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TITLE: Mechanism of Systemic Inflammation-Associated Endothelial and Epithelial Cell Dysfunction Following Acute Pancreatitis, Trauma, and Burns

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Fort Detrick, Maryland 21702-5012

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UNIVERSITY OF PITTSBURGH, THE
3520 FIFTH AVENUE
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14. ABSTRACT

Systemic inflammatory response syndrome (SIRS) is difficult to study in humans because the response to injury in humans is heterogeneous, development of SIRS is unpredictable and progression to vascular leak syndrome (VLS) is highly variable, suggesting multiple hidden variables between people. Obesity and hypertriglyceridemia (HTG) increase risk of multi-organ dysfunction syndrome (MODS), giving some clues to pathophysiology, but these factors account for a minority of variability. Endothelial cell injury and VLS [leading to intravascular hypovolemia and shock], appear to link SIRS to MODS, which is prolonged by gut leak syndrome (GLS). We show that "toxic serum" affects the endothelial cells by at least three specific mechanisms: free fatty acids (FFAs), cytokines, and high molecular weight proteins. We are investigating if similar mechanisms lead to epithelial cell injury. **We hypothesize** that one or more mechanism(s) may be the major contributor(s) to VLS in *individual* patients, and that parallel processes cause gut permeability (allowing bacterial products to enter the circulation) and that **these mechanisms are targetable.**

15. SUBJECT TERMS
Pancreatitis, systemic, inflammation, vascular leak, multiple organ dysfunction, hypertriglyceridemia, biomarkers, endothelium, epithelium, viability

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	20	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The **problem** being addressed is the unknown mechanism(s) in patients with multiple trauma, severe burn, sepsis or acute pancreatitis (AP) responsible for the variable, unpredictable progression from **tissue injury** to **systemic inflammation** to the **vascular leak syndrome** (VLS) and **gut leak syndrome** (GLS) which in turn leads to **multi-organ dysfunction syndrome** (MODS), major morbidities and risk of death. The primary observation is that serum from patients suffering from severe acute pancreatitis induced by various etiologies or trauma decreases cellular viability of endothelial and epithelial cells in culture.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Pancreatitis, systemic, inflammation, vascular leak, multiple organ dysfunction, hypertriglyceridemia, biomarkers, endothelium, epithelium, viability

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Aim 1. Determine the mechanism of *endothelial cell* dysfunction caused by circulating factors in patients with persistent SIRS and VLS. (months 4-36)

