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Diagnosis of Systemic Inflammation Using Transendothelial Electrical Resistance and Low-Temperature Co-fired Ceramic Materials

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DIAGNOSIS OF SYSTEMIC INFLAMMATION USING TRANSENDOTHELIAL ELECTRICAL RESISTANCE AND LOW-TEMPERATURE CO-FIRED CERAMIC MATERIALS

THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Chemical Engineering
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By

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Lexington, Kentucky

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2013

ABSTRACT OF THESIS

DIAGNOSIS OF SYSTEMIC INFLAMMATION USING TRANSENDOTHELIAL ELECTRICAL RESISTANCE AND LOW-TEMPERATURE CO-FIRED CERAMIC MATERIALS

Systemic inflammation involves a complex array of cytokines that can result in organ dysfunction. Mortality remains high despite the vast amount of research conducted to find an effective biomarker. The cause of systemic inflammation can be broad and non-specific; therefore, this research investigates using transendothelial electrical resistance (TEER) measurements to better define systemic inflammatory response syndrome (SIRS)/sepsis within a patient. Results show a difference in TEER measurements between healthy individuals and SIRS-rated patients. This research also displays correlations between TEER measurements and biomarkers currently studied with systemic inflammation (tumor necrosis factor-α, C- reactive protein, procalcitonin). Furthermore, this research also presents the groundwork for developing a microfluidic cell-based biosensor using low temperature co-fired ceramic materials. An LTCC TEER-based microfluidic device has the potential to aid in a more effective treatment strategy for patients and potentially save lives.

KEYWORDS: Transendothelial Electrical Resistance, Sepsis, Systemic Inflammatory Response Syndrome, Low Temperature Co-fired Ceramics, Diagnosis

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This thesis is dedicated to the loving memories of Jack Switzer Hurley and Mary Lewis Mercke.

“To laugh often and much, to win the respect of intelligent people and the affection of children, to leave the world a better place, to know even one life has breathed easier because you have lived. This is to have succeeded.”
--Ralph Waldo Emerson

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CHAPTER 1

INTRODUCTION

A. BACKGROUND

In recent years, extensive research has been conducted to better understand systemic inflammatory response syndrome (SIRS). SIRS is defined as a severe response to an insult that triggers a systemic acute inflammatory reaction. When SIRS is caused by a severe infection, it is classified as sepsis. Since SIRS is a syndrome that arises from a wide variety of pathological cues (e.g., sepsis, ischemia, trauma), there is currently no biomarker available for detecting SIRS.[1] Further, the current symptoms that define a patient as having SIRS does not effectively correlate with the patient's outcome.[2,3] Any of these pathological cues (infection, ischemia, trauma, etc.) can trigger a response in the immune system, which initially maintains the vascular integrity. However, the immune system can start to produce too many cytokines, thereby creating a harmful environment which leads to the loss of vascular integrity and organ dysfunction.[4,5] In order to streamline the diagnosis and treatment of SIRS, it would be beneficial to develop a device that could effectively measure the vascular response of the patient's circulatory system in "real-time". It is hypothesized that an endothelial cell-based biosensor will react to the imbalance of cytokines and provide a novel diagnostic aid, assisting the clinician in the rapid detection, and staging of SIRS/sepsis prior to the presentation of physiological symptoms (fever, heart rate, hypotension, etc.).

The endothelium is a monolayer of confluent endothelial cells (ECs) that line the circulatory system. This monolayer acts as a barrier that regulates the flow of molecules throughout the body. The primary way to analyze an EC monolayer is through its barrier properties. Barrier properties of ECs are primarily maintained by the formation of tight junction and adheren junction protein complexes between the cells in the monolayer. The dynamic modulation of endothelial permeability, regulated by these junctions, can be quantified through the use of resistance measurements.[6-9] Studies have shown that when these cells are exposed to increased proinflammatory cytokines, which have been shown to be upregulated in patients with SIRS [10], a disruption of these junctions will occur causing a decrease in resistance allowing for stratification of varying degrees of endothelium dysfunction. It is hypothesized that this change in resistance could aid in the diagnosis of SIRS and ultimately in the formulation of an appropriate treatment strategy.

A common method employed in biomedical/pharmaceutical research to measure changes in endothelial cell permeability includes well plate studies. Due to the need for large sterile testing equipment like a laminar flow hood as well as trained personnel to run these studies, they are not practical for the clinical atmosphere. The incorporation of microfluidics into these static, commonly used techniques will allow for testing in a closed, controlled environment. Microfluidic technology also allows the use of small sensing elements that can produce greater sensitivity. Glass and ceramics have been widely used in biomedical applications for their chemical inertness, ability to be

sterilized, and robust mechanical properties.[11] Separately, ceramics have been applied in electronic applications for similar reasons (eg- high electrical resistivity, hermeticity, and durability).[12] In particular, low temperature co-fired ceramics (LTCCs) combine many of the above features in microfluidic systems with electronic elements leading to an all-in-one multiple application device. Such a device has the potential to analyze multiple scenarios within the circulatory system simultaneously, which can lead to more effective studies and ultimately produce better patient outcomes.

B. INNOVATION

Since the causes of SIRS are broad and non-specific, the goal of this research is to show endothelial cells' innate response to the uncontrolled cascade of the cytokine storm that is produced during SIRS/sepsis can be measured using transendothelial electrical resistance measurements. Furthermore, these measurements can effectively detect SIRS/sepsis within a patient.

Chapter 2 will review literature on endothelial cell junctions, SIRS, sepsis, biomarkers studied with SIRS/sepsis, and biosensors. Chapter 3 will discuss the TEER and biomarker studies performed on septic serums. Chapter 4 will address the research on developing a microfluidic cell-based biosensor using low temperature co-fired ceramic materials. By incorporating microfluidics, this research can enable continuous aseptic testing of endothelial cell permeability with the capability of generating automated results thereby reducing the need for trained personnel. Chapter 5 will talk about the conclusions of these studies.

CHAPTER 2

LITERATURE REVIEW

A. ENODTHELIAL CELL JUNCTIONS AND INFLAMMATION

The vascular endothelium is composed of an endothelial cell monolayer that lines the inner surface of a person's entire circulatory system, separating the underlying tissue from the circulating blood and helping to maintain vascular homeostasis. The endothelium is in charge of regulating the flow of blood, nutrients, and multiple types of biologically active molecules. Basal permeability throughout the endothelium varies since there are various physical properties and flow rates in the circulatory system. Research has shown in post capillary venules to be more permeable than capillaries and arterioles, with arterioles being the least permeable.[13] Endothelial cell permeability can occur either through the cell (transcellular) or between cells (paracellular). Paracellular permeability deals with the disruption of the junctions between two adjacent endothelial cells.[14] Paracellular permeability is highly regulated by adheren junction (AJ) and tight junction (TJ) protein complexes as seen in Figure 1. These protein complexes depend upon complex interactions amongst cytoskeletal rearrangement, junction adhesion molecules, and cellular adhesive forces.[15-17] TJ protein complexes are formed from occludin, claudins, junction associated molecules (JAMs), and zonula occludens (ZO) proteins.[13,17,18] Claudin-5 is specific to endothelial cells.[17] ZO-1 proteins bind the JAMs, occludin, and claudin protein to the actin cytoskeleton. AJ protein complexes are formed from cadherin proteins interacting with catenins.[13,14,17,19] In endothelial

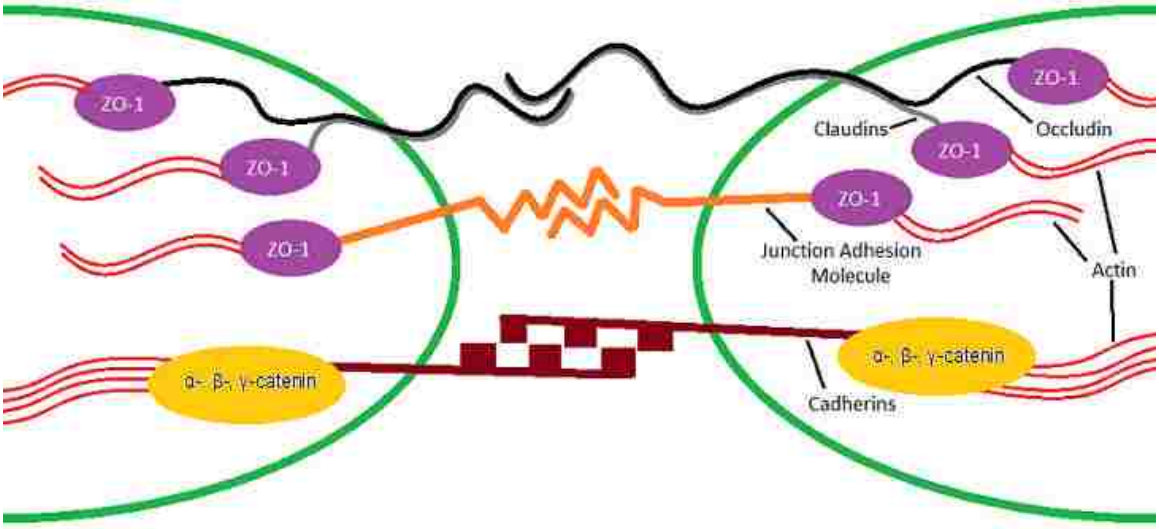

Figure 1: Interactions of the proteins associated with AJs and TJs

cells, cadherin interacts with cytoplasmic proteins (α-, β-, γ-catenin, p120). These proteins anchor vascular endothelial (VE)-cadherin to the actin cytoskeleton.[13,14,17,19] Research has been conducted in order to understand the way in which these junctions function. One study shows the E-twenty six (ETS)-related gene controling endothelial cell TJ stability through transcriptional regulation of claudin-5 as indicated in increased permeability and reduced expression of claudin-5 when the ETS-related gene was knockdown.[17] Another study indicates that specific cell lines are controlled by different pathways. In this study, human dermal microvascular endothelial cells (HDMECs) are TJ regulated through the expression and organization of claudin-5, while human umbilical vein endothelial cells (HUVECs) are AJ controlled via VE-cadherin.[13] Another study shows the importance of the connection between JAMs (specifically CD31) over their signaling functions in endothelial cell barrier integrity.[16]

Vascular inflammation has been shown to occur in rheumatoid arthritis, atherosclerosis, diabetes, acute lung injury, acute respiratory distress syndrome, SIRS, and sepsis.[4,14,16,17,20-22] Inflammation can lead to increased endothelial cell permeability. Once vascular stability is compromised, the interstitial space is filled with a collection of plasma and cells. Many pro-inflammatory stimuli like cytokines have been shown to disrupt junctions between endothelial cells and cause cytoskeleton rearrangement. Factors such as histamine, thrombin, vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), and interleukins (IL-1,-6,-8,-10) can lead to gap formation.[14,17,18,23] Studies have shown rapid disruption of the endothelial cell barrier due to exposure from the

factors thrombin and histamine.[17] On the other hand, factors like TNF-α and endotoxins have been shown to cause a gradual change that occurs over the course of several hours.[14,17] One study shows PIP3 dependent Rac Exchanger 1 is activated downstream of TNF-α. This study also shows that targeting it can potentially protect vascular inflammation induced by TNF- α and lipopolysaccharide.[14] Additional research has also been conducted in an attempt to further the understanding of these factors on endothelial cell barrier dysfunction.[14,16,18-20,23] However, time and time again these articles state that the mechanism in which these factors cause endothelium dysfunction is either too complex or not clearly understood. An improved understanding of these mechanisms can lead to the development of novel treatment to prevent endothelial cell permeability caused by various diseases. Unfortunately, there is a current demand for rapid detection of such diseases, and the understanding of these mechanisms is not complete. This research suggests the use of endothelial cells' innate response in order to better define and detect such diseases.

B. SIRS AND SEPSIS

Systemic inflammatory response syndrome (SIRS) has been shown to be present in one third of in-hospital patients, greater than 50% of ICU patients, and greater than 80% of surgical ICU patients with mortality rates nearing 20%.[24,25] Patient are diagnosed with SIRS when they exhibit at least two of the following symptoms: 1) temperature above 100.4°F or below 96.8°F, 2) heart rate in excess of 90 beats per minute, 3) respiratory rate greater than 20 breaths per

minute or arterial carbon dioxide tension ($PaCO_2$) below 32 mm Hg, and 4) abnormal white blood cell count. Studies have shown cytokine levels to increase as the severity of SIRS increases. [2,3,10,26,27] The cause of SIRS is broad, involving insults such as: trauma, inflammation, infection, ischemia, surgical procedures, or any combination of these. There is currently no gold standard biomarker for the detection of SIRS[1]; by definition SIRS attempts to define a wide range of patient immune responses to wide range of injuries.

When SIRS is caused by a severe infection, it is classified as sepsis. Sepsis is a complex and dynamic disorder. Research has shown sepsis leads to elevated levels in cytokines; this proinflammatory environment results in severe deregulations of various systems throughout the body.[4,16,22,28] To keep inflammatory diseases in check, vascular permeability must be strictly regulated. Current treatment for sepsis includes targeting specific pathogens with antimicrobials.[22] The problem with antimicrobial treatment is that time is needed to identify the specific pathogen; time the patient does not have. Another possible treatment for sepsis is to modulate the patient's immune system.[22] The problem with this approach is that our knowledge regarding inflammation is in its infancy and response varies greatly from patient to patient.

After the onset of an infection or trauma, cytokines are produced in order to provoke an inflammatory response from the immune system.[4,28] This response is typically beneficial and necessary for maintaining vascular stability; however, when it gets out of control, it can be harmful and potentially life-threatening. The immune response, when uncontrolled, has been defined as the

"cytokine storm". During the cytokine storm, massive amounts of cytokines are circulated throughout the body which can cause organ dysfunction.[5] This overactive immune response can lead to the increased severity of SIRS/sepsis, multiple organ dysfunction syndrome (MODS), shock, and even death.[2,3,10] When SIRS/sepsis progresses and induces MODS, the mortality rate increases to above 50%.

