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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

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1. INTRODUCTION:

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:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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?

Aim 1. Determine the mechanism of *endothelial cell* dysfunction caused by circulating factors in patients with persistent SIRS and VLS.

Aim 2. Determine the mechanism of *epithelial cell* dysfunction caused by circulating factors in patients with persistent SIRS and VLS.

1. Patient Enrollment

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. 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).

Table 1 - Severity categories following the Revision of Atlanta Classification[3].

Acute pancreatitis severity	Organ failure and local or systemic complications
Mild acute pancreatitis	- No organ failure - No local or systemic complications
Moderately severe acute pancreatitis	- Transient organ failure (resolves in 48 hours) Local or systemic complications without persistent organ failure
Severe acute pancreatitis	- Persistent organ failure (single or multiple)

A total of 26 subjects were enrolled into this study. The COVID-19 pandemic impacted our ability to recruit the target of 36 new patients. Due to this phenomena we also included patient samples from our previous DOD study (W81XWH-14-1-0376). The goal was to enroll subjects with acute pancreatitis demonstrating systemic inflammation response syndrome (SIRS) putting the patient into a high-risk category. The subjects were further split into groups of SIRS with and without organ failure. Of the 26 subjects enrolled, 11 demonstrated severe, 4 moderate, and 11 mild acute pancreatitis (AP) according to the severity categories in Table 1. Of the 26 subjects, 15 exhibited organ failure - 4 transient and 11 persistent organ failure (Table 2). Of the 15 subjects demonstrating organ failure, 2 exhibited (multi-organ dysfunction syndrome) MODS and 13 exhibited single organ failure. Primary organs that failed were respiratory, renal, and cardiovascular. All of the patients with severe acute pancreatitis demonstrated vascular leak syndrome as indicated by edema, low serum total protein and albumin levels, and high levels of hemoglobin and hematocrit (as described below).

2. Serum Sample Characterization

Plasma Albumin/Plasma Proteins

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

Table 2. Demographic data

DOD ID	Age	Sex	BMI	Etiology	Severity
DOD032	27	M	42.6	HTG	Mild
DOD033	82	F	45.3	Gallstone	Severe
DOD034	43	F	34.1	Alcohol & hyperlipidemia	Mild
DOD035	45	M	26.8	Alcohol & HTG	Severe
DOD036	33	F	51.5	Gallstone	Moderate
DOD037	80	F	33.2	Alcohol	Mild
DOD039	62	F	23.8	Gallstone	Mild
DOD040	55	M	33	Alcohol	Mild
DOD041	38	M	32.1	Gallstone	Mild
DOD042	45	M	30	Idiopathic	Moderate
DOD043	37	M	26.4	Biliary	Severe
DOD044	32	F	25.9	IRAP	Mild
DOD045	63	M	31.7	Biliary	Severe
DOD046	53	M	18.3	Biliary	Severe
DOD047	51	F	38.7	IRAP-Gallstone	Severe
DOD048	69	M	38.2	IRAP	Mild
DOD049	36	F	44.6	IRAP	Mild
DOD050	65	M	27.2	HTG & RAP	Severe
DOD051	46	M	25.8	HTG & Alcohol	Severe
DOD052	40	F	29.8	HTG & RAP	Severe
DOD053	70	F	37.1	Biliary	Moderate
DOD054	56	M	37.3	Gallstone	Severe
DOD055	41	M	30.1	HTG & Alcohol	Severe
DOD056	48	F	25	HTG	Moderate
DOD057	19	M	35.2	HTG	Mild
DOD058	72	F	29.9	Alcohol	Mild

IRAP=idiopathic recurrent acute pancreatitis, HTG=hypertriglyceridemia

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%). 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 (Figure 1). 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 (Figure 1). 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. Seventeen subjects developed multiorgan failure (MOF), 8 had single OF, and 17 subjects did not develop any OF.

Metabolomics – Fatty Acids

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 (Figure 2). 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. 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 (Figure 3). 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.