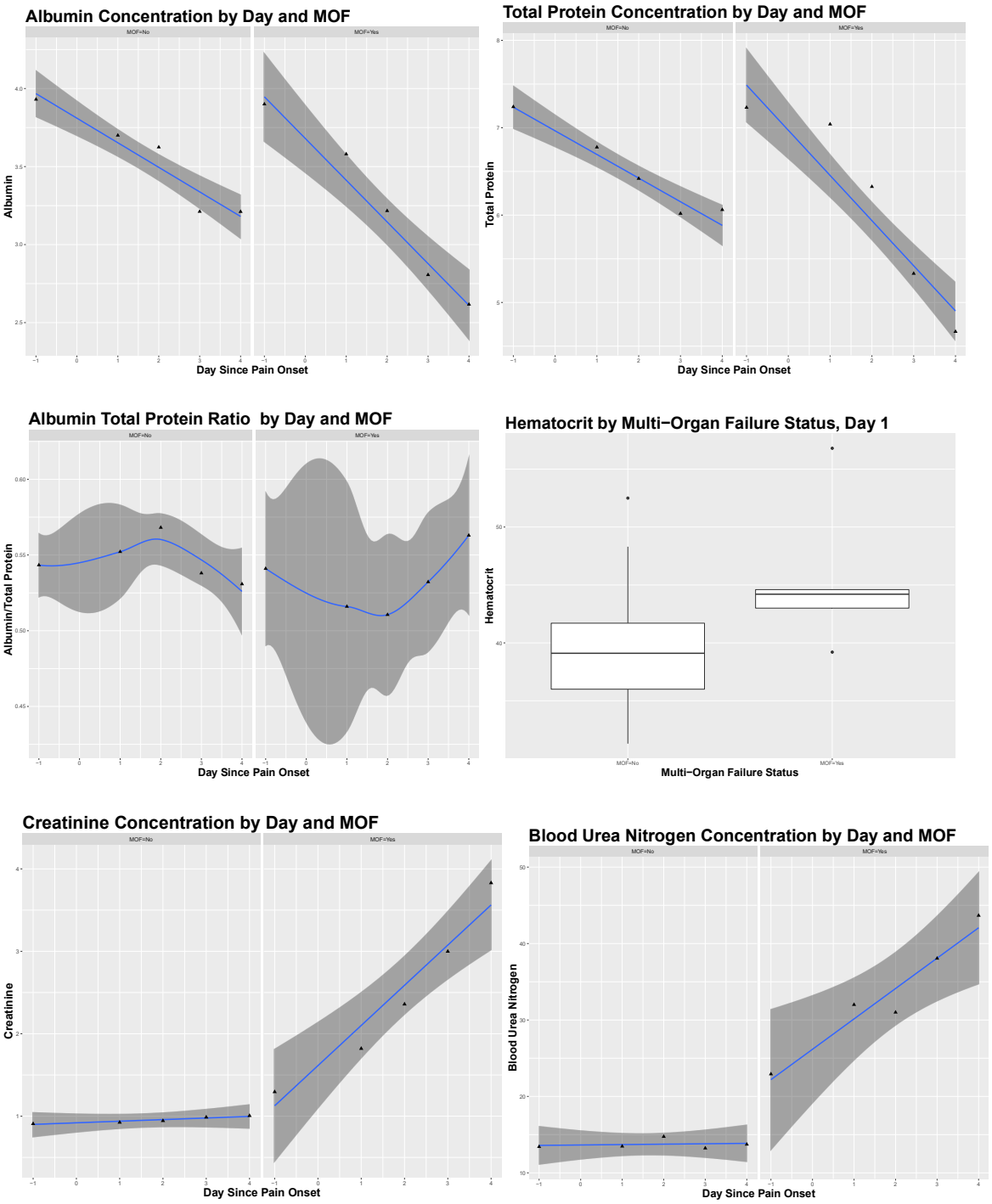
Aim 2. Determine the mechanism of *epithelial cell* dysfunction caused by circulating factors in patients with persistent SIRS and with/without VLS. (months 4-36)

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

An important specific objective under both **Aims 1 and 2** is to recruit patients into this study. The protocol was approved as Minimal Risk by University of Pittsburgh HRPO December 15, 2017. Approval was received from USAMRMC HRPO January 18, 2018. The study was approved for the enrollment of 61 subjects. Seven subjects were enrolled the third year of the study. The information from case report forms and pertinent demographics, physiological data and medical information related to disease were entered into a secure database using Research Electronic Database Capture (REDCap).