C. SIRS AND SEPSIS MARKERS

Many biomarkers have been studied in an attempt to effectively predict the severity of inflammation as well as patient outcome. Some studies point to cytokine concentrations as being effective biomarkers. One such study examined how inflammation evolves during early phase sepsis by measuring multiple cytokines. Using cecal ligation and puncture (CLP) in animals to emulate polymicrobial human sepsis, this study found that early death (within five days) could be predicted within 24 hours via increased levels of both pro- and anti-inflammatory cytokines. However, after five days, they were unable to accurately predict the outcome.[27] Another study compared multiple cytokine levels and the Acute Physiology and Chronic Health Evaluation (APACHE) II score of 174 patients meeting the SIRS criteria. This research indicated an increase in all cytokine concentrations in patients with SIRS; IL-6 was most often correlated with increased severity as well as poor outcomes.[26] A review of Medline articles searching for the mechanisms involved in SIRS development looked at data from both animal models and human studies. This review concludes that both pro- and anti- inflammatory cytokines are released after

injury; furthermore, the way in which these cytokines excite the immune system is too complex and unpredictable.[10]

Procalcitonin (PCT) and C-reactive protein (CRP) have been the most widely studied biomarkers for sepsis. A study of 18 patients being examined daily for the presence/absence of SIRS in connection to CRP concentrations reports no difference in CRP concentrations between patients with multiple or single occurrences; additionally a decrease of 25% in CRP values from the day before signifies the end of a septic episode.[29] Another study followed 23 ICU patients each day and classified them according to their infectious status. The results indicated that CRP values were significantly lower in patients that were "negative" or "unlikely" to have an infection when compared to those that were "probable" or "definite".[30] Research was also conducted over a 15 day period on a population of 40 patients diagnosed with systemic inflammation and MODS. The collected data included PCT, CRP, Sequential Organ Failure Assessment (SOFA) score, APACHE II score, and survival. This study shows that CRP concentrations were raised in all the SOFA scores and PCT concentrations increased significantly with higher SOFA scores. Furthermore, CRP values were similar between survivors and non-survivors whereas non-surviving patients produced a significant increase in PCT values indicating that PCT provides more information on the severity of the inflammation than CRP.[31] Another study compared PCT, CRP, and infection on 150 ICU patients being examined for 10 days. This research indicates PCT and CRP concentrations were higher in patients with an infection; additionally, PCT was a better marker for

approximating the severity, prognosis, and time course of infection.[32] However, research on the daily PCT and CRP values of 190 ICU patients contradicts these previous studies stating that CRP concentrations have a higher sensitivity and specificity when compared to PCT. This study also concludes that while PCT values were significantly higher in non-survivors, CRP values were not.[33] A review of Pubmed articles searching combinations of PCT, critical(ly), intensive, biomarkers, and sepsis focused on publications from 2000-2010 supporting the claim that PCT concentrations are a better marker for sepsis than CRP. This review determined that PCT is more effective in ruling out systemic sepsis than confirming infection and that recent evidence suggests PCT values could give effective aid in the length of antibiotic treatment.[34] Another review of over 3,000 studies in the Medline database when searching for “sepsis” and “biomarker” indicates that 178 biomarkers have been evaluated experimentally, clinically, or both. This study reports that all of these biomarkers lack the specificity and/or sensitivity necessary to be used as a single biomarker for developing treatments; however, combinations of these biomarkers may be more effective.[35]

Regardless of the extensive research, clinical trials using anti-inflammatory treatments for SIRS/sepsis have resulted in failure.[4,22] A recent study of over 1,800 patients suggests that the classic model of using anti-cytokines for treatment is impractical because cytokine activation progresses longer than the model anticipated. Furthermore, by the time most patients pursued treatment, the cytokine cascade was in full effect. Furthermore, the patients who had a higher risk of developing severe sepsis and mortality had

higher concentrations of both proinflammatory AND anti-inflammatory cytokines. This indicates that targeting cytokines might not be beneficial in treatment therapies.[28] Additionally, contradicting studies show that PCT and CRP are unable to solely distinguish between SIRS and sepsis or predict patient outcome.[31,33] Most of the studies suggest PCT values are indicative of the severity of sepsis and can aid in antibiotic treatment.[31,32] Some studies have also shown that following recovery from the initial trauma, a patient's life is more easily threatened when exposed to a minor second incident of SIRS.[7] Unfortunately, an ideal biomarker will not likely be discovered for sepsis in the near future due to the complexity of the disease. However, a cell-based biosensor evaluating a cell's intrinsic response to the inflammatory agents produced in real-time has the potential to better define SIRS/sepsis by way of vascular dysfunction, as well as aiding in the rapid diagnosis and treatment before the patient's life is threatened.

D. BIOSENSORS

The medical diagnostic field can be greatly expanded through the use of cell-based biosensors. Cell-based biosensors are devices that use living cells as sensing elements. One benefit of cell-based biosensors is their ability to provide insight into complex and potentially unknown physiological cell receptor-ligand interactions that occur in disorders such as SIRS. Research has shown an ability to alter cellular DNA so that it fluoresces in the presence of cytokines, but there is no correlation between the concentrations of cytokines and the luminescence intensities .[36,37] Studies have also shown an ability to detect changes in

transcellular permeability using fluorescent tags. A study was conducted where an array of HUVEC monolayers were grown on a microporous membrane which was located between a polydimethylsiloxane (PDMS) layer and a glass substrate. Microchannels and microvalves perfused fluorescein isothiocyanate conjugated (FITC) albumin and measured the permeability of the HUVEC monolayers by measuring fluorescent intensities.[38] Other studies generated microfluidic chips to apply fluid flow to endothelial cells while analyzing permeability using FITC albumin. These studies indicate an ability to test endothelial permeability under physiologically relevant shear stresses.[39,40] However, this type of analysis can become expensive and requires large fluorescent equipment not desired or practical in each ICU room.

Biosensors using electrical analysis methods such as transendothelial electrical resistance (TEER)[6,7,41-43], electrical cell substrate impedance spectroscopy (ECIS)[8,9,44-49], electrochemical impedance spectroscopy (EIS)[50-52], and ion sensitive electrodes (ISE)[53] have shown the ability to retrieve information rapidly regarding the response of cells. One study presented TEER measurements being used to show that reactive oxygen species produced during hypoxia regulates secretion of cytokines that can induce endothelial permeability.[41] Another study demonstrated how a cost-effective EIS for cytokine IL-12 could be used for diagnosis of diseases with known biomarkers in real-time.[52] The ECIS has been established as a great way to analyze endothelial cells using impedance. It has been used to correlate endothelial cell permeability to cytochalasin B[49], ZO-1 protein[45], rickettsial infection[54], and even establish

synthetic histatins as a skin wound-healing agent[47]. The ISE has shown that a cell-based biosensor can be used to detect and differentiate between the existences of small quantities of cytokines that correlate with stages of cancer.[53] Each of these devices has also shown accurate cellular responses due to proinflammatory cytokines.[6,41,52-55] They have the ability to provide the quick, broad range, natural cell permeability response desired in a disease like SIRS/sepsis. These simple techniques when performed statically are effective. However, they do not analyze cells in their natural environment.

Since research shows that ECs have the ability to change physically with their environment[56-59], the incorporation of microfluidic technology can allow cell based diagnostic methods to be used in controlled conditions and a closed sterile environment with constant nutrient and waste removal during experimentation; this removes the need for trained personnel and large testing equipment.[60] The use of microfluidics analyzes a smaller sample size which is advantageous since anemia is a concern in the ICU.[71] Microfluidic systems also have the capability to apply physiological shear stress with a constant laminar flow, replicate cellular environments, and function with automated processing.[38,61-66] Microfluidic devices have become tools for analyzing and expanding research in: DNA analysis, chemical synthesis, cellular analysis, protein analysis, biochemical analysis, electrokinetic separation, biomedical monitoring, and clinical diagnostics.[61-63,67] Transendothelial electrical resistance measurements have shown an ability to effectively analyze endothelial barrier function without the cost of fluorescent tags. Some studies have incorporated this technique into the

microfluidic world. One such study constructed a microfluidic TEER chip by sandwiching a semiporous membrane between two PDMS channels containing Ag/AgCl electrodes and was able to successfully monitor changes in TEER measurements in real-time.[68] Another study used bipolar pulse square wave potential to measure TEER across endothelial cells grown in wells with a microporous polycarbonate membrane base overtop of a microfluidic channels containing an aluminum electrode. The results showed an ability to evaluate endothelial permeability via TEER while interacting with a flowing stream of blood components; however, the cells in this device are not directly exposed to the flowing stream of blood.[69] A recent study cultured endothelial cells directly onto small gold electrodes arranged within a flow channel and different shear stresses were applied to the cells. In this study, TEER measurements were taken during these shear stresses to determine the change in barrier function. The results show an increase in resistance while the cells dynamically alter their morphology in response to an increase in shear stress as well as a drop in resistance as the shear stress in decreased.[70] For electrical impedance measurements used in the previous study, the microfluidic geometry allows use of smaller sensing elements leading to a greater change in impedance in the cell monolayer. This can result in a wider range of detection limits when compared to studies conducted in macroscopic well plates. One study presents a “trap-and-measure” device that is able to differentiate between HeLa, A549, MCF-7, and MDA-MB-231 cells by measuring impedance at multiple frequencies and voltages. This study showed that cells are unique in their tolerances to electric fields.[62]

A multilayered ceramic microfluidic device that incorporates an array of confluent EC-based sensors to monitor electrical impedance changes can study endothelial cell permeability in real-time under physiological conditions. The production of devices with this ceramic material is quicker and simpler than that of a silicon device as well as being more durable and cost efficient. Most importantly, the proposed ceramic material can integrate microstructures with optic and electronic components leading to the analysis of multiple applications that can be automated within one device.[72-75] These ceramic devices have the potential to analyze multiple physiological scenarios within the circulatory system simultaneously, which can lead to more effective and efficient EC permeability studies.

CHAPTER 3

SEPSIS STUDIES

Due to the complexity of sepsis, an ideal biomarker will not likely be discovered in the near future; therefore this research looks at a different way of analyzing the systemic inflammation that occurs with this disease. This chapter looks at the practicality of a cell-based biosensor evaluating a cell's intrinsic response to the inflammatory agents produced in real-time using TEER measurements. This type of sensor has the potential to better define SIRS/sepsis by way of vascular dysfunction, as well as aiding in the rapid diagnosis and treatment before the patient's life is threatened.

A.) MATERIALS AND METHODS

A-i. CELL CULTURE

The Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Switzerland) at passage 1. The HUVECs were grown in 75 cm^2 tissue culture flasks. EGM-2 media (Lonza) was used to grow the cells inside the flasks in an incubator at 37ºC and 5%CO_2. Once HUVECs were close to confluency (>85%), they were split and seeded onto Transwell inserts (Corning) at a concentration of 25,000 cells/cm^2. Passages 4-8 were used in these experiments. Following the seeding of cells, the well plates were placed in the incubator under the same conditions as the cell culture flasks. For trials lasting longer than one day, the media was exchanged for fresh media within the first 24 hours post-seeding and then every other day.

A-ii. TRANSENDOTHELIAL ELECTRICAL RESISTANCE MEASUREMENTS

Transwell inserts (Corning) were filled with media and incubated overnight. Afterwards, background resistance measurements were obtained using the Endohm12 TEER cup. HUVECs were then seeded into each insert at a concentration of 25,000 cells/cm^2. Once a confluent layer of HUVECs was obtained in the inserts (>10 $\Omega*cm^2$), the inserts were treated with serum samples and analyzed with the Endohm12 cup. Measurements were obtained every 5 seconds for the first 12 minutes. Measurements were also obtained at 20 min, 30 min, 1 hour, 4 hours, and after overnight incubation.

A-iii. RAT SERUM STUDIES

The primary validation of the current approach has been demonstrated in an animal model of SIRS/Sepsis. In collaboration with Drs. Callahan and Supinski (UK Medical Center), a preliminary proof-of-concept study was performed using a rat CLP model of sepsis. The ligation of the cecum resulted in acute polymicrobial peritonitis within the animal.[76,77] In this study, 1 mL of blood was drawn from two healthy animals prior to surgery. The animals were then sedated and CLP surgery performed. At 24 hours following surgery, another 1 mL blood sample was drawn. Following this, the animals were sacrificed by a euthanizing dose of Phenobarbital. The blood samples were spun down and the serum was obtained and stored at -20°C immediately. For the TEER study, a 1:1 dilution of the serum into culture media was used. This mixture was then placed in the luminal portion of a transwell insert containing a confluent HUVEC monolayer. TEER values were collected at varying time points for an hour. One-

way ANOVA was used to make comparisons between the blood samples taken before surgery (healthy control) and after (septic).

A-iv. HUMAN SERUM STUDIES

A-iv-1. HUMAN SERUM STUDY PROCEDURES

Once SIRS-rated samples were obtained, HUVECs were seeded into Transwell inserts and grown to confluency. When confluent, Healthy serum (negative control), Healthy serum+histamine (positive control), or SIRS-rated serum samples were equilibrated to 37°C in the incubator and exposed to the HUVEC monolayer. The healthy serum control was used to display the response of the endothelial cells to serum without increased inflammatory stimuli. The healthy serum spiked with 2mM histamine control was used to mimic the rapid and significant inflammatory response displayed in the rat serum study. Resistance measurements were obtained every 5 seconds for the 12 minutes. Measurements were also obtained at 20 min, 30 min, 1 hour, 4 hours, and after overnight incubation. The data obtained from the inserts exposed to different SIRS-rated serums were compared to the data obtained when exposed to healthy serum. The %TEER data is indicative to the endothelial cell monolayer's "leakiness" associated with the exposure to the sample. One-way ANOVA was used to compare the %TEER of the different samples as well as the patient outcome (e.g. days in ICU/ mortality).

A-iv-2. COLLECTION OF BLOOD

Collaboration was achieved between the medical center and our lab through the CCTS Pilot: Cell Based Endothelium Activation Potential (EAP)

Sensor for the Diagnosis and Monitoring of Sepsis, Severe Sepsis, and Septic Shock. This was essential for this portion of the research as blood samples were collected from healthy volunteers as well as sick patients. Through the pilot study, admitted patients were screened for SIRS and fourteen patients were enrolled. The following criteria was matched with the number of patients indicated: 1) non-infectious SIRS- 4 patients, 2) sepsis (SIRS + confirmed infection)- 5 patients, and 3) severe sepsis (sepsis + organ dysfunction)- 5 patients. These criteria were chosen to show the response to increased inflammation severity. 10 mL of blood was withdrawn by qualified clinic and nursing staff.

A-iv-3. CLINICAL DEFINITIONS

Subjects were defined as SIRS if they displayed two or more criteria from Table 1 without an infection. Subjects were defined as septic if they met the SIRS criteria and were suspected or confirmed to have an infection. Subjects were defined as severe sepsis if they met the sepsis criteria and displayed one or more criteria from Table 2.

