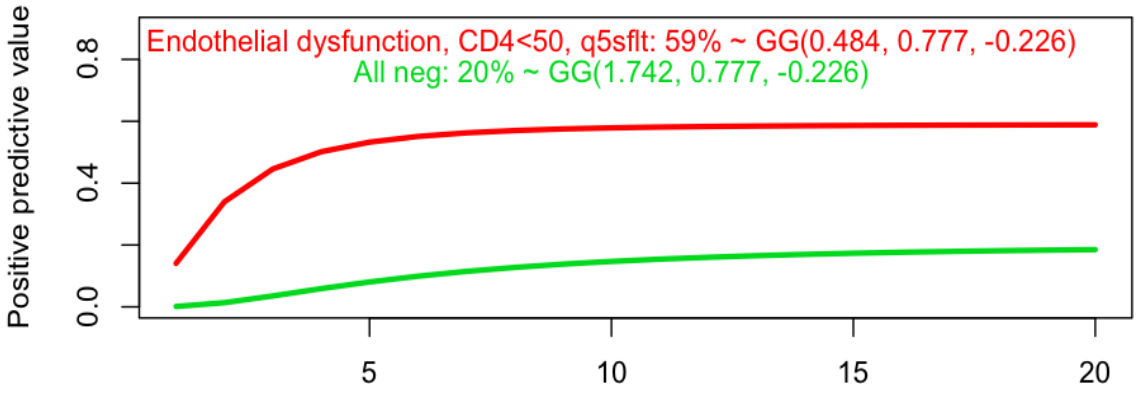

Figure 12a. Positive predictive value over time

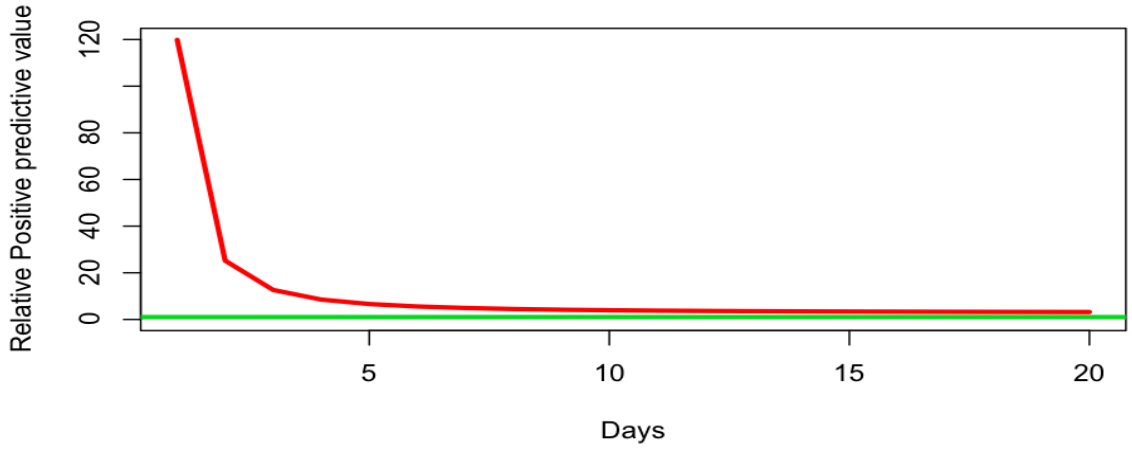

Figure 12b. Relative positive predictive value over time

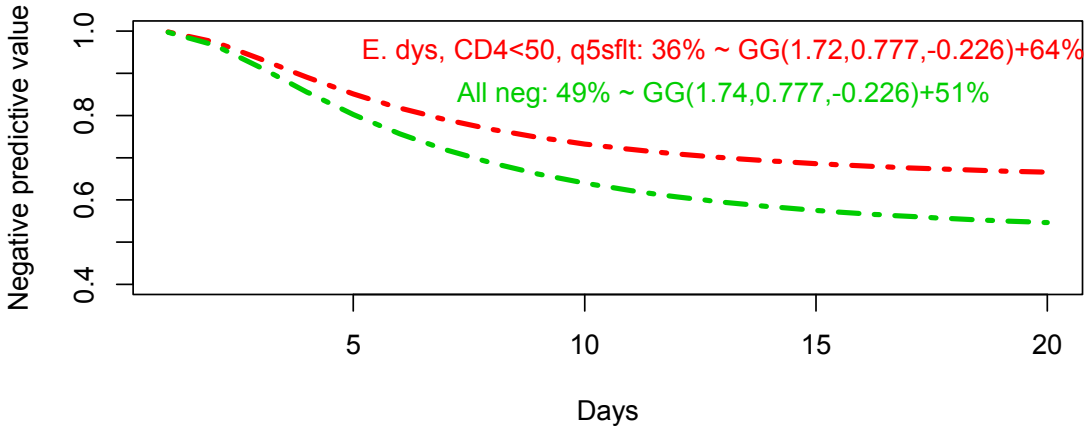

Figure 13a. Negative predictive values over time

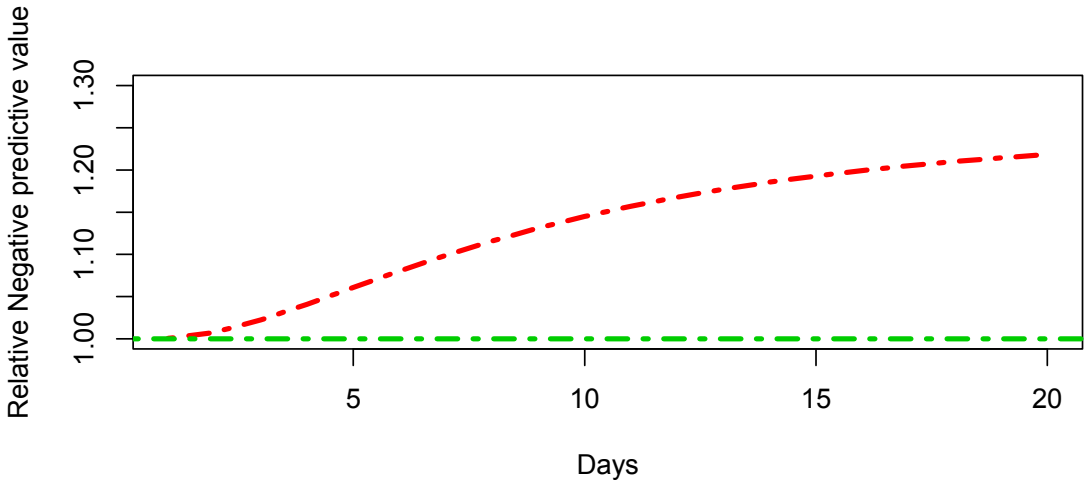

Figure 13b. Relative negative predictive values over time

Discussion

The ability to differentiate patients with severe sepsis who have a fulminant clinical course from patients who progress more slowly could provide important information for clinical decision-making. Decisions regarding the level of care required, how aggressive

to be with fluid resuscitation, and potentially therapeutic options could be tailored based on the patient's expected clinical course. Patients with endothelial dysfunction die substantially sooner than patients with endothelial repair or quiescence, with a median time to death of 2 days versus 4 or 6 days, respectively. This difference in survival was demonstrated with Kaplan-Meier survival curves and the relative time to death was quantified using a generalized gamma parametric failure time model. The survival time for patients with endothelial dysfunction was approximately half that of patients with similar $CD4^+$ T cell counts, Karnofsky scores, and sFlt-1 concentrations but without endothelial dysfunction. The biomarker sFlt-1 independently contributed to the prediction of the time of death, while $CD4^+$ T cell count and Karnofsky score were not informative. Patients with both endothelial dysfunction and high sFlt-1 concentrations had a 72% reduction in their survival times compared to otherwise similar patients without the two indicators of poor prognosis.