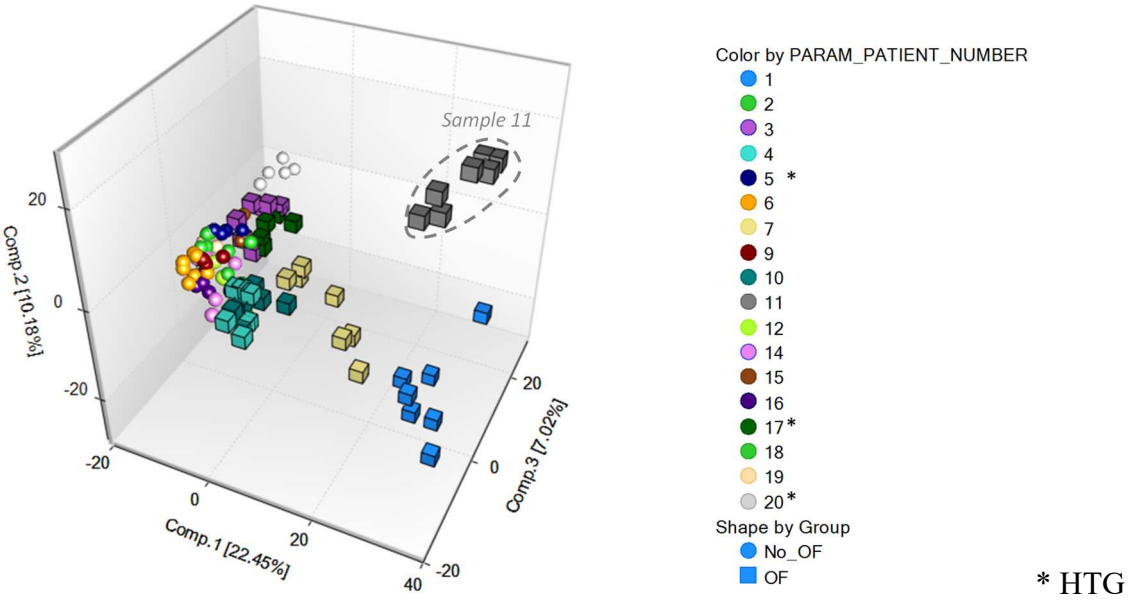
A specific objective under **Aims 1 and 2** is also to isolate and characterize human organ-specific endothelial and epithelial cells for use in this project. This will be an important ongoing process throughout the project in order to meet the goals of this study. Both human intestinal vascular endothelial cells and human dermal microvascular endothelial cells are utilized in experiments along with colonic epithelial cell lines Caco-2 and HT-29.

One of the underlying mechanisms implicated in pathogenesis of severe AP is the disturbance of systemic microcirculation leading to endothelial cell dysfunction, capillaries that leak plasma proteins and the vascular leak syndrome (VLS). Loss of plasma proteins diminishes the colloid osmotic gradient in the post-capillary venules and inadequate reabsorption of fluid from the tissues. Failure to reabsorb tissue fluid, and abnormal pooling of fluid outside of the vascular and normal interstitial fluid compartments (e.g. third space) results in significant loss of intravascular fluid, cardiovascular volume depletion, hemoconcentration, hypotension and acute injuries to kidney (pre-renal azotemia) and pulmonary edema and hypovolemic shock. Albumin, hematocrit, and blood urea nitrogen (BUN) have been studied as biomarkers that are associated with predicting persistent organ failure in AP. Albumin is a negatively charged plasma protein that is synthesized in the liver and excreted into the bloodstream. It is a flexible, ellipsoid-shaped molecule with a molecular weight of 66.5 kDa and a diameter of 3.8 nm by 15 nm, typically modeled as a diameter of ~7 nm. Albumin is a significant contributor to total serum proteins and represents a protein of intermediate size that is above the typical peri-endothelial cell filtration cut-off size in non-sinusoidal non-fenestrated blood capillaries of ~5 nm as seen in skin, muscle, adipose tissue, intestinal mesentery and lung. About 30-40% of albumin is found in the intravascular compartment, while the remaining albumin is distributed in the extravascular compartment and returns to circulation via the lymphatics system. Much of the albumin in interstitial spaces is transferred directly through the endothelial cells by a transcellular pathway by caveolae via an absorptive (receptor-mediated) or fluid-phase pathways that are also highly regulated. But the concentration of albumin and other proteins in the plasma exceeds the concentration of these proteins the interstitial space to maintain the colloid osmotic gradient. BUN and hematocrit are common clinical laboratory analyses that are used to assess kidney function and can be used to estimate intravascular volume depletion with a disproportional rise in BUN (i.e. prerenal azotemia). The dynamic changes in levels of serum albumin, total protein (TP), albumin/TP ratio (A/TP), BUN, creatinine, and hematocrit will be useful as biomarkers of vascular biology in AP and provide further insights into the pathogenesis of MODS. The specific laboratory values that were collected for comparison included hematocrit, hemoglobin, albumin, TP, creatinine (Cr), and BUN. Lab values were collected from baseline, admission, 24, 48, 72 and 96 hours after admission. A total of 54 subjects enrolled in the DOD and Pancreatitis-associated Risk Of Organ Failure (PROOF) studies met inclusion criteria with a minimum of pre-admission, day of pain and 24h after pain onset of pain values, and preferably continuing until 96 hours of pain onset. Median age of our studied cohort was 57 and female to male ratio of 1.2. Three main etiologies were biliary (54%), idiopathic (7%), and post-ERCP pancreatitis (7%). Seventeen subjects developed multiorgan failure (MOF), 8 had single OF, and 17 subjects did not develop any OF.