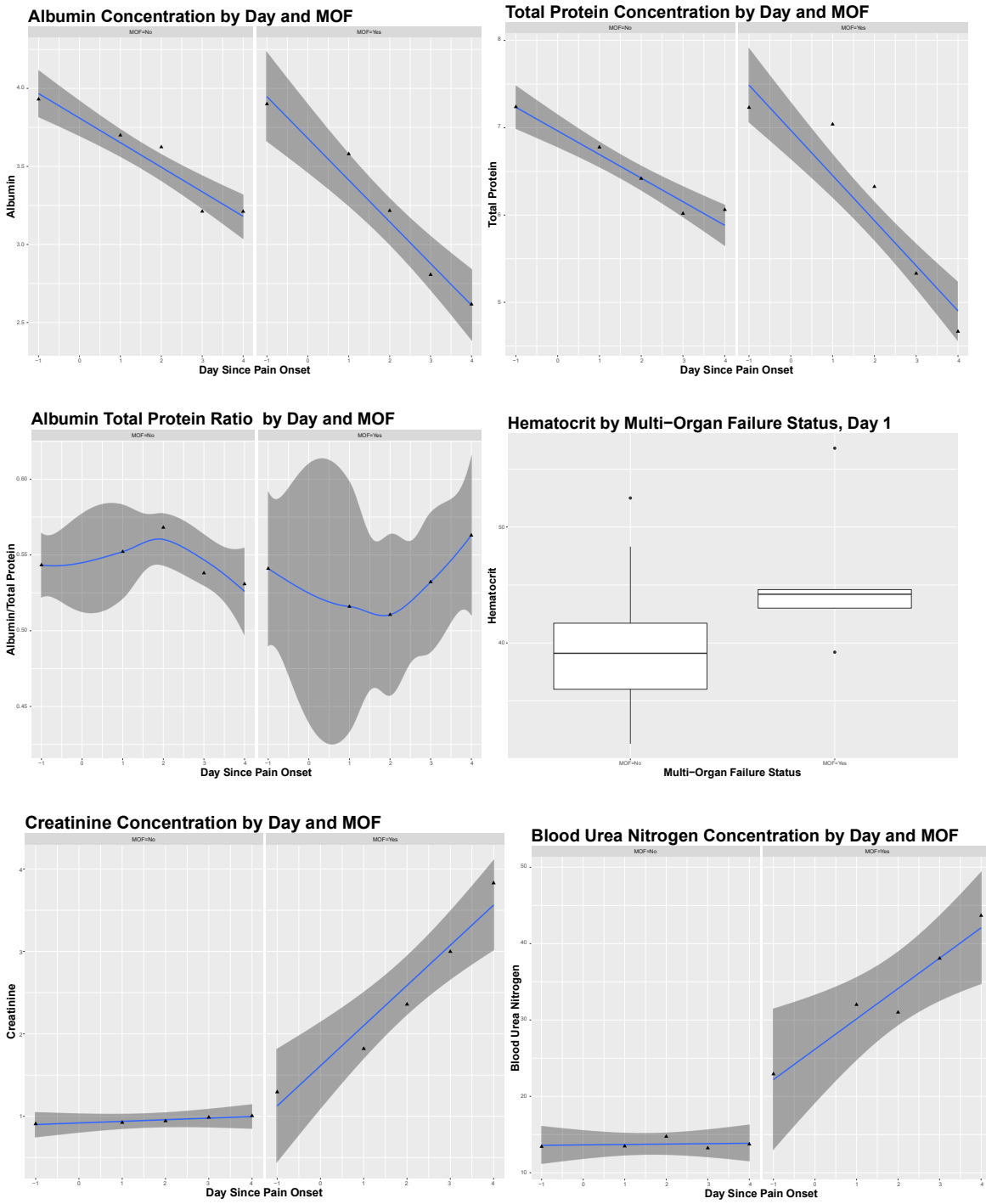
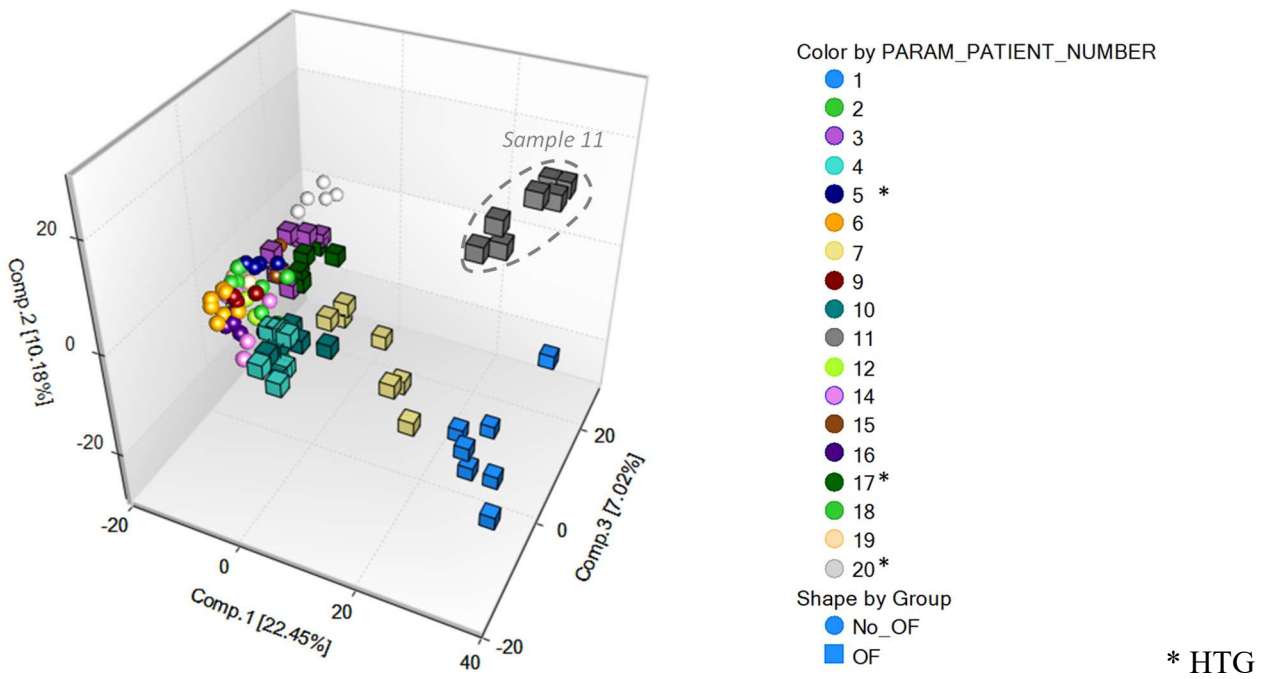