Clinical studies of patients with sepsis typically use 28-day mortality as the primary end point;[99] few studies explicitly investigated the time to death. Macias and Nelson divided hospitalization days for patients with severe sepsis into three segments, day 0 to 5, day 6 to 15, and day 16 to 28, and evaluated biomarkers and clinical variables potentially related to the time of death.[99] The investigators found that the rate and cause of death differs over the 28-day follow-up period, with refractory shock as the major cause of death in the first 5 days, and respiratory failure or multi-organ dysfunction predominating in the later periods. Severe protein C deficiency and elevated IL-6 concentrations were associated with death in the early time period. These results support the hypothesis that

the optimal treatment strategy may differ depending on the clinical course, which emphasizes the need for methods to accurately predict the clinical course as early as possible.

PPV and NPV are standard measures of predictive accuracy used with binary outcomes, such as 28-day mortality. However, there are no standard methods to quantify predictive accuracy for predicting a failure time outcome. We adapted a recently published method for quantifying PPV and NPV for failure time outcomes.[98] This approach allows for quantification of the accuracy of a biomarker (or a set of biomarkers) for predicting the probability of the outcome over the course of time, as opposed to status at one point in time (such as at 28-days). By plotting the positive predictive value of the model as a function of time, we were able to illustrate that the PPV of the model is highest within the first 5 days of hospital admission. This result suggests that endothelial dysfunction combined with $CD4^{+}$T cell count and Karnofsky score measured at baseline are most informative for events occurring within approximately 5 days. Future studies are needed to investigate the utility of serial testing and to establish the optimal timing of the repeat measurements.

Our study had several limitations. Our scope was limited to biomarkers involved in the endothelial response to sepsis. While the endothelial response is an important component of sepsis pathogenesis, there are likely other biologic processes that influence outcome. Future studies incorporating a broader set of indicators may improve the accuracy of the prediction. Secondly, the biomarker concentrations were dichotomized into above or

below the fifth quintile for the study population. This approach is not optimal but was chosen over approaches where the cut-offs are determined with respect to the outcome of interest, which could lead to inflated estimates of model accuracy. Few studies of endothelial biomarkers for prediction of mortality report the biomarker cut-offs used, or the discriminative value of the biomarker.[40] Research aiming to establish and standardize approaches for biomarker measurement and reporting of the results would greatly facilitate the analysis of future studies and allow for meaningful comparisons across studies. Our results were derived from patients with severe sepsis in Uganda. This patient population differs from most studies of patients with sepsis in that the patients are young (median age of 35) and predominantly HIV infected. The infectious etiologies also differ from those found in developed countries. Therefore, further research is needed to determine the generalizability of our results to other patient populations.

In our study, patients with an endothelial dysfunction profile and elevated sFlt-1 concentrations died significantly sooner than patients without those indicators, adjusting for $CD4^+$ T cell count and Karnofsky score. These patients are at high risk of imminent death, and therefore may be candidates for more aggressive therapy. Future clinical trials could target this subgroup of patients, as treatment efficacy may differ depending on the patient's endothelial response profile.

V. Summary and conclusions

Sepsis is a leading cause of death in both developed and developing countries. Despite thousands of clinical and laboratory-based studies, we still lack a prognostic test with sufficient accuracy to be clinically useful. This project aimed to identify endothelial biomarkers that could accurately predict the time to death for patients with severe sepsis in Uganda.

The endothelial response to an infection is complex. Using a panel of 11 biomarkers involved in the endothelial response, we evaluated whether the response is one-dimensional or comprised of multiple biologic processes. The LFA results suggested four latent processes, interpreted as "inflammation", "vessel stability", "leukocyte recruitment", and "vessel instability" based on the known biologic functions of the constituent biomarkers. Many of the biomarkers investigated have pleiotropic effects, depending on the context. For example, the presence of VEGF may indicate vessel instability, unless Ang-1 is present, in which case the destabilizing effect of VEGF is inhibited. Examining VEGF alone would not provide the correct interpretation. Analyzing the correlation structure of the biomarker panel provided a context allowing us to interpret the effect of the group of biomarkers.