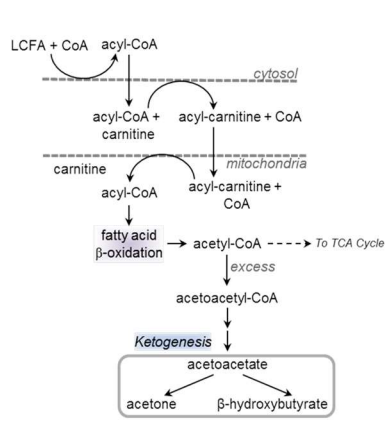
There is a steeper decline of albumin ($p<0.01$) and total protein ($p<0.001$) in MOF patients with AP compared to the non-multi-organ failure (NOF) subset. There was no significant difference between the albumin/total protein ratio, however there was a distinct trend in the MOF subset with a steep decline within the first 24 hours. The MOF patients had a higher admission hematocrit ($p<0.05$). The MOF patients had a sharper increase in BUN ($p<0.001$) and creatinine ($p<0.001$) than NOF. The BUN/creatinine ratio did not show a significant difference between MOF and NOF patients.

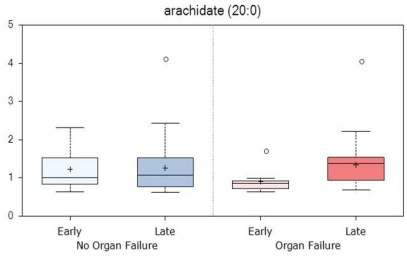
Biochemical profiling of serum from subjects with and without OF, were assessed by LC-MS/MS. A mixed model ANOVA was used to identify metabolites that differed significantly between experimental groups. There were 912 named biochemicals in this set of 99 human serum samples from 20 patients collected at early and late time points. At a $p<0.05$, 46 significant differences can be expected from random chance. For the comparisons involving organ failure (OF) and hypertriglyceridemia (HTG), the number of significant differences was above this level (292 and 106, respectively), suggesting distinct metabolic profiles. Sub-grouping the OF samples into Early (Days 1 and 2) and Late (Days 3 and beyond) also give a large number of differences related to both time and OF status (>250 for all comparisons). There is one patient (Subject_ID = 11) that was deemed a potential statistical outlier, and statistics were performed both with and without these samples. Principal components analysis (PCA) of all metabolites was performed comparing the patients with and without organ failure. PCA is a method used to transform a large number of variables into a smaller number of components, thereby providing a high-level overview of differences within the dataset. Samples from the same patient are predicted to be more similar to each other than to the other samples (due to high inter-subject variation in human studies). Overlap by PCA does not rule out that specific biochemicals or pathways are different between the groups. We therefore are analyzing the metabolites by super pathways to tease out differences between the groups.