A-iv-4. BIOMARKER ASSAY

Systemic responses to factors within the blood are complex and patient dependant. It is unclear if access to precise concentrations of circulating factors within the blood would benefit in generating effective treatment strategy as many studies show increased levels of cytokines with many failed anti-cytokine treatments failing in clinical trials. All samples were analyzed for concentrations of inflammatory signaling factors displayed in sepsis as well as biomarkers used

Table 1- SIRS criteria

Temperature	**>38ºC or <36ºC**
Heart Rate	**>90 beats/minute**
Respiratory Rate	**>20 breathes/minute**
$PaCO_2$	**<32 Torr**
WBC	**>12,000 or <4,000 cells/mm^3**

Table 2- Severe Sepsis criteria

Respirations	PaO_2/FiO_2	**250 Torr**
Coagulation	Platelets	**<50x10^3/mm^3**
Liver	Bilirubin	**>4.0 mg/dl**
Cardiovascular	Vapressor	**prescribed**
Renal	Creatinine	**>4.0 mg/dl**
Oliguria	Urine output	**<500 mL/day**

for sepsis. These factors are: tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), procalcitonin (PCT), C-reactive protein (CRP), and granulocyte/macrophage colony stimulating factor (GM-CSF). CRP and PCT concentrations were measured by ELISA (ALPCO and RayBiotech respectively) assay plates. A milliplex human magnetic high sensitivity panel luminex assay was used to measure the concentrations of IL-6, IL-8, GM-CSF, and TNF-α. These assays were conducted and analyzed by trained personnel at the University of Kentucky biochemical analysis laboratory.

B.) RESULTS

B-i. VALIDATION OF TEER AS PROBE FOR SIRS/SEPSIS

A background resistance (R_b) of each insert and media was obtained before seeding of the HUVECs. Once the cells were confluent, a baseline resistance (R_c) was recorded for each insert. Rat serum was then exposed to the endothelial cell monolayer and resistance measurements (R_t) were taken after 2.5, 7.5, 15, 30, 60 minutes of exposure. %TEER was calculated by removing the background resistance for both the baseline and time point resistances and dividing the time point by the baseline [$(R_t-R_b)/(R_c-R_b)*100$ =%TEER]. The results from the rat CLP study show a quick and significant decrease in %TEER with septic serum when analyzed against the values of its healthy counterpart (Figure 2). Figure 2 shows the average %TEER of serum taken before surgery (Control) on the rats were all above 60% during the first hour of exposure to the serum while the average %TEER values of the serum taken after surgery (Septic) were all below 60%. These results indicate an ability

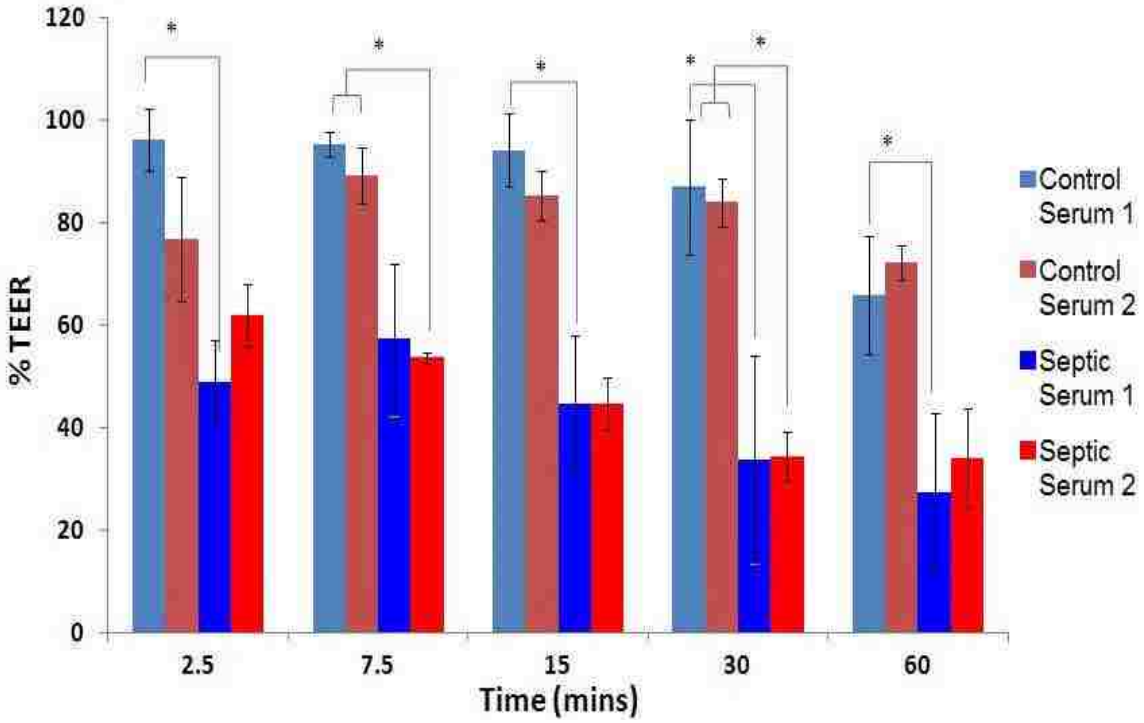

Figure 2: Graph showing the change in %TEER of HUVECs for the first hour after exposure to rat serum before (control) and after (Septic) CLP

*N=3; error bars represent SE; *p<0.05*

to quickly detect the difference between healthy rat serum and rat serum with increase inflammatory agents caused by CLP using TEER measurements.

B-ii. %TEER RESPONSE SIRS-RATED HUMAN SERUM

The same technique as mentioned earlier was used to calculate %TEER. The results from the SIRS-rated human serums differed from the rat CLP results. During the first 30 minutes of exposure to the serum, the data obtained displays no statistically significant serum difference found between the healthy serum samples and the SIRS-rated serum samples (Figure 3). Figure 3 does show a significant difference between the negative control (Healthy) and the positive control (Healthy+Histamine). After exposing the cells to the serum for an hour, statistical differences were found in the average %TEER measurements between the healthy serum samples (147.8%±4.5% SE) and the SIRS (130.2%±6.7%) and Severe Sepsis (131.3%±5.1%) serum samples (Figure 4). After 4 hours of exposure, all of the different levels of SIRS-rated samples(SIRS- 102.1%±6.3%; Sepsis- 97.9%±4.9%; Sev. Sepsis- 96.4%±3.1%) displayed a statistically lower %TEER value compared to the healthy serum (128.8%±5.3%) (Figure 4). After overnight incubation only the SIRS (96.6%±7.0%) and severe sepsis (92.1%±6.0%) samples remained significantly lower than the healthy serum (123.9%±5.8%). Unfortunately, no significant differences were found between the different SIRS-rated serums throughout this experiment. These results indicate an ability to detect the difference between healthy human serum and human serum with systemic inflammation after an hour of exposure using TEER measurements.

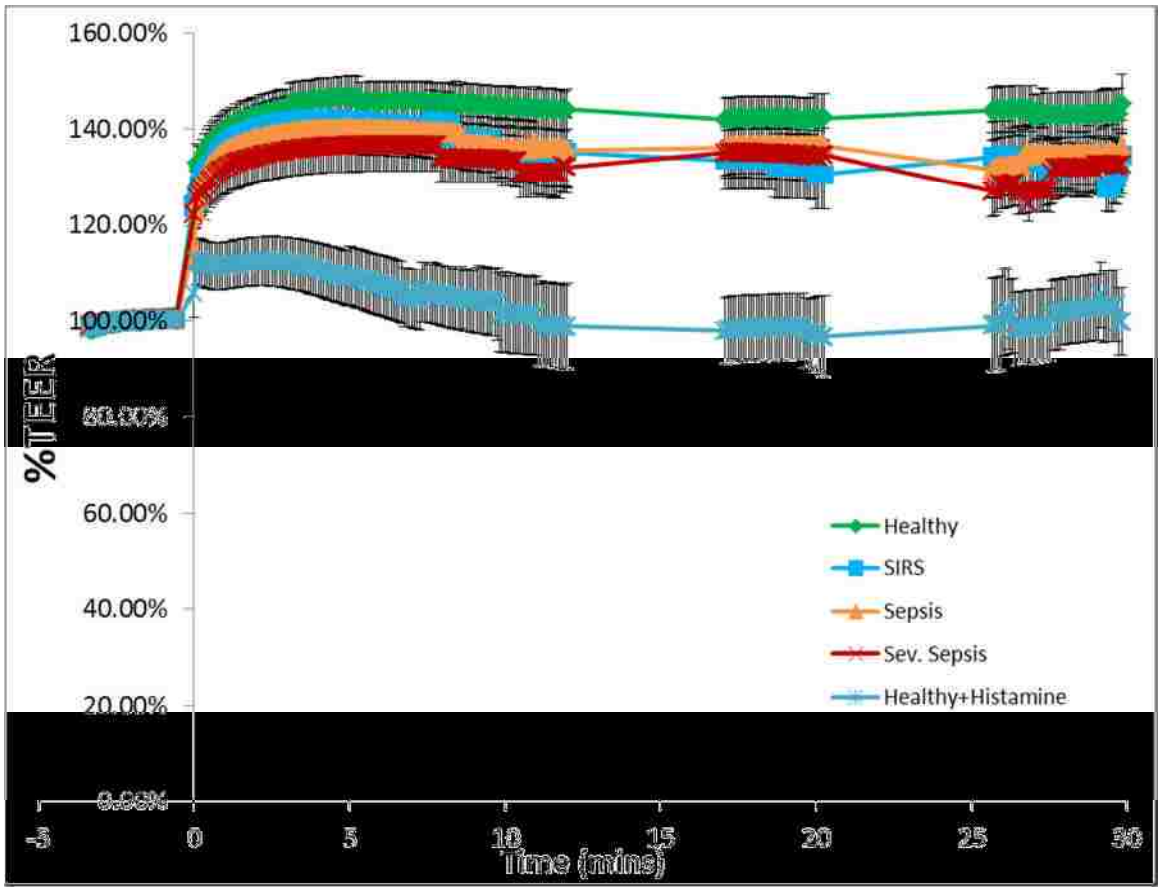

Figure 3: Graph showing the change in %TEER for the first 30 minutes of exposure to serum collected from patients that were healthy or showing symptoms of SIRS, Sepsis, or Severe Sepsis
N=5 for Healthy,Sepsis,Sev. Sepsis,Healthy+Histamine; N=4 for SIRS; error bars represent SE

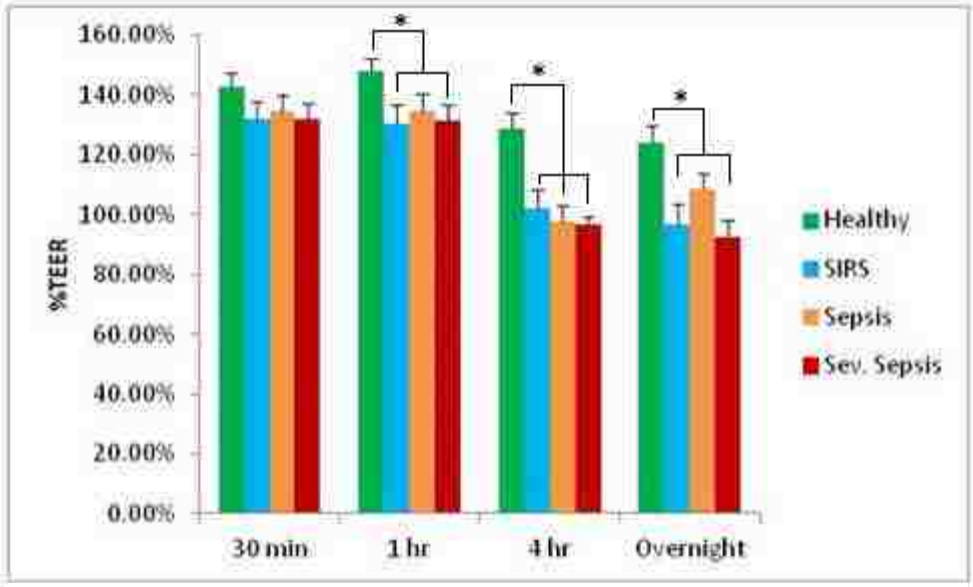

Figure 4: Graph showing the change in %TEER of exposure to serum collected from patients that were healthy or showing symptoms of SIRS, Sepsis, or Severe Sepsis
*N=5 for Healthy,Sepsis,Sev. Sepsis; N=4 for SIRS; error bars represent SE; *p<0.05, compared to control*

B-iii. BIOMARKER ANALYSIS

The results show in Figure 5 from ELISA assays indicate that concentrations of TNF-α and IL-6 were significantly higher in patients labeled SIRS (13.6±2.4 SD and 1,096±905 SD respectfully) or severe sepsis (24.5±6.3 and 1,669±661 respectfully) than healthy individuals (7.1±2.2 and 4.4±1.8 respectfully). Furthermore, TNF-α was significantly higher in severe sepsis patients than SIRS patients. Due to the scatter of the IL-8 (Figure 5C) and GM-CSF (Figure 5D) concentrations, no significant differences were found between healthy individual and sick patients. All of the healthy individuals displayed PCT levels around or below the detectable limit (10pg/mL) (Figure 5E). The patients in the SIRS and sepsis categories, however, contained PCT concentrations all over the spectrum (Figure 5E). Therefore, no significant difference can be seen between the healthy controls and the patients rated with SIRS or sepsis. However, there was a significant difference between healthy individuals (13.2±3.9) and patients under the severe sepsis (1,103±542) criteria due to all of those patients having elevated PCT levels. All of the sick patients had severely elevated CRP values (>98.6μg/mL) when compared to healthy individuals (<8.3μg/mL) (Figure 5F). However, there was no significant difference between the different SIRS-rated groups. This agrees with literature that CRP concentrations increase drastically once a patient became ill; however, these values do not change significantly as the illness progresses.