We next investigated whether patients with severe sepsis had one common endothelial response pattern, versus a patient population comprised of a mixture of latent subgroups. Using LPA, we identified three subgroups of patients with endothelial response patterns that were homogenous within the group and distinct from the other groups. The patterns were interpreted as "quiescent", "endothelial dysfunction", and "endothelial repair". Patients with endothelial dysfunction were characterized by elevated concentrations of all

biomarkers except for those identified through the factor analysis as belonging to the vessel stabilization process (Ang1, PF4, and VEGF). We hypothesized that this subgroup would have a poor prognosis, as they had high concentrations of biomarkers associated with mortality and low concentrations of biomarkers involved in what was likely a protective process. The three endothelial response groups were similar in their demographic characteristics, yet their clinical laboratory values were significantly different. These results suggest that profiling patients with severe sepsis into subgroups based on their endothelial response may provide a clinically meaningful way to categorize patients into more homogeneous groups. These profiles may prove useful in future clinical trials, where homogeneous study populations are needed to detect potential treatment effects.

In Chapter 3 we assessed the ability of the endothelial dysfunction profile and other candidate predictors to accurately predict which patients would die within 28 days of hospital admission. The predictors with the highest AUC were selected for inclusion in the final model. Endothelial dysfunction was significantly associated with 28-day mortality, but had poor discriminative value on its own. The final model consisted of endothelial dysfunction, $CD4^+$ T cell count less than 50 cells/mm^3, Karnofsky score of 20 or less, and the 5^{th} quintile of sFlt-1 concentration. The AUC for the model for 28-day mortality was 0.73 in the derivation set, and 0.77 in the validation set. The discriminative value of this model was not accurate enough for use in the clinical management of patients. There are several likely reasons for the low accuracy. Our analysis was limited to 11 endothelial markers, 6 clinical laboratory values, and the Karnofsky score. There are likely other key determinants of outcome not included in our analysis. In particular,

the immune response is known to be an important contributor to the pathogenesis of sepsis. Another possible explanation of the poor prognostic value is that 28 days may not be an appropriate timeframe for an accurate prediction. Endothelial biomarkers measured at baseline may be informative for deaths occurring within five days, but later deaths are likely influenced by other events, such as treatment effects and nosocomial infections. Shortening the timeframe used to evaluate the prognostic value of endothelial biomarkers to 3-day or perhaps 5-day mortality would likely give more accurate predictions for which patients are at high risk of death.

In Chapter 4 we investigated whether the model developed in Chapter 3 and other candidate predictors could accurately predict the time to death, among those who died. We found that patients with endothelial dysfunction died twice as fast as otherwise similar patients. Patients with both endothelial dysfunction and high sFlt-1 concentrations had a 72% reduction in their survival times. The positive predictive value for the prediction was highest within the first 5 days of hospital admission. The time to death prediction could provide clinically meaningful information for patient care. For example, a prognostic test could inform the physician that the patient had a predicted probability of death in the next 5 days of 86%, and if the patient did die, the expected time of death would be on day 2. Given this information, the treating physician may choose a more aggressive treatment strategy.

Further studies are needed to externally validate our findings and to improve the predictive model. Our patient population was comprised of patients with severe sepsis from two hospitals in Uganda. The latent factors and endothelial response profiles that we identified may be specific to the spectrum of pathogens found in Uganda, and may be

different in patient populations with lower HIV prevalence. In particular, the endothelial dysfunction profile was associated with MTB bacteremia, which is uncommon in patients with severe sepsis in developed countries. The accuracy of our models for predicting which patients are at high risk of death and the expected time of death may be improved by including other biologic processes known to influence clinical outcome.