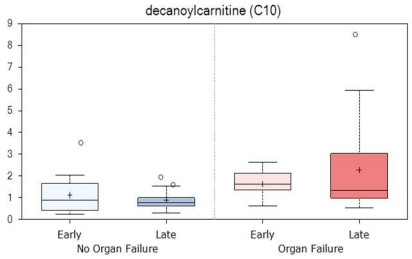


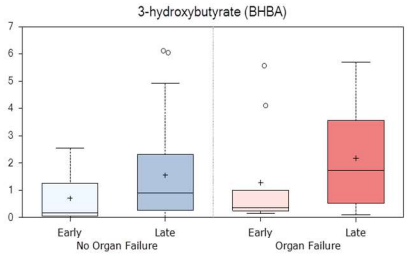
* HTG

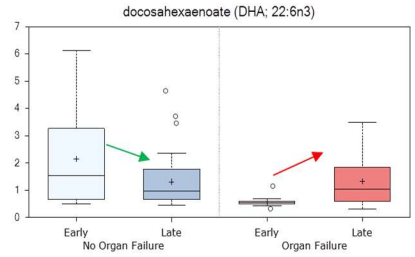
Those patients who exhibited OF had significantly higher levels of medium chain, long chain, polyunsaturated, and branched chain fatty, as well as acyl carnitines at later time points. In patients that did not progress to OF, the levels of many of these classes of fatty acids were lower. Additionally, **acetoacetate** and **BHBA**, ketone bodies that act as markers of lipid β-oxidation, were elevated at the later times in both groups. These findings are consistent with higher levels of β-oxidation occurring as time progresses, with a markedly higher level of lipolysis occurring in the patients that progressed to organ failure. There are also a number of fatty acid dicarboxylates that become elevated in the later time points in the OF group. These compounds are generated during ω-oxidation, and can be further subject to β-oxidation in the mitochondria. The absence of free fatty acid and acyl carnitine changes in the No OF group is consistent with a more moderate increase in that group.

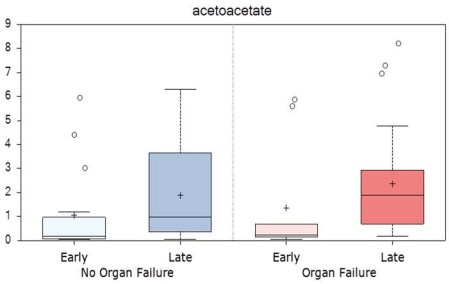












The Image-it DEAD Green Viability assay (Molecular Probes) was performed following exposure of human dermal microvascular endothelial cells (HMEC-1) and human colonic epithelial cells (HT-29) to serum from a patient with severe acute pancreatitis and hypertriglyceridemia for 24 hours. The XTT Cell Viability assay (Biotium) was also performed. The XTT assay measures cell viability by measuring mitochondrial enzyme activity in live cells. The XTT cell viability showed a 51% decrease in HMEC-1 viability and an 18% decrease in HT-29 cell viability. The DEAD Green assay measures cytotoxicity by the uptake of the green dye that is otherwise impermeable to cells.