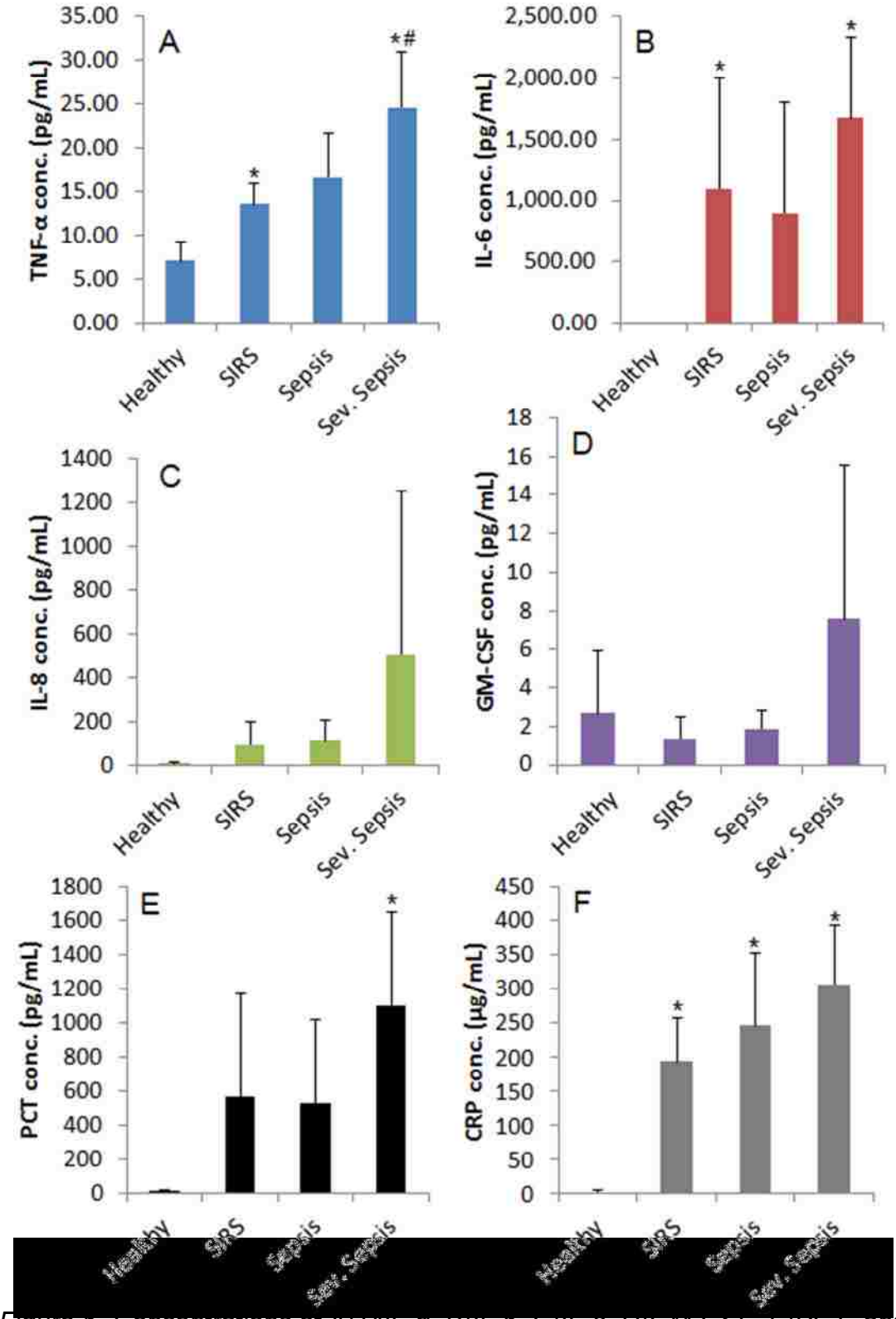

Figure 5- Concentrations of A)TNF-α, B)IL-6, C)IL-8, D)GM-CSF, E)PCT, and F)CRP in patients of different SIRS-rated categories
*N=5 for Healthy,Sepsis,Sev. Sepsis; N=4 for SIRS; error bars=SD; *p<0.05 compared to control, #p<0.05 compared to SIRS*

Looking at the patients individually, Table 3 shows the four hour % TEER measurements correlated the highest to cytokines TNF-α, IL-6, and IL-8 (-0.706, -0.451, and -0.575 respectfully) as well as biomarkers PCT and CRP (-0.604 and -0.854 respectfully) while the one hour %TEER measurements correlated the highest to ICU days (-0.462). Figures 6, 7, 8, and 9 show the significant linear correlations between %TEER after four hours of exposure to different human serums and TNF-α, PCT, CRP, and IL-8 concentrations (respectfully). Figures 10, 11, and 12 shows the scatter between %TEER after four hours of exposure and the time spent in the ICU, IL-6, and GM-CSF (respectfully). Table 4 indicates TNF-α (0.627) as the highest assay correlated to the length of stay in the ICU. The average stay of SIRS, sepsis, and severe sepsis patients were 6 days, 2 days, and 8 days respectively. One patient out of each SIRS-rated group passed away. Each of these three patients contained a PCT concentrations above 1,000 pg/mL, maximum IL-6 concentrations (>2,000 pg/mL), and IL-8 concentrations above 200 pg/mL. None of the survivors met all three of these criteria. These results indicate taking TEER measurements after four hours as the time point to differentiate between healthy and SIRS-rated samples.

C.) DISCUSSION

In these studies, a method for assessing systemic inflammation using transendothelial electrical resistance (TEER) measurements was established. The rat CLP model displayed an ability to quickly and effectively see a difference between healthy and septic rat serum. These results indicated that a device capable of monitoring impedance of endothelial cells in “real-time” can show a

Table 3- %TEER/Biomarker Pearson Correlation Data

	30 min %TEER	1 hr %TEER	4 hr %TEER	Overnight %TEER
ICU Days	-0.248 (0.355)	-0.462 (0.072)	-0.400 (0.124)	-0.398 (0.127)
TNF-α	-0.380 (0.146)	-0.551* (0.027)	-0.706* (0.002)	-0.688* (0.003)
PCT	-0.219 (0.414)	-0.375 (0.152)	-0.604* (0.013)	-0.581* (0.018)
CRP	-0.494 (0.052)	-0.586* (0.017)	-0.854* (0.000)	-0.668* (0.005)
IL-6	0.076 (0.779)	-0.165 (0.541)	-0.451 (0.080)	-0.406 (0.118)
IL-8	-0.468 (0.067)	-0.544* (0.030)	-0.575* (0.020)	-0.377 (0.150)
GM-CSF	-0.308 (0.247)	-0.293 (0.271)	-0.058 (0.831)	-0.199 (0.460)

Cell Contents: Pearson correlation
(P-Value)
**p<0.05*

Table 4- Biomarker Pearson Correlation Data

	GM-CSF	IL-6	IL-8	CRP	ICU Days	TNF-α
IL-6	-0.014 (0.960)					
IL-8	0.346 (0.189)	0.670* (0.004)				
CRP	0.350 (0.184)	0.491 (0.053)	0.648* (0.007)			
ICU Days	0.433 (0.094)	0.250 (0.351)	0.579* (0.019)	0.565* (0.023)		
TNF-α	0.653* (0.006)	0.248 (0.355)	0.488 (0.055)	0.817* (0.000)	0.627* (0.009)	
PCT	0.250 (0.350)	0.507* (0.045)	0.423 (0.102)	0.676* (0.004)	0.441 (0.087)	0.603* (0.013)

Cell Contents: Pearson correlation
(P-Value)
**p<0.05*

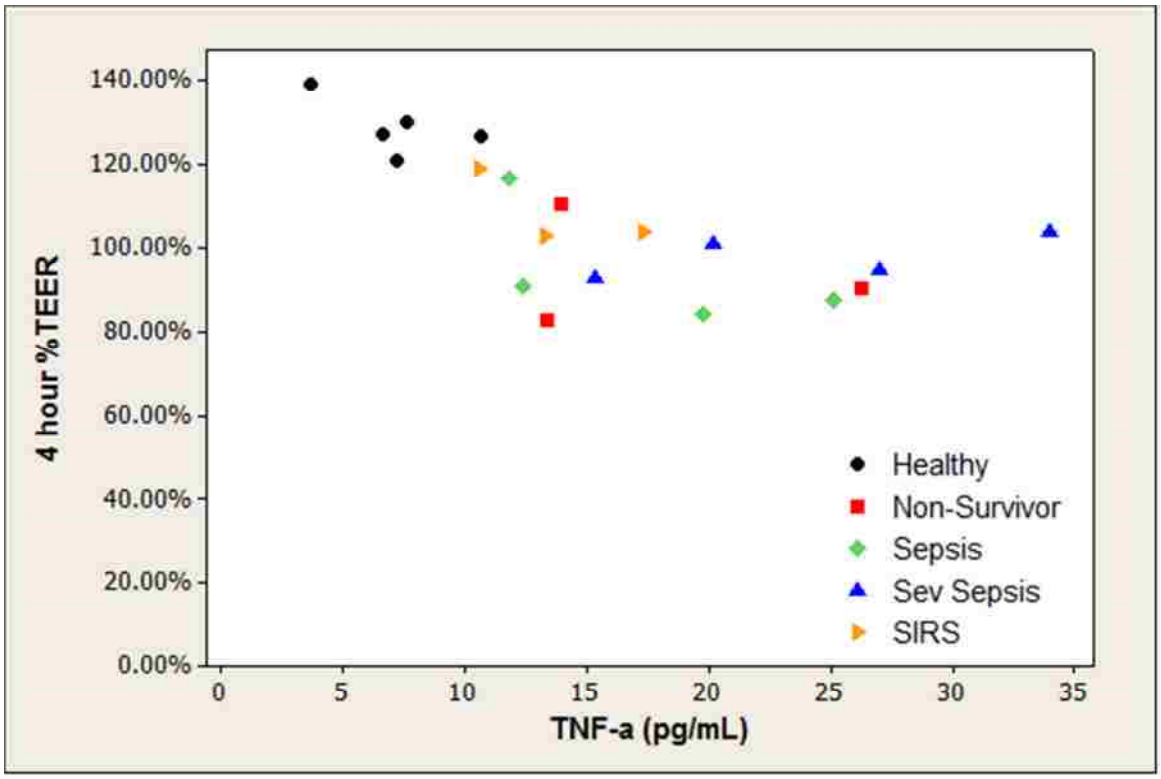

Figure 6- Graph showing %TEER after 4 hours of exposure to serum collected from patients versus TNF-α concentrations

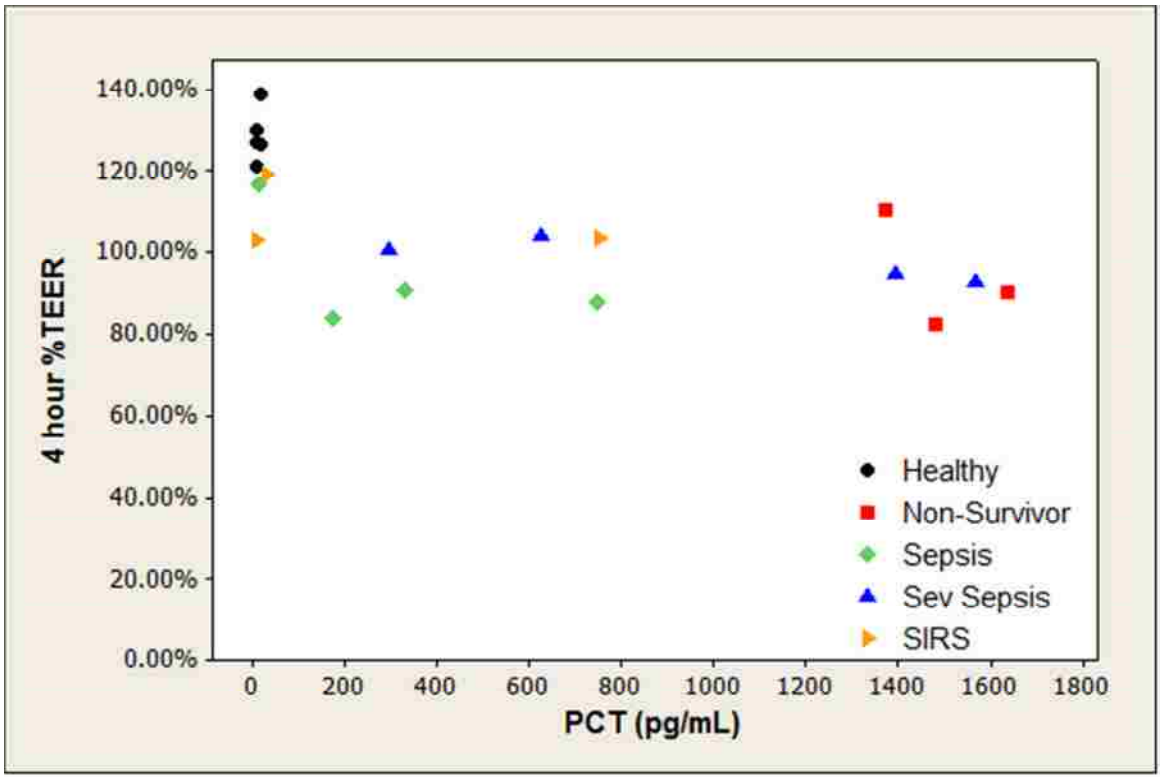

Figure 7- Graph showing %TEER after 4 hours of exposure to serum collected from patients versus PCT concentrations

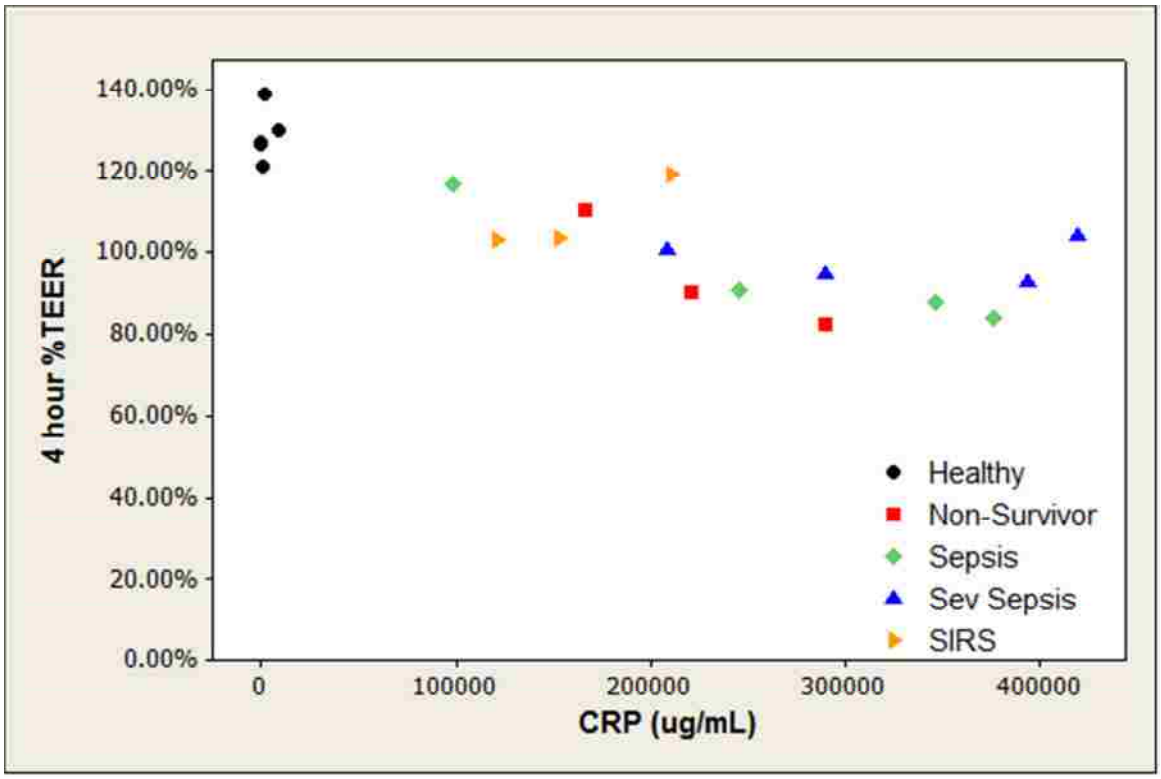

Figure 8- Graph showing %TEER after 4 hours of exposure to serum collected from patients versus CRP concentrations