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Appendix 1. Definitions

Term	Criteria
SIRS*	2 out of the 4 following criteria:
	Temperature >38°C or < 36°C
	Heart rate >90/min
	Hyperventilation evidenced by respiratory rate >20/min or arterial CO_2 lower than 32 mmHg
	White blood cell count >12000 cells/ul or lower than 4000 cells/ul
Sepsis	SIRS criteria with presumed or proven infection
Severe sepsis	Sepsis with organ dysfunction
Septic shock	Sepsis with hypotension despite adequate fluid resuscitation

*SIRS: systemic inflammatory response syndrome

Table A.1. Definitions from the 1991 Consensus Conference[2,5]

Term	Criteria
Sepsis	Documented (or suspected) infection with any one of the following clinical or laboratory criteria
General parameters	Fever, hypothermia, tachycardia, tachypnea, altered mental status, arterial hypotension, decreased urine output, significant peripheral edema, or positive fluid balance
Inflammatory parameters	Leukocytosis, leukopenia, hyperglycemia, increased C-reactive protein, procalcitonin or creatinine, coagulation abnormalities, increased cardiac output, reduced mixed venous oxygen saturation
Hemodynamic parameters	Hypotension, elevated mixed venous oxygen saturation, elevated cardiac index
Organ dysfunction parameters	Arterial hypoxemia, acute oliguria, increase in creatinine level, elevated international normalized ratio or activated partial thromboplastin time, ileus, thrombocytopenia, hyperbilirubinemia
Tissue perfusion parameters	Hyperlactatemia, decreased capillary refill or mottling

Table A.2. Definition for sepsis from the 2001 Consensus Conference[3,5]

Curriculum Vita

Education

Johns Hopkins Bloomberg School of Public Health Baltimore, MD

Doctor of Philosophy, Epidemiology, Infectious Diseases Concentration *Pending*

Rollins School of Public Health, Emory University Atlanta, GA

Master of Public Health, International Health, Infectious Diseases Track *05/2004*

Worcester Polytechnic Institute Worcester, MA

Bachelor of Science, Biotechnology and International Studies (Double Major) *05/2002*

Experience

Naval Medical Research Center, Frederick Frederick, MD

Principal Investigator, Henry M. Jackson Foundation *08/2012 – present*

- Served as the Deputy Director and Southeast Asia Regional Director for a program aimed at improving clinical outcomes and understanding the pathogenesis of sepsis in austere environments.
- Designed randomized controlled trials and cohort studies of patients with sepsis in Uganda, Cambodia, and US Military Treatment Facilities.
- Managed program implementation, including development and oversight of university partnerships, budgets, and statements of work.
- Engaged and coordinated with a multi-disciplinary team of university, DoD, and US government partners.

Integrated Research Facility (IRF/NIAID/NIH) Frederick, MD

Clinical Research Portfolio Manager, Lovelace Respiratory Research *9/2010–8/2012*

- Contributed to the development of an international program researching sepsis in low resource settings.
- Designed clinical studies of sepsis and viral hemorrhagic fevers in Africa, with planned expansion to South East Asia.
- Analyzed clinical data to determine prognostic markers indicative of poor sepsis outcomes among patients hospitalized in Uganda.

Walter Reed Army Institute of Research (WRAIR) Silver Spring, MD

Research Lead – Biological Threat Reduction Program (BTRP) *01/2007 – 08/2010*

- Designed and conducted epidemiologic studies in the former Soviet Union primarily aiming to define the relative importance of select arthropod-borne and zoonotic pathogens, characterize their clinical manifestations, and identify high risk groups.
- Served as principal investigator on 1 research protocol, project lead on 7 protocols, and investigator on an additional 8 protocols.
- Trained over 100 epidemiologists and clinicians in Georgia, Azerbaijan and Ukraine in study design, study procedures, database design, and data analysis.
- Resided in Azerbaijan to supervise ongoing studies and establish a sustainable epidemiologic research capability (2/2008 – 5/2009)

Science Officer – BTRP *03/2006 – 01/2007*

- Worked on a multi-disciplinary team to design and implement a congressionally funded program aiming to reduce the threat of biologic agents in the former Soviet Union through infectious diseases surveillance and collaborative research.
- Served as the liaison between the funding organization and teams of epidemiologists, entomologists, infectious disease physicians, and microbiologists.

Lead Surveillance Epidemiologist – BTRP *11/2005 – 03/2006*

- Worked closely with institute directors and health professionals in Georgia and Azerbaijan to develop surveillance guidelines, including the development of case definitions and case report forms.
- Lead representative on a multi-agency integrated project team formed to coordinate training in epidemiology, microbiology, and disease recognition.