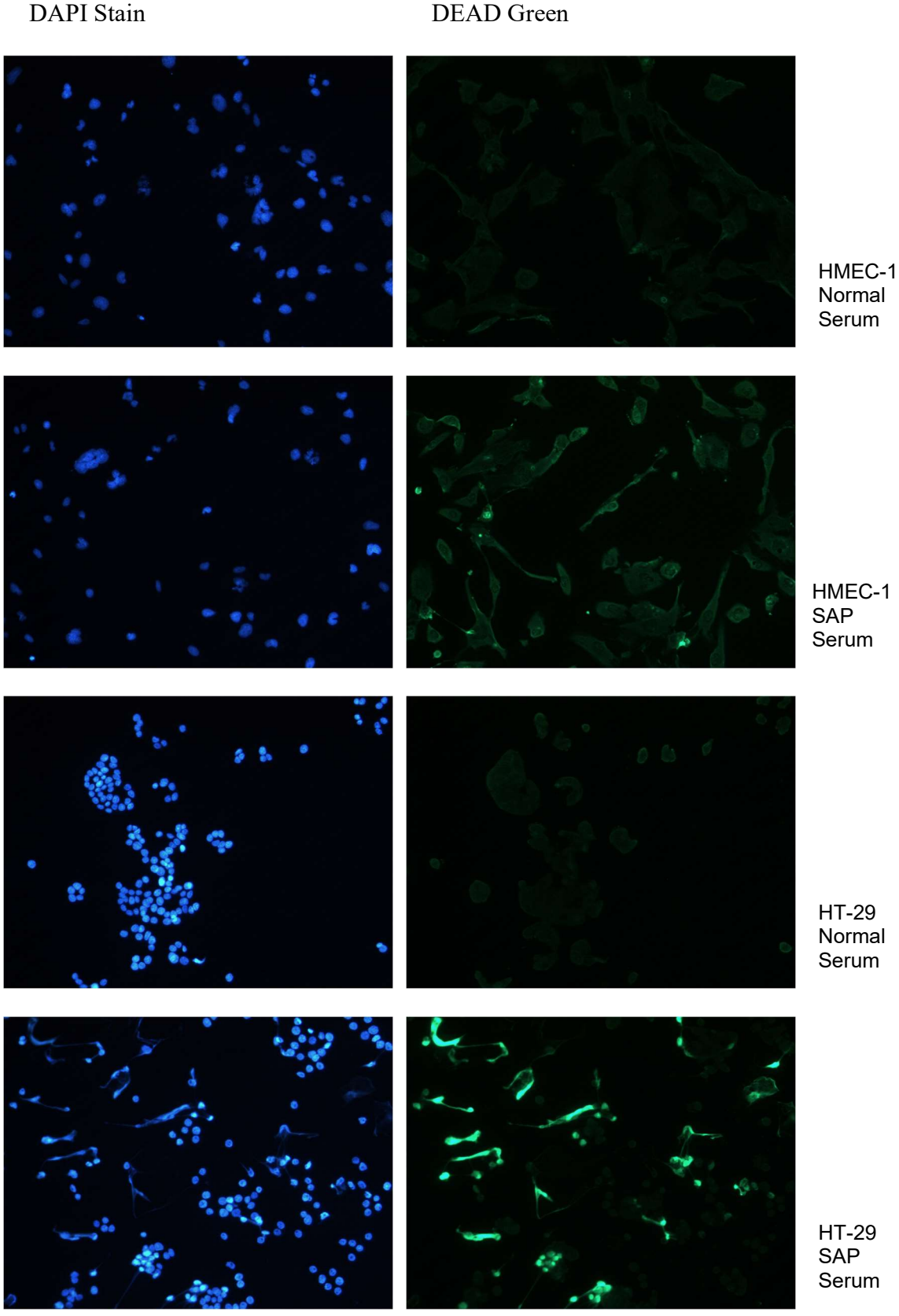

DAPI Stain
DEAD Green
HMEC-1
Normal
Serum
HMEC-1
SAP
Serum
HT-29
Normal
Serum
HT-29
SAP
Serum

Table 1. Demographic Data

DOD ID	Age	Sex	Etiology	Severity
DOD033	82	F	Gallstone	Severe
DOD034	43	F	Ethanol	Mild
DOD035	45	M	HTG	Severe
DOD036	33	F	Gallstone	Moderate
DOD037	80	F		Mild
DOD039	62	F		Mild
DOD040	55	M		Mild
DOD041	38	M		Mild
DOD042	45	M		Moderate
DOD043	37	M	Biliary	Severe
DOD044	32	F	IRAP	Mild
DOD045	63	M	Biliary	Severe
DOD046	53	M	Biliary	Severe
DOD047	51	F	IRAP	Severe
DOD048	69	M	IRAP	Mild
DOD049	36	F	IRAP	Mild
DOD050	65	M	HTG RAP	Severe
DOD051	46	M	HTG-Ethanol	Severe
DOD052	40	F	HTG RAP	Severe
DOD053	70	F	Biliary	Moderate

IRAP=idiopathic recurrent acute pancreatitis, HTG=hypertriglyceridemia

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We plan to continue enrolling subjects into this project. In the meantime, we will continue to test potential compounds of interest such as fatty acids, cytokines, Ang 2, LDH, HMGB1 – to determine if they are cytotoxic to the isolated human intestinal vascular endothelial cells, human dermal microvascular endothelial cells, and colonic epithelial cell lines. Due to the shut down of labs during pandemic experiments have been delayed. We are now up and functioning. We will begin testing the serum samples on the different cell types now that we are reaching the volumes required for high throughput analyses.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Nothing to report.