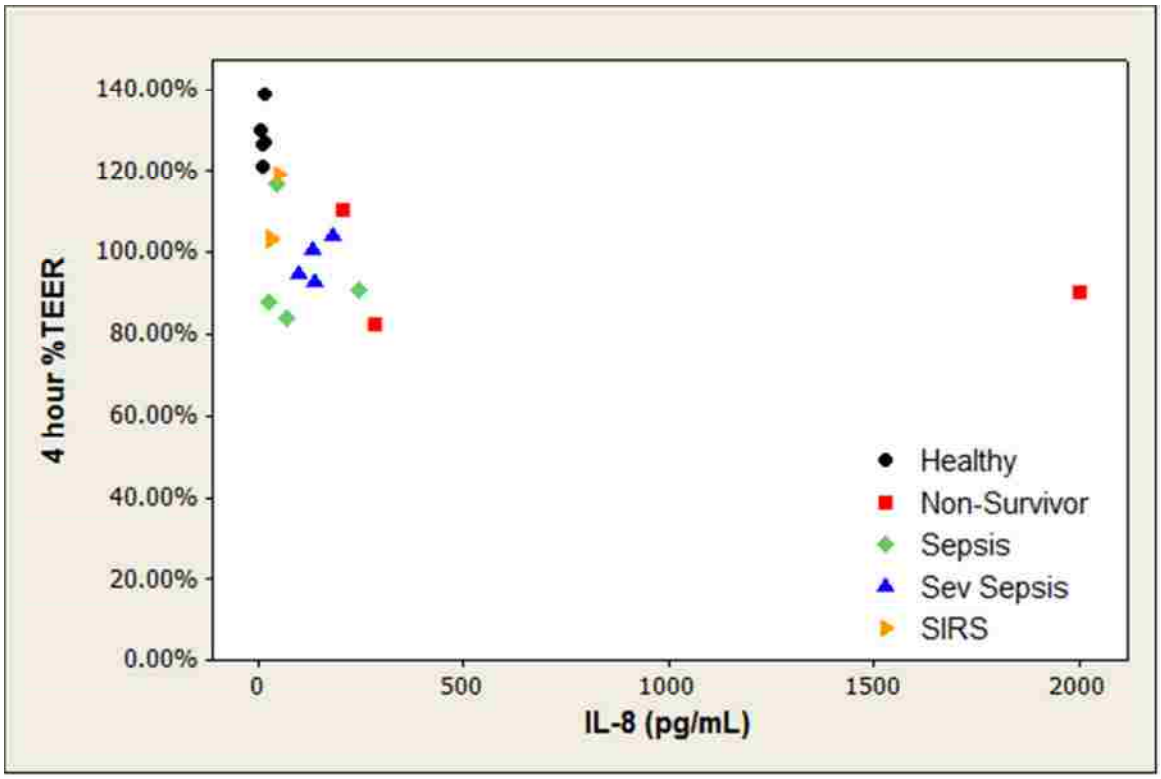

Figure 9- Graph showing %TEER after 4 hours of exposure to serum collected from patients versus IL-8 concentrations

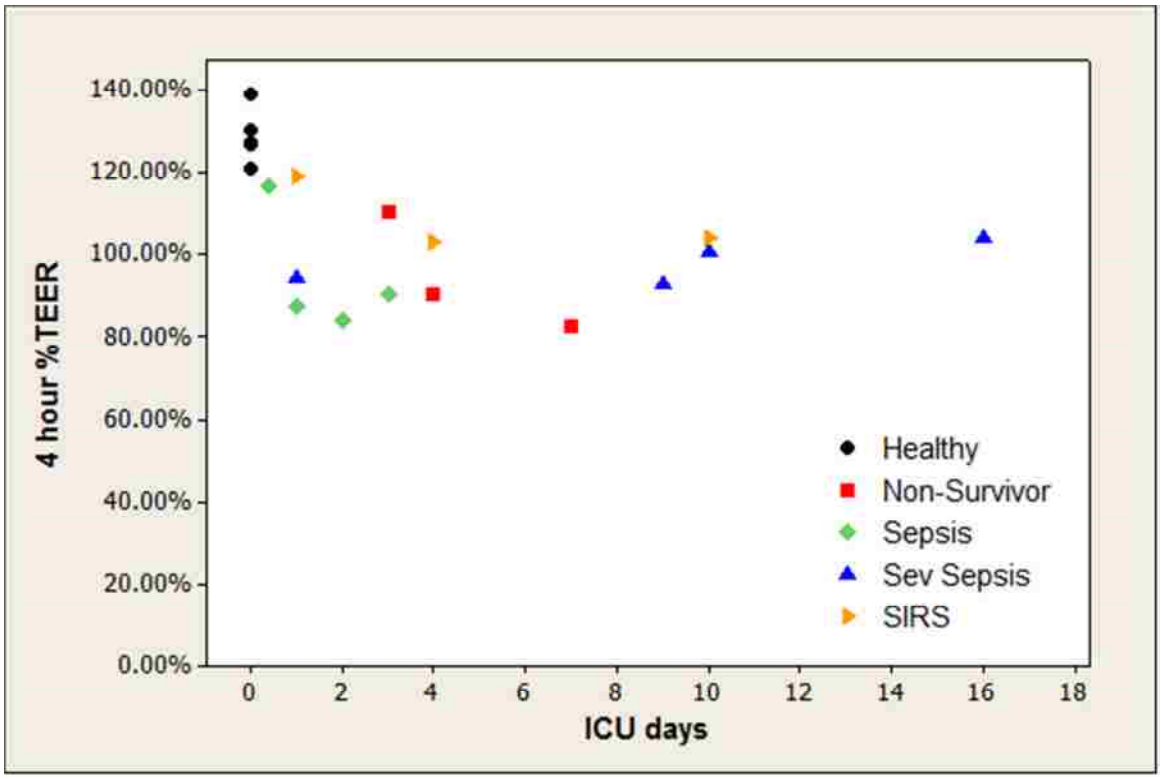

Figure 10- Graph showing %TEER after 4 hours of exposure to serum collected from patients versus time spent in the ICU

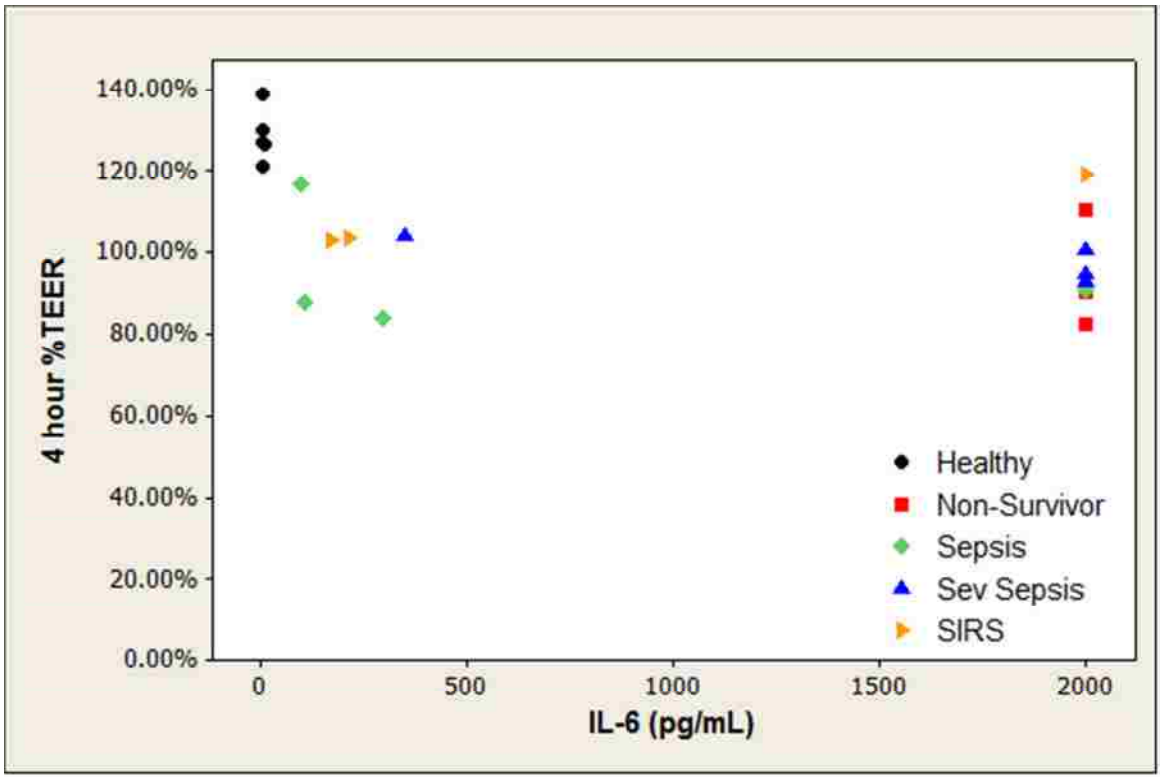

Figure 11- Graph showing %TEER after 4 hours of exposure to serum collected from patients versus IL-6 concentrations

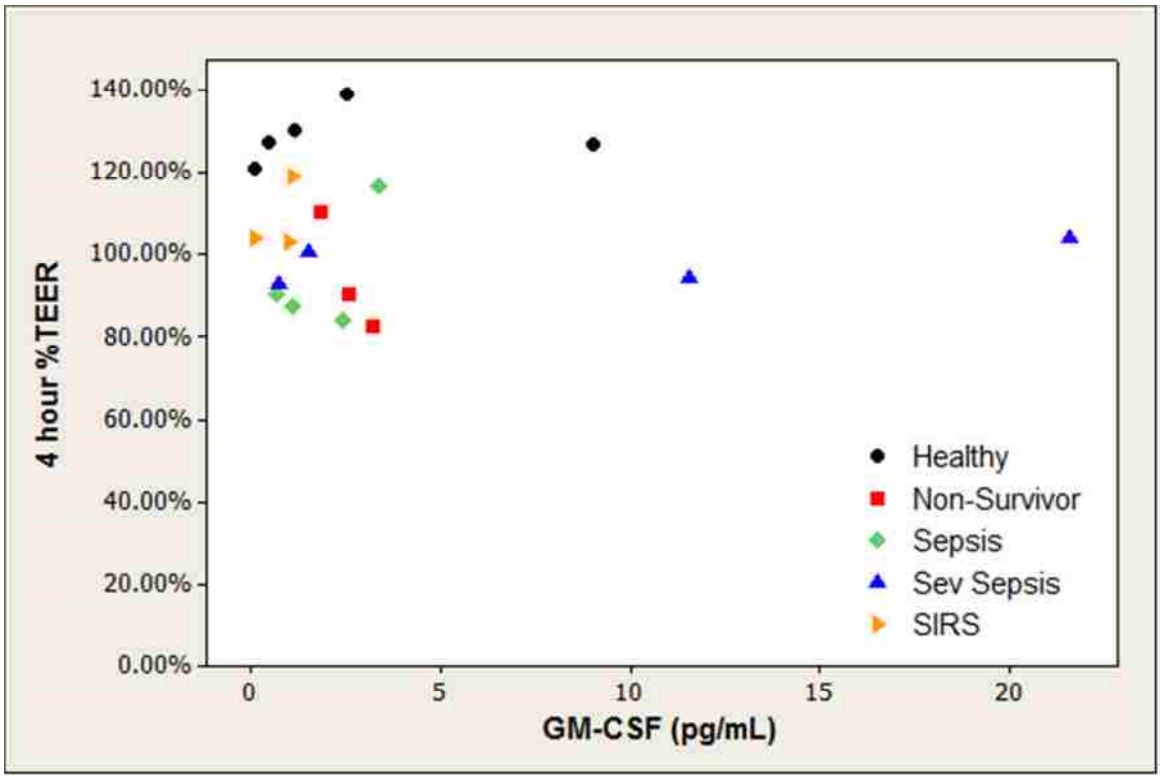

Figure 12- Graph showing %TEER after 4 hours of exposure to serum collected from patients versus GM-CSF concentrations

fast response. It is important to note that this experiment was performed in a rat model and the serum being analyzed is an extremely severe SIRS/sepsis specimen. This data corroborates the notion that TEER measurements have the potential for aiding in SIRS detection and treatment.

It has been reported that cytokines cause an increase in permeability and a drop in TEER measurements after four hours of exposure.[80] This study demonstrated such a significant drop in TEER after four hours of exposure to human serum with increased levels of cytokines. This study also displayed significant correlations between the %TEER measurements taken after four hours of exposure to serum and TNF-α, IL-8, PCT, and CRP. The results gathered by the human serum samples showed a difference in %TEER values between healthy serum and SIRS-rated serum as early as one hour post exposure. Unfortunately, the differences in %TEER amongst the different SIRS-rated samples were not significant indicating only the ability to detect healthy serum from unhealthy serum.

It has been reported that SIRS/sepsis causes an increase in TNF-α, IL-6, IL-8, PCT, and CRP.[26, 32] Literature shows TNF-α concentrations are significantly higher from healthy individuals (10.5±0.7 pg/mL) to SIRS (16.7±1) and from SIRS to people with severe sepsis (48.4±17).[26] The results from this study demonstrated such a trend from healthy (7.15±2.23 pg/mL) to SIRS (13.63±2.40) and from SIRS to severe sepsis (24.55±6.34) with similar concentrations. The literature on IL-6 and IL-8 show an increase in concentrations between healthy individuals (2.8±0.3 and 20±3 pg/mL) and

people with SIRS (93±15 and 141±74), sepsis (183±66 and 114±80), and severe sepsis (409±131 and 325±271).[26] The results from this study displayed a low concentration for healthy people (4.38±1.82 and 11.36±3.40 pg/mL) with scattered elevated concentrations for SIRS (1,096±905 and 97.27±106.92), sepsis (900±900 and 116.06±89.68), and severe sepsis (1669±661 and 509.10±745.95). Furthermore, the CRP results from this study show a similar trend bow low healthy concentrations and elevated SIRS, sepsis, and severe sepsis (2.06±3.17, 193.6±64.0, 246.4±104.9, 306.4±86.6 µg/mL) to that of literature values (2.7±1, 109±7, 141±12, 189±17 µg/mL).[26] The PCT concentration reported in literature (healthy-104, SIRS-380, sepsis-1,580, severe sepsis-5,580)[32] are higher than those shown in this study (13.2, 567.7, 526.4, 1,103.0). The scatter of concentrations obtained by the ELISA assays within the different SIRS-rated categories reflects the inability of the clinician to detect and stage the SIRS/sepsis criteria.