Surveillance Epidemiologist – BTRP *11/2004 – 11/200**5***

- Collaborated with host-country health professionals and the Centers for Disease Control and Prevention to develop an epidemiology training curriculum.
- Conducted assessments of the public health systems in Georgia and Azerbaijan.

Pro-Agua y Desarrollo Integral de Lempira Lempira, Honduras

Masters Thesis Research, Rollins School of Public Health *01/2003 – 04/2004*

- Designed and implemented a study evaluating the impact of a water and sanitation program in rural Honduras.
- Assessed hygiene practices, sanitary conditions, bacterial contamination of household drinking water, diarrhea prevalence, and anthropometric status of children under five.

Centers for Disease Control and Prevention Atlanta, GA

Research Assistant: HIV/AIDS Branch *09/2002 – 05/2003*

- Worked in a team aiming to engineer a DNA vaccine for HIV-2.
- Demonstrated ability to design experiments and follow laboratory protocols.

Abbott Bioresearch Center Worcester, MA

Research Intern: Molecular Biology Department *06/2001 – 07/2002*

- Cloned and expressed protein kinases for use in drug specificity assays.
- Developed laboratory skills in molecular biology techniques such as PCR.

Armed Forces Research Institute of Medical Sciences Bangkok, Thailand

Major Qualifying Project, Worcester Polytechnic Institute *12/2001 – 04/2002*

- Designed and conducted a study assessing the economic burden of dengue fever in Thailand using Disability Adjusted Life Years (DALYs).
- Administered over 200 household surveys in the province of Kamphaeng Phet examining the direct and indirect costs of illness due to dengue hospitalization.
- Winner of the Provost's Major Qualifying Project Award.

University of Massachusetts Medical School Worcester, MA

Major Qualifying Project, Worcester Polytechnic Institute *09/2001 – 05/2002*

- Worked in the Infectious Diseases and Immunology laboratory at the University of Massachusetts Medical Center on a project aiming to understand the role of virus-specific T lymphocytes in the clinical manifestations of dengue fever.
- Derived a cell line expressing HLA alleles thought to influence disease severity.

Publications

1. Ortiz JR, Rudd KE, **Clark DV**, Jacob ST, West TE. Clinical research during a public health emergency: a systematic review of severe pandemic influenza management. Critical Care Medicine 2013; 41(5):1345-52.

2. **Clark DV**, Jahrling PB, Lawler JV. Clinical management of filovirus-infected patients. Viruses, 2012; 4(9):1668-86**.**

3. **Clark DV**, Ismayilov A, Seyidova E, Hajiyeva A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Aslanova A, Debes AK, Qasimov M, Hepburn MJ. Seroprevalence of Tularemia in Rural Azerbaijan. *Vector Borne Zoonotic Dis,* 2012 Mar 27 [E-pub].

4. **Clark DV**, Ismayilov A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Aslanova A, Qasimov M, Hepburn MJ. Under-utilization of health care services for infectious diseases syndromes in rural Azerbaijan. *BMC Health Serv Res,* 2011 Feb 11;11:32.

5. Akhvlediani T, **Clark DV**, Chubabria G, Zenaishvili O, Hepburn MJ. The changing pattern of human brucellosis clinical manifestations, epidemiology, and treatment outcomes over three decades in Georgia. *BMC Infect Dis.* 2010 Dec 9;10:346.

6. Chitadze N, Kuchuloria T, **Clark DV**, Ekaterine T, Chokheli M, Tsertsvadze N, Trapaidze N, Lane A, Bakanidze L, Tsanava S, Hepburn M, Imnadze P. Water-borne outbreak of oropharyngeal and glandular tularemia in Georgia: investigation and follow-up. *Infection*, 2009 Dec;37(6):514-21.

7. Kuchuloria T, **Clark DV,** Hepburn MJ, Pimentel G, Imnadze P. Hantavirus infection in the Republic of Georgia. *Emerging Infectious Diseases*, 2009;15(9):1489-1491.