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

The pandemic required us to stop all research procedures for 3 months (March through June). Because of this we filed a no cost extension and will complete the project by the end of 2021.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use of biohazards and/or select agents

Not applicable.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  1. Komara NL, Paragomi P, Greer PJ, Wilson AS, Breze C, Papachristou GI, Whitcomb DC. Severe acute pancreatitis: Capillary permeability model linking systemic inflammation to multiorgan failure. Am J Physiol Gastrointest Liver Physiol. 2020 Sep 2. doi: 10.1152/ajpgi.00285.2020. Epub ahead of print. PMID: 32877220.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Manuscript in Process:
1. Severe Acute Pancreatitis: Multi-organ failure is associated with elevated proteolysis products in serum and endothelial cell toxicity; Tang X[1], Paragomi P[1], Wilson AS[1], Phillips AE[1], Castaneda JC[1], Hall K[1], O'Keefe SJ[1], Papachristou GI1 and Whitcomb DC[1-3]

1. Division of Gastroenterology, Hepatology and Nutrition, Department of Medicine, University of Pittsburgh, Pittsburgh PA 2. Departments of Cell Biology and Molecular Physiology, University of Pittsburgh, Pittsburgh, PA 3. Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA.

DDW 2020: Biochemical and Protein Profiling to Identify Potential Markers of Progression from Acute Pancreatitis with Systemic Inflammatory Response Syndrome to Organ Failure; Annette Wilson, Juan Castaneda, Pedram Paragomi, Georgios Papachristou, David Whitcomb

- **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance

progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: David C. Whitcomb, MD
Project Role: PI
Nearest person month(s) worked: 2 months
Contribution to Project: Dr. Whitcomb oversaw all research in this project. Weekly research meetings were held to disseminate progress. In addition, interviewed candidates for the Nurse Research Coordinator position.

Name: David G. Binion, MD
Project Role: Co-Investigator
Nearest person month(s) worked: 2 months
Contribution to Project: Dr. Binion provided assistance with experiments in this project and participates in research meetings.

Name: Lansing Taylor, PhD
Project Role: Co-Investigator
Nearest person month(s) worked: 1 month
Contribution to Project: Dr. Taylor is Director of the Drug Discovery Institute.

Name: Mark Shurdak, MD
Project Role: Co-Investigator
Nearest person month(s) worked: 1 month
Contribution to Project: Dr. Shurdak coordinates the high throughput assays in the Drug Discovery Institute.

Name: Annette S. Wilson, PhD
Project Role: Laboratory Manager
Nearest person month(s) worked: 6 months
Contribution to Project: Dr. Wilson coordinated the experiments and performed imaging, chromatography, ELISA assays, and data analysis. She participates in the weekly research meetings. In addition, Dr. Wilson assisted Dr. Whitcomb with writing the IRB renewal application.

Name: Tong Ying Shun, PhD
Project Role: Statistician
Nearest person month(s) worked: 1 month
Contribution to Project: Provides statistical assistance at Drug Discovery Institute.

Name: Harold Takya, PhD
Project Role: Information Systems Manger
Nearest person month(s) worked: 1 month
Contribution to Project: Oversees database at Drug Discovery Institute.

Name: Shari Reynolds
Project Role: Clinical Research Coordinator
Nearest person month(s) worked: 6 months
Contribution to Project: Ms. Reynolds has consented patients currently in the study. She has transported the blood samples to the research lab and assisted in processing, aliquotting, and storing samples. She attends the weekly research meetings.

Name: Seia Comsa
Project Role: Assay Implementation Specialist
Nearest person month(s) worked: 12 months
Contribution to Project: Mrs. Comsa was responsible for cell culture propagation.

Name: Kristen Hall
Project Role: Research Technician
Nearest person month(s) worked: 12 months
Contribution to Project: Ms. Hall was responsible for performing experiments and data compilation.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.