Overall, this study showed TEER measurements detecting systemic inflammation. It displayed the trends between % TEER and biomarkers found with systemic inflammation. Lastly, It demonstrated similar concentrations and trends between healthy individuals and SIRS-rated serums to that of literature for biomarkers: TNF-α, IL-6, IL-8, and CRP. These results confirm that a device monitoring impedance of endothelial cells can effectively detect a difference between healthy serum and unhealthy serum.

CHAPTER 4

LOW TEMPERATURE CO-FIRED CERAMIC STUDIES

A.) MATERIALS AND METHODS

A-i. CELL CULTURE AND STAINING

The Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Switzerland) at passage 1. The HUVECs were grown in 75 cm^2 tissue culture flasks. EGM-2 media (Lonza) was used to grow the cells inside the flasks in an incubator at 37ºC and 5%CO_2. Once HUVECs were close to confluency (>85%), they were split and seeded onto either the test samples, or Transwell inserts at a concentration of 25,000 cells/cm^2. Passages 4-8 were used in these experiments. Following the seeding of cells, the well plates were placed in the incubator under the same conditions as the cell culture flasks. For trials lasting longer than one day, the media was exchanged for fresh media within the first 24 hours post-seeding and then every other day.

The Dulbecco's phosphate-buffered saline (DPBS) solution used in these experiments was generated by adding 4g NaCl, 0.5g Na_2PO_4, 0.1g KCl, and 0.1g KH_2PO_4 to 500 mL of DI water from the Milli-Q® Advantage A10 system (Millipore). The solution was sterilized via autoclave before it was used. The Live/Dead solution used in these experiments was 0.2 (v/v%) Calcein AM and 0.34 (v/v%) Ethidium Homodimer-1 in DPBS. For imaging, the well or channel was rinsed once with DPBS to remove loosely affixed cells. The well or channel was then filled with the Live/Dead solution and incubated for 20-30 minutes.

A-ii. MATERIAL BIOCOMPATIBILITY

The LTCC materials used were Heralock® HL2000 (Heraeus, West Conshohocken, PA). Four (3” x 3”) HL2000 layers were laminated together using a hydraulic press (Model 3851, Carver, Wabash, IN) equipped with heated platens. The platens were heated up to 70ºC and the layers were placed under 5,000 lbs of pressure for 10 minutes followed up by 10,000 lbs for 15 minutes. Following the lamination, 1 cm x 1 cm LTCC test samples were patterned via laser ablation. Three sample sets were created. The first set was only the HL2000 LTCC material bare, which from here on out will be called “Blank”. The other two sets had conductive pastes that were then hand painted onto one side of these HL2000 LTCC test samples. The conductive pastes that were evaluated are TC0306 (Heraeus) or TC8101 (Heraeus), which from here on out will be called “silver” and “gold” respectively. After patterning with conductor inks, each test set was sintered following the recommendations by the manufacturer.

Eighteen HL2000 LTCC test samples were constructed for each sample set (Blank, Gold, and Silver). Half of each sample set was soaked for 24 hours in DI H2O from the Milli-Q® Advantage A10 System (Millipore), these samples will be considered “Leached” samples while the others will be “Unleached”. Each sample set was placed into a resealable autoclave pouch and was autoclaved to 121ºC for 30 minutes. Afterwards, the pouches were sprayed with 70% ethanol and placed under a laminar flow hood. The pouches were opened and UV light was exposed to the surface of the LTCC test samples on for an hour to aid in the sterilization. Three of each sample set were placed into a 12 well plate and were

either soaked in 0.5 mL of fibronectin solution at a concentration of 25ìg/mL and incubated for 45 minutes or not soaked with fibronectin. The fibronectin wells were then rinsed three times with DPBS and HUVECs were seeded into the wells as described earlier with three wells on each plate being a control without any LTCC material. The well plates were then cultured for 1-2 hours to look at the initial attachment, one day, and three days. Following these culture periods, the wells were rinsed once with DPBS, stained with Live/Dead solution, and imaged. The images were analyzed using NIS-Elements BR 3.0 Object Count software.

A-iii. DEVICE FABRICATION AND BIOCOMPATIBILITY

Three simple layers were patterned via laser ablation (top, channel, and bottom). The top layer contained inlet and outlet ports of 1.5 mm in diameter as well as an electrode pattern. The channel layer contained a rectangular channel with dimensions of 4.44 cm x 3 cm x 0.8 cm. The bottom layer contained the electrode pattern. The electrode pattern was screen-printed onto the top of the bottom layer and bottom of the top layer. Lamination of the HL2000 LTCC tape was accomplished using a hydraulic press (Model 3851, Carver, Wabash, IN) equipped with heated platens. The platens were heated up to 70ºC. Individual HL2000 layers were placed under 5,000 lbs of pressure for 10 minutes followed up by 10,000 lbs for 5 minutes. Following this, the layers were then laminated together at the same temperature under 5,000 lbs of pressure for 10 minutes followed up by 8,000 lbs for 15 minutes. After laminating the layers together, the device was sintered following the recommendations by the manufacturer. For

viewing purposes, a PDMS window was attached via oxygen plasma after sintering the device (See Figure 13).

The device was soaked in DI H_2O to remove leachates. Afterwards, the device was sterilized via autoclave and UV light exposure. The device was then filled with media and incubated overnight. Afterwards, the device was soaked in FN and HUVECs were seeded as previously described. The fittings were filled with media and the device was placed in the incubator. The media in the channel was replaced each day with fresh media. At the end, the device was disconnected, stained with Live/Dead solution, and imaged.

A-iv. DEVICE SENSITIVITY

A waveform generator (Agilent 33220A) was connected to a resistor box and then to a data acquisition/switching unit (Agilent 34970A) which was connected to a pair of clips. Data was then transferred to the computer through the switching unit (see Figure 14). A power supply of 1 volt at a frequency of 1000 Hz was used in these studies. A 100 kOhm resistor was placed into the resistor box to generate a 10 µA current through the clips.

The LTCC device was then tested for its sensitivity in resistance values and compared devices currently on the market. The device was compared to the Endohm12 TEER cup and the STX2 TEER chopsticks (World Precision Instruments, Sarasota, FL) which are static TEER devices that are commonly used in research. The resistance values for the STX2 were obtained with an EVOM^2 Voltohmmeter (WPI). The resistance values for the LTCC device were

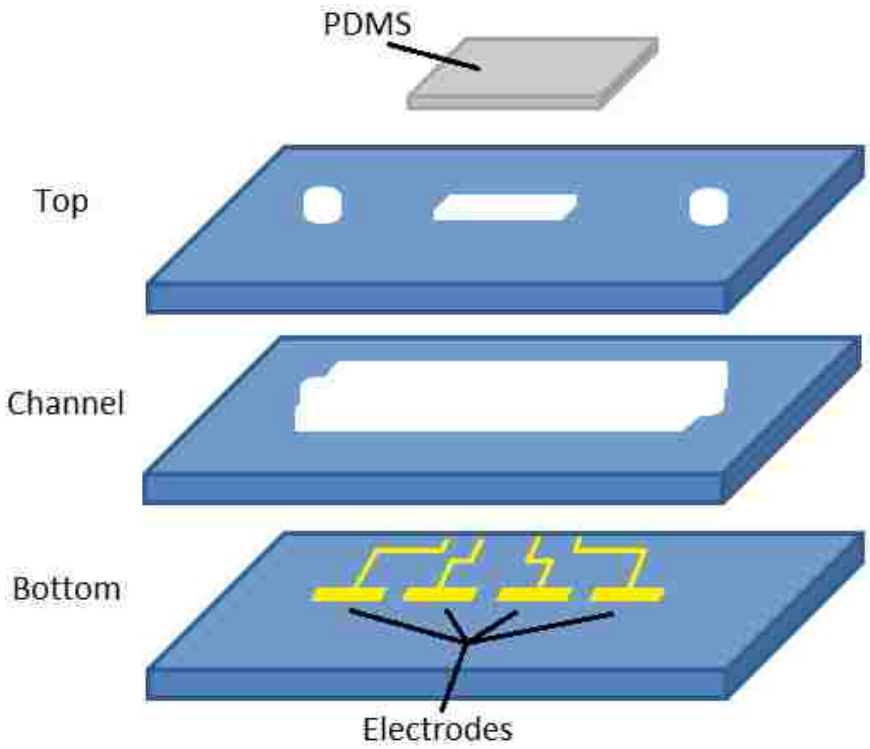

Figure 13: Diagram of LTCC device

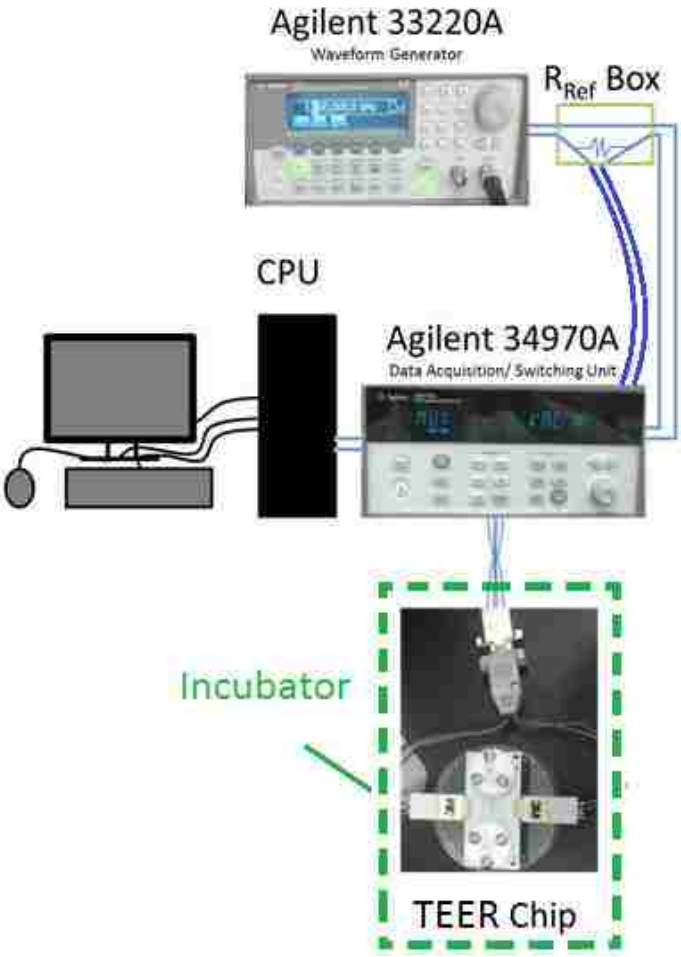

Figure 14: Diagram of In-house set-up

acquired with the Agilent set-up while the resistance values for the En_dohm12 were retrieved by both the Voltohmmeter and the Agilent set-up.

A-v. SYSTEM IMPROVEMENTS

The In-house system aided in the start-up of the LTCC device for TEER measurements as it proved to be beneficial in the detection of an endothelial cell monolayer. However, the noise of this system was too great to detect small TEER responses of an endothelial cell monolayer. To reduce this noise, the Agilent systems were replaced with a Lock-in amplifier (See Figure 15). The LTCC device was then tested for its sensitivity in resistance values and compared to the previously obtained data from the In-house system.

The electrode geometry was also altered to generate suitable impedance graphs of a cell monolayer for TEER detection. The electrode size was reduced from 4 mm^2 rectangles to a 0.05 mm^2 circle vias to focus the impedance on the interface of the electrode. The electrodes (4 mm^2 rectangles/0.05 mm^2 circles) were then tested for their ability to detect a “pseudo-cell” (a parallel resistor and capacitor to mimic a cell monolayer). In this analysis, the LTCC devices were filled with media and impedance measurements were obtain at varying frequencies. After obtaining this background impedance, the “pseudo-cell” was connected in series with the device and impedance measurements were obtained at the same frequencies.

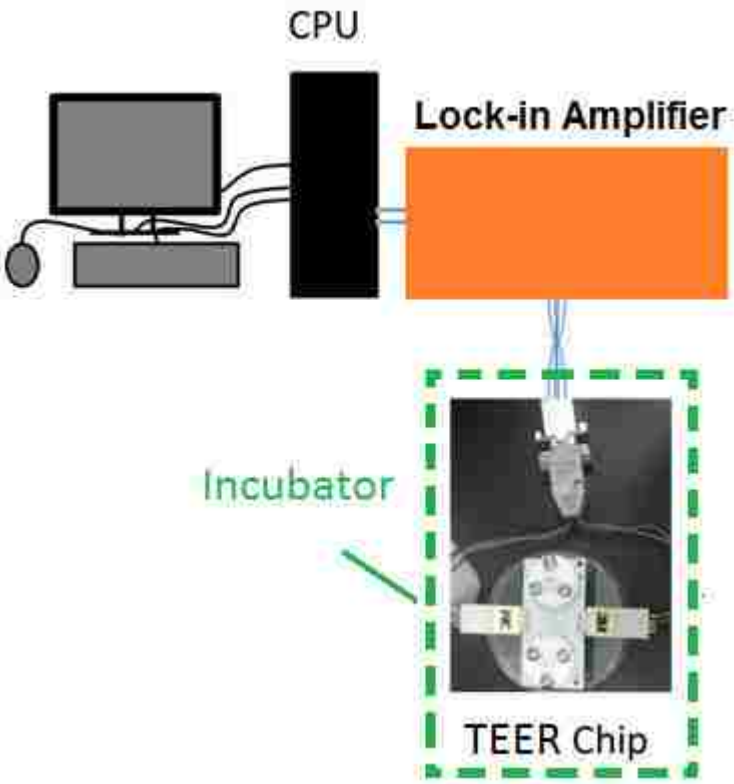

Figure 15: Diagram of Lock-in Amplifier set-up

B. RESULTS

B-i. MINIMAL ATTACHMENT AND GROWTH ON HL2000 LTCC

The biocompatibility images for Blank LTCC test samples are shown in Figure 16. When comparing Blank test samples that were soaked in DI H_2O ("Leached") to those that were not ("Unleached"), the images show that soaking in DI H_2O facilitated in cellular attachment and spreading. The images show more HUVECs spread onto the leached samples indicating healthier cells for growth and attachment. The data in Figure 17A shows no significant decrease in viability of the HUVECs on each of the Blank LTCC materials. However, the data in Figure 17B shows significant decreases in the number of cells per area when compared to the control well. The data in Figure 17B also illustrates an increase in the number of HUVECs per area in the leached test samples over the unleached test samples. Figure 17B also exhibits that HUVECs did attach onto the Blank test samples as well as grow on the surface as exhibited by the increase in the number of cells per area from Day 1 to Day 3. Therefore, HUVECs spread and grew better onto leached Blank LTCC test samples compared to unleached Blank LTCC test samples.