8. **Clark DV,** Mammen MP Jr, Nisalak A, Puthimethee V, Endy TP. Economic Impact of Dengue Fever/Dengue Hemorrhagic Fever in Thailand at the Family and Population Levels. *American Journal of Tropical Medicine and Hygiene*, 2005 Jun;72(6):786-91.

Presentations

1. **Clark DV**. Long-term sequelae among Ebola virus survivors in Bundibugyo, Uganda. *American Society of Microbiology Biodefense and Emerging Diseases Research Meeting*. Washington DC, February 6-9, 2011.

2. Ismayilov A, **Clark DV,** Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Aslanova A, Lane A, Hepburn MJ. Human seroprevalence of brucellosis in three regions of Northern Azerbaijan. *12th World Congress on Public Health*. Istanbul, April 27-30, 2009.

3. Akhmedova MD, Imomaliev UN, Butaev MK, Yaraev RG, Ismatova RA, Nikolich M, **Clark DV**, Elzer P. Epidemiological Surveillance of Human and Animal Brucellosis in the Republic of Uzbekistan. *Biologic Threat Reduction Program Science Review*. Garmisch, Germany, February 11-15, 2007.

4. **Clark DV**. Invited Speaker: Measuring the Impact of Dengue in Thailand at the Family and Population Levels. *Workshop on Dengue Burden Studies, Convened by the Pan American Health Organization, the Rockefeller Foundation, and the Pediatric Dengue Vaccine Initiative.* Washington DC, November 5-7, 2002.

5. **Clark DV**, Mammen MP Jr, Nisalak A, Puthimethee V, Endy TP. Economic Impact of Dengue Fever/Dengue Hemorrhagic Fever in Thailand at the Family and Population Levels. *American Journal of Tropical Medicine and Hygiene 51st Annual Meeting.* Denver CO, November 10-14, 2002.

Poster Presentations

1. **Clark DV**, Ismayilov A, Seyidova E, Hajiyeva A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Richards A, Habashy EE, Maksoud MA, Rahman BA, Pimentel G, Koehler J, Lane A, Qasimov M, Hepburn MJ. Seroprevalence of Select Arthropod-borne and Zoonotic Infections in Rural Azerbaijan. *International Conference on Emerging Infectious Diseases*. Atlanta GA, July 11-14, 2010.

2. **Clark DV**, Ismayilov A, Seyidova E, Hajiyeva A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Aslanova A, Lane A, Qasimov M, Hepburn MJ. Tularemia in Azerbaijan. *American Society of Microbiology: Biodefense and Emerging Diseases.* Baltimore, MD, February 21-24, 2010.

3. **Clark DV**, Kuchuloria T, Akhvlediani T, Hepburn MJ, Pimentel G, Chokheli M, Mamuchishvili N, Imnadze P. Leptospirosis in the Republic of Georgia. *American Society of Tropical Medicine and Hygiene 58th Annual Meeting*. Washington DC, November 18-22, 2009.

4. **Clark DV**, Ismayilov A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Aslanova A, Qasimov M, Hepburn MJ. Under-utilization of Health Care Services for Infectious Disease Syndromes in Rural Azerbaijan. *American Society of Tropical Medicine and Hygiene 58th Annual Meeting.* Washington DC, November 18-22, 2009.

5. **Clark DV**, Ismayilov A, Seyidova E, Hajiyeva A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Aslanova A, Lane A, Qasimov M, Hepburn MJ. Emerging Viral Zoonoses in Azerbaijan: A Cross-Sectional Study. *American Society of Tropical Medicine and Hygiene 58th Annual Meeting.* Washington DC, November 18-22, 2009.

6. **Clark DV**, Ismayilov A, Seyidova E, Hajiyeva A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Aslanova A, Lane A, Qasimov M, Hepburn MJ. Identification of an endemic infection: Q fever in rural Azerbaijan. *Infectious Diseases Society of America 47th Annual Meeting*. Philadelphia, October 29-November 1, 2009.

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