B-ii. HUVEC ATTACHMENT AND GROWTH ON CONDUCTIVE PASTES

The biocompatibility results for both Silver and Gold conductive pastes were very favorable for TEER applications. The viability of the HUVECs grown on both pastes was similar to those of the control wells as demonstrated in Figure 18A and 18C. Both Silver and Gold test samples show a significant decrease in cell density compared to that of the control wells after one day of

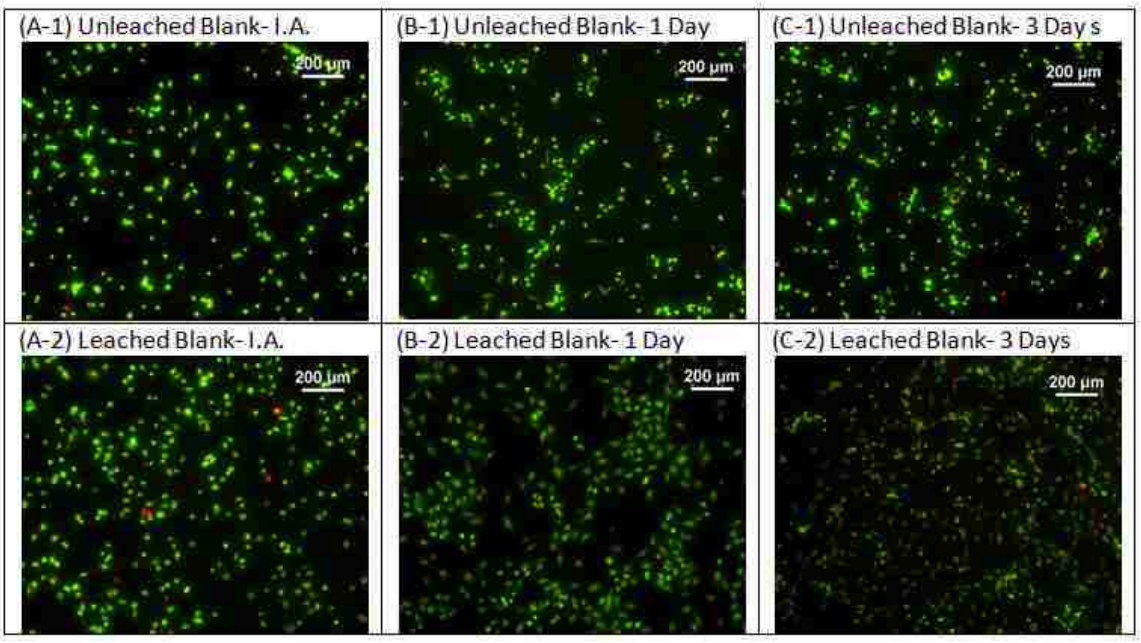

Figure 16: Images of HUVECs on Blank LTCC test samples at (A) Initial Attachment, (B) one day cell culturing, or (C) three days of cell culturing on either (1) unleached or (2) leached samples

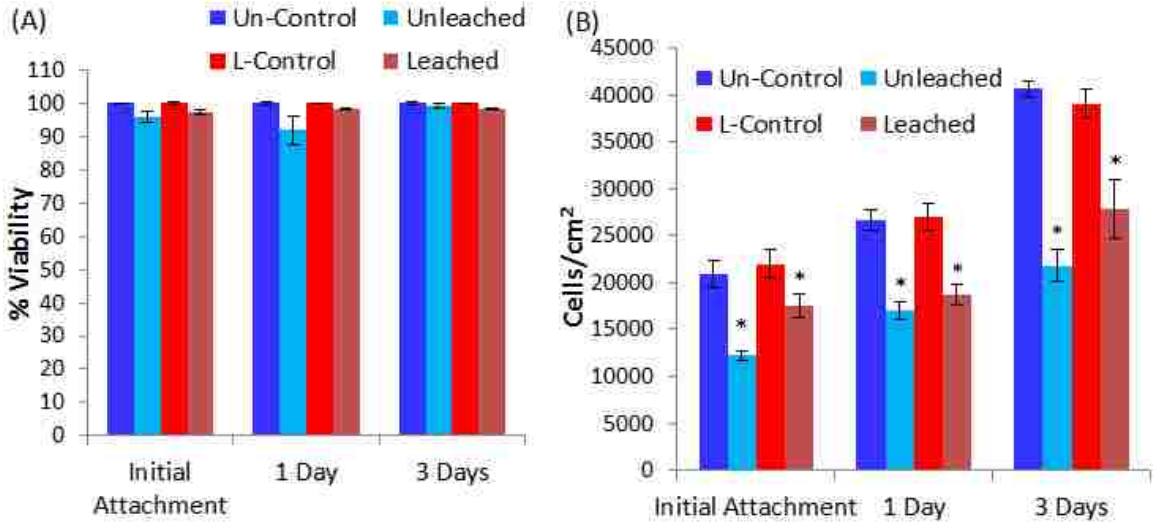

*Figure 17: Biocompatibility results for unleached and leached Blank LTCC test samples (A) percentage of viable HUVECs compared to the control group (B) Cells/area compared to the control group; *p<0.05*

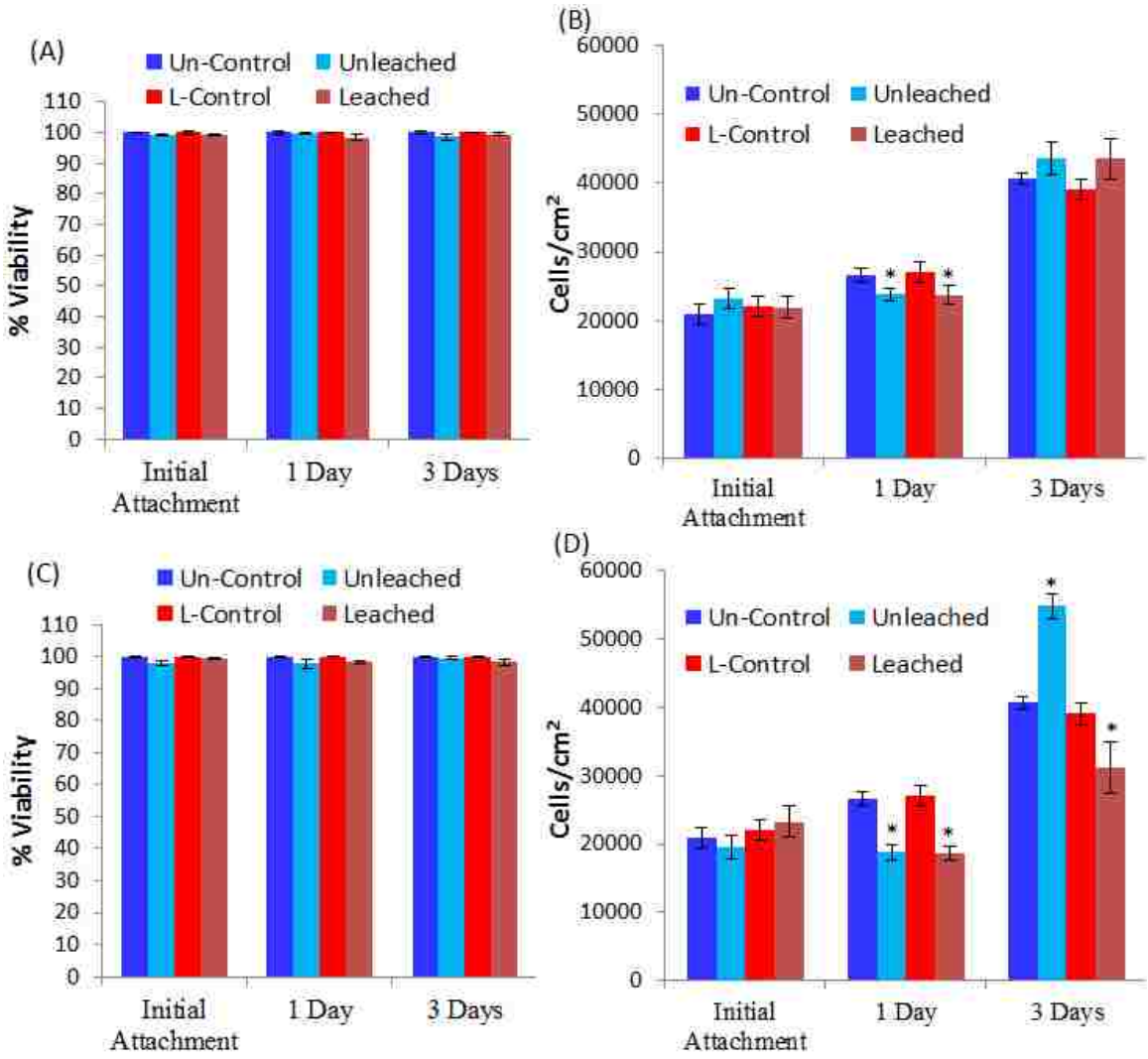

*Figure 18: Biocompatibility results for unleached and leached Gold LTCC test samples (A) percentage of viable HUVECs compared to the control group (B) Cells/area compared to the control group as well as Silver LTCC test samples (C) percentage of viable HUVECs compared to the control group (D) Cells/area compared to the control group; *p<0.05*

culturing HUVECs. However, the HUVECs on both of the conductive pastes demonstrated similar growth to the control after three days of culturing. The data in Figure 18 indicates that both unleached and leached test samples for the conductive inks were similar to each other in both viability and cells per area. The only exception was that the unleached Silver sample set on the third day showing significantly higher cells per area than both the control and its leached counterpart. The Figure 18 data also shows inconsistency of cell growth on the Silver samples as the data of cells per area went down in day 1 but shot up in day 3. The images in Figure 19 (C-1) confirmed a high cell density onto the unleached Silver LTCC sample by the displaying a nice confluent monolayer of HUVECs on the surface of the test samples after three days of culturing. Images from both conductive pastes (Figure 19 and Figure 20) are in agreement with the blank sample set and show more spreading of the HUVECs on the leached samples compared to those of the unleached samples. Therefore, these studies demonstrate the benefit of a leaching period as indicated by the HUVECs ability to attach and grow properly along the surface of conductive pastes in which this occurs.

B-iii. CONFLUENT HUVEC MONOLAYER INSIDE CHANNEL OF LTCC DEVICE

The results from this study show that a confluent HUVEC monolayer can be obtained within the channel of an LTCC system that was soaked in fibronectin. The images obtained from this study display a viable endothelial cell monolayer on both the LTCC bare ceramic material as well as the gold

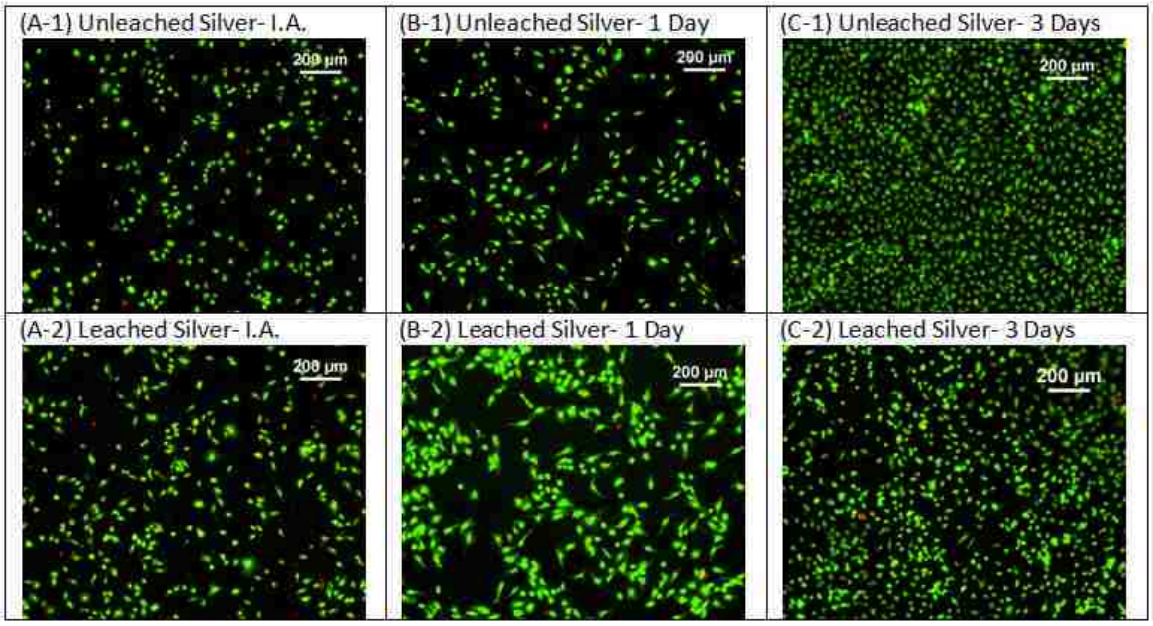

Figure 19: Images of HUVECs on Silver LTCC test samples at (A) Initial Attachment, (B) one day cell culturing, or (C) three days of cell culturing on either (1) unleached or (2) leached samples

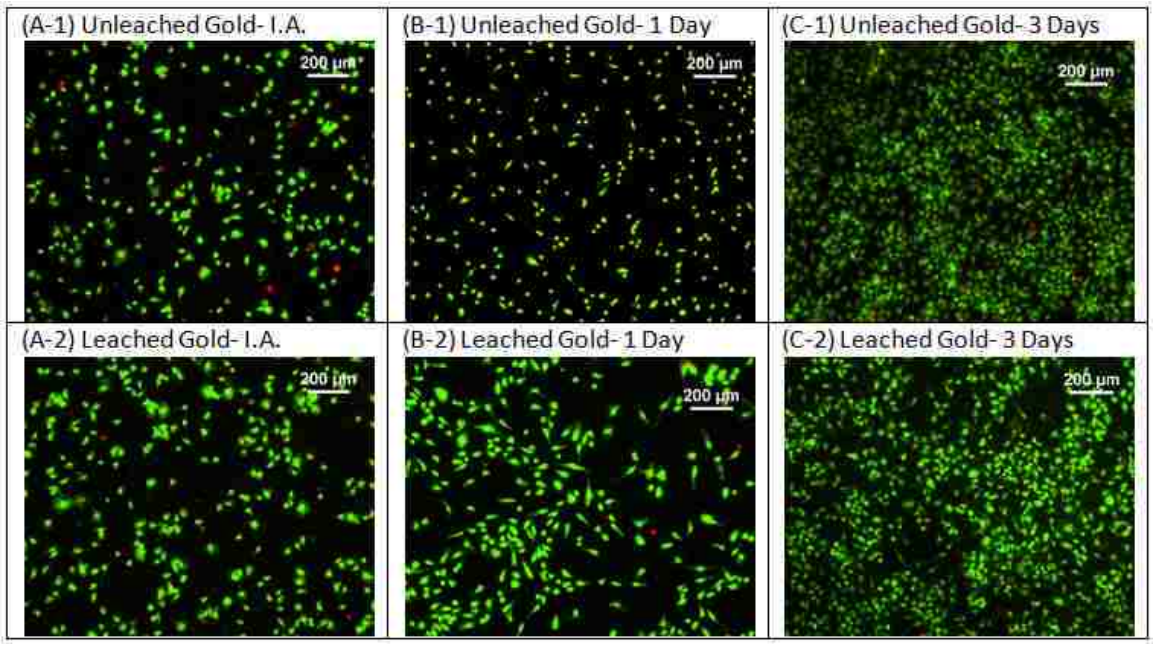

Figure 20: Images of HUVECs on Gold LTCC test samples at (A) Initial Attachment, (B) one day cell culturing, or (C) three days of cell culturing on either (1) unleached or (2) leached samples

Figure 21: Images of confluent HUVEC monolayer inside an LTCC channel containing two rectangular gold electrodes

conductive paste used to make the rectangular electrodes (Figure 21). This further supports the idea that these materials can be used to test endothelial cells with electronic applications such as TEER while the cells are exposed to a more physiological environment. With a device like this, endothelial cells can be exposed to shear stresses that would augment the cells in the direction of fluid flow as well as take TEER measurements.

B-iv. LTCC DEVICE SYSTEM PERFORMANCE COMPARISON

The instrument detection limit (IDL) is the smallest signal differentiated from the background noise of a system. It is calculated by taking three standard deviations. The IDL is used to compare different instruments. The IDL data in Figure 23 indicates the Endohm12 TEER cup with the EVOM2 Voltohmmeter is the most precise instrument followed by the LTCC TEER device with the In-house system and lastly the STX2 chopsticks. However, the data in Figure 22 also shows that the LTCC TEER device performed better than the Endohm12 TEER cup when the cup was connected to the In-house system the LTCC TEER device was using. This data reveals that the LTCC device is suitable for the detection of an endothelial cell monolayer. The LTCC device performance is also mirroring that of the commonly used and reliable Endohm12 TEER cup.

B-v. IMPROVED IMPEDANCE RESPONSES

Electrical impedance experiments, using standard circuit elements to simulate the response and characteristics of a cultured cell monolayer, confirm the ability to isolate and accurately measure the expected changes in cellular permeability occurring in the sensor array. To confirm that the sensors were

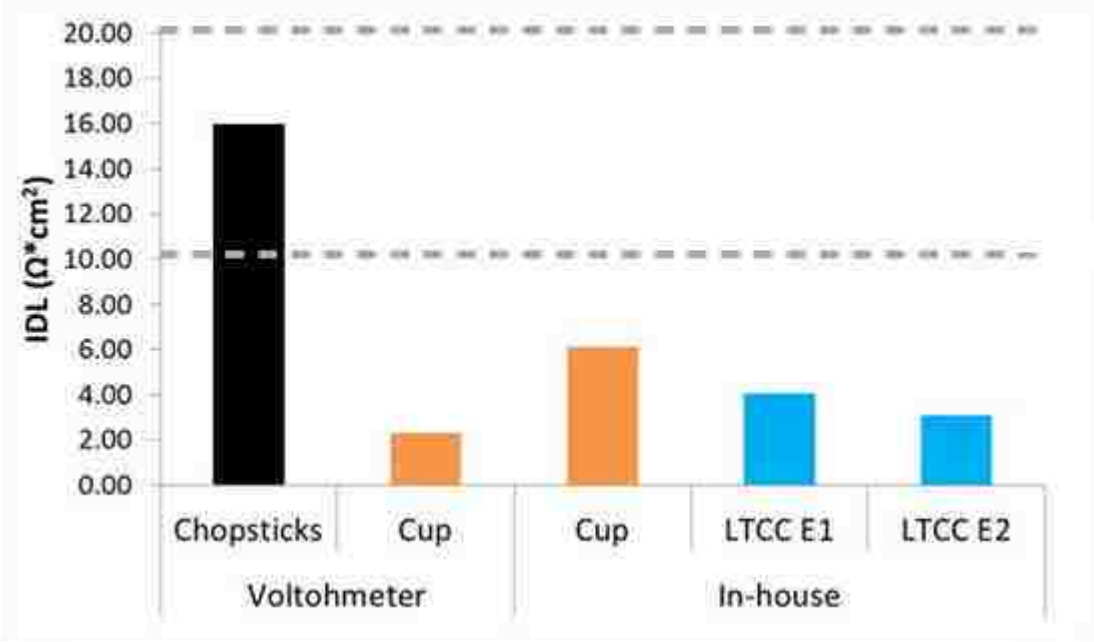

Figure 22: Instrument detection limits and anticipated endothelial cell monolayer values (dotted lines)

sensitive enough to detect the expected impedance values of a HUVEC monolayer, a preliminary study was conducted on two ceramic chips. One chip was constructed with an electrode array of 4mm^2, while the other was 0.05mm^2. The channel of these chips was then filled with media and background impedance measurements were obtained. Afterwards, a circuit was attached to the chips to mimic an EC monolayer and impedance measurements were obtained. Results indicate that by decreasing the size of the electrode, the double layer capacitance caused by the media-electrode interface could be overcome (Figure 23). Figure 23 also illustrates an improvement isolating the contributions of the cell layer from the interfacial effects for the small electrode (B) as opposed to the larger electrode (A). Previous experiments have also shown an improvement in system sensitivity by reducing electrode size and adding in a lock-in amplifier. This resulted in the detection limit of the ceramic device to drop to 0.198 Ω*cm^2 (see Figure 24). This allows for effective determination of minimal changes (~1-2% of a HUVEC monolayer, 10-20 Ω*cm^2)[78-80] in the permeability of the EC monolayer being analyzed within the device.

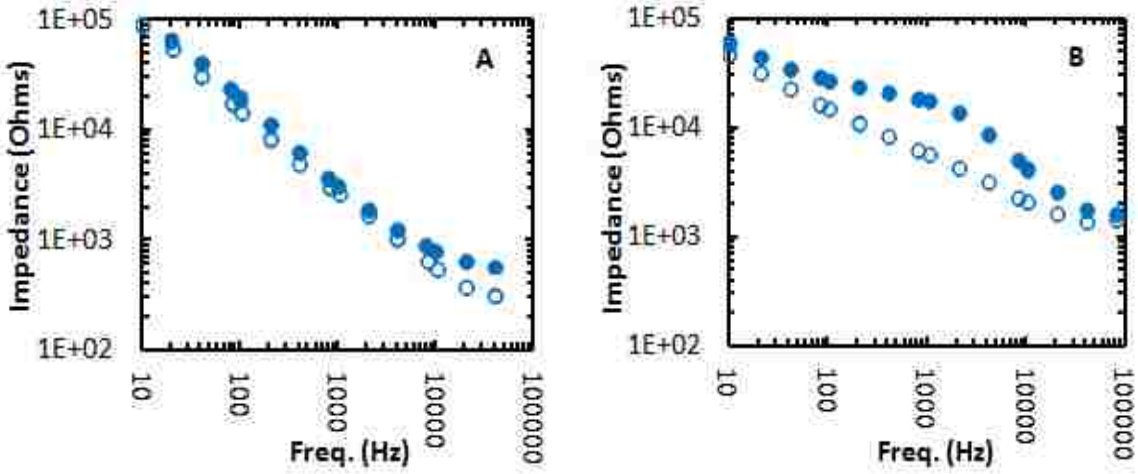

Figure 23: Graphs showing impedance values of a ceramic device with a 4mm^2(A) and 0.05mm^2(B) electrode array exposed to media (open dots) and a parallel resistor and capacitor to mimic a cell monolayer (closed dots) at varying frequencies

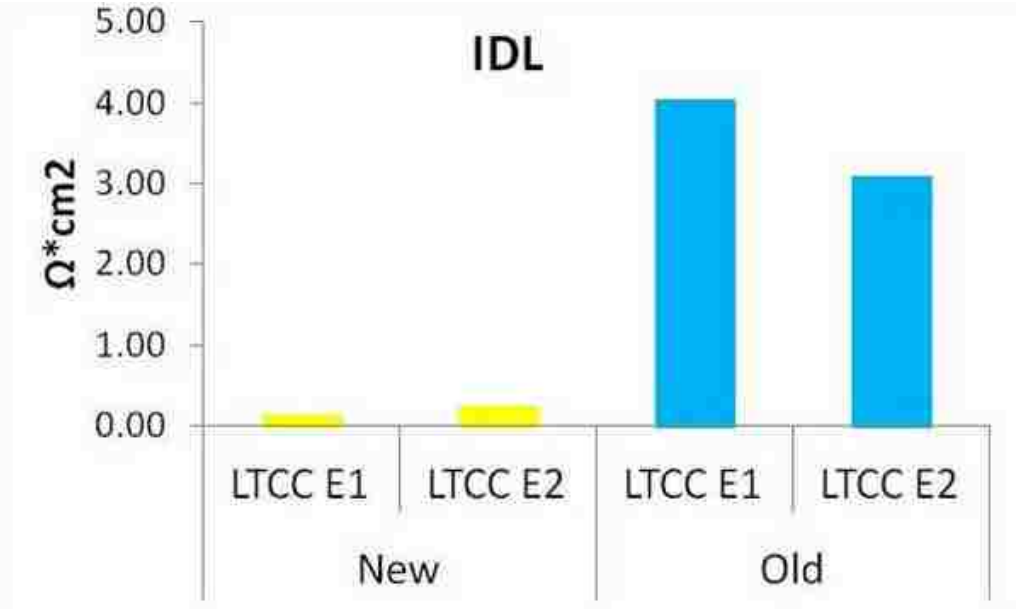

Figure 24: Instrument detection limits with lock-in amplifier and LTCC device with smaller electrode array (New) and without (Old)

C. DISCUSSION

The results from the HL2000 biocompatibility studies indicate a poor initial attachment of endothelial cells to Blank LTCC surfaces followed by growth of endothelial cells once initially attached. These experiments also show attachment and growth of endothelial cells on top of conductive pastes used by LTCC devices. The minimal differences in biocompatibility between “leached” and “unleached” LTCC materials suggest leachates do not deter HUVEC proliferation and growth on LTCC materials. However, HUVECs are shown to spread easier along the surface of these LTCC materials after a 24 hour soak period in DI water was employed. These experiments have also shown the performance of an LTCC TEER-based device. This device expands the application of TEER measurements into a microfluidic platform, with improved detection limits compared to static cell culture well experiments. HUVECs were also shown to attach and grow confluently within the channel of an LTCC-based device. Improvements have also been made and shown to aid in the detection of TEER response within the device.

CHAPTER 5

CONCLUSION

This research has introduced a possible new approach to better define and detect SIRS/sepsis. Initial results show a rapid, significant difference between healthy and extremely septic TEER values in the rat CLP model. Following the analysis of varying SIRS-rated serums, a more delayed response in TEER values was found. Even though this large static model was unable to display a statistical difference amongst the different SIRS-rated serums, measurements can be improved and optimized through electrode geometry, cell type, and environment in which the cells are analyzed. The mechanisms in which endothelial cell junctions are disrupted due to SIRS/sepsis is quite complex and poorly understood today. There is still a need for a better definition and early detection of this disease. The ELISA assays displayed an insight into the TEER response and further indicates the complexity of this disease. Research also looked into the use of low temperature co-fired ceramics as a potential microfluidic base that can incorporate 3-D microstructures and electrical applications. This research shows biocompatibility of endothelial cells on LTCC materials as well as improved measurements for impedance testing. Therefore, a foundation for cell based “in-vitro” devices using LTCC materials has been established. Due to the nature in which endothelial cells are affected by SIRS/sepsis, an LTCC TEER-based microfluidic device has the potential to detect varying degrees of SIRS and aid in a more effective treatment strategy for patients thereby potentially saving lives.

RECOMMENDATIONS

This research does not analyze cells in their natural environment. Therefore, the construction of a microfluidic device is recommended; incorporating an array of confluent EC-based sensors that monitor electrical impedance changes would be the most beneficial and practical approach to expanding into the biomedical industry. Generating such a device will allow for testing of the endothelial cells in a more natural fluidic environment. The incorporation of a smaller electrode in such a device will produce larger impedance values while analyzing a smaller group of cells. A greater sensitivity will be achieved when compared to the static studies displayed in this research. Microfluidic technology will enable continuous aseptic testing of EC permeability with the capability of automated results.

Furthermore, research has shown that different cell lines within the endothelium use different pathways to respond to inflammation.[13] These cells also contain different amounts of protein complexes within the TJs and AJs. [80-87] Therefore, the impedance responses of these cells will differ amongst the varying inflammatory agents associated with SIRS/sepsis. It is recommended that future research looks into the impedance effects of different endothelial cell lines to the inflammatory agents associated with SIRS/sepsis to determine the most effective cell line for use in the impedance device.

APPENDICES

Appendix A: Abbreviations

Systemic inflammatory response syndrome ……(SIRS)
Endothelial cell ……(EC)
Low temperature co-fired ceramic ……...(LTCC)
Adheren junction ……(AJ)
Tight junction ……...(TJ)
Junction associated molecule ……...(JAM)
Zonula occludens ……..(ZO)
Vascular endothelial ……..(VE)
E-Twenty Six ……(ETS)
Human dermal microvascular endothelial cell ……(HDMEC)
Human umbilical vein endothelial cell ……(HUVEC)
Vascular endothelial growth factor ……...(VEGF)
Tumor necrosis factor ……(TNF)
Interleukin ……..(IL)
Multiple organ dysfunction syndrome ……..(MODS)
Cecal litigation and puncture ……(CLP)
Acute physiology and chronic health evaluation ……(APACHE)
Procalcitonin ……(PCT)
C-Reactive Protein ……(CRP)
Sequential organ failure assessment ……(SOFA)
Polydimethylsiloxane……(PDMS)
Fluorescein isothiocyanate conjugated……(FITC)
Transendothelial electrical resistance……(TEER)
Electrical cell substrate impedance spectroscopy……(ECIS)
Electrochemical impedance spectroscopy……(EIS)
Ion sensitive electrode……..(ISE)
Dulbecco's phosphate-buffered saline……...(DPBS)
Granulocyte/macrophage colony stimulating factor……(GM-CSF)

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