Albumin Concentration by Day and MOF
MOF=No
MOF=Yes
Albumin
Day Since Pain Onset
Total Protein Concentration by Day and MOF
Total Protein
Albumin Total Protein Ratio by Day and MOF
Albumin/Total Protein
Hematocrit by Multi−Organ Failure Status, Day 1
Hematocrit
Multi−Organ Failure Status
Creatinine Concentration by Day and MOF
Creatinine
Blood Urea Nitrogen Concentration by Day and MOF
Blood Urea Nitrogen

Figure 1



* HTG

Figure 2

Other Potential Biomarkers

Free fatty acid levels were analyzed in patient serum samples. In addition, biomarkers of angiogenesis – tyrosine kinase-2 (Tie-2), vascular endothelial growth factor-A (VEGF-A), basic fibroblast growth factor (bFGF), and fms-like tyrosine kinase-1 (sFlt-1) were analyzed. Angiogenesis is essential to the process of neovascularization under both physiological and pathological conditions including cancer, diabetic retinopathy, rheumatoid arthritis, and ischemia. Angiogenesis is modulated by both initiatory and inhibitory molecules. Angiogenic markers are potential prognostic markers for disease activity. Tie-2 is a tyrosine receptor kinase expressed in the endothelium of actively growing blood vessels. Ang-1 and Ang-2 bind to Tie-2; Ang-1 initiates branching of blood vessels and Ang-2 antagonizes this effect. VEGF-A is a potent tumor-secreted cytokine that initiates signal transduction cascades by binding tyrosine receptor kinases expressed on endothelial cells (Flt-1 and Flk-1) and endothelium neurons (neurophilin-1 and neurophilin-2). bFGF is a heparin-binding mitogen that induces signal transduction by binding to tyrosine kinase FGF receptors. It enhances angiogenesis by stimulating endothelial cell proliferation and migration and producing collagenase and plasminogen activator. FGFs play critical roles in adult tissue repair and maintenance. Flt-1, also known as VEGF receptor 1(VEGFR1), is a transmembrane tyrosine receptor kinase that is highly expressed in endothelial cells. VEGF/Flt-1 transduced signaling is required for the recruitment of hematopoietic precursors and endothelial cell progenitors, migration of monocytes and macrophages, and release of growth factors from hepatic endothelial cells. Tie-2 and sFlt-1 were found to be higher in the serum samples of patients with severe (Tie-2: 1,793 +/- 82 pg/mL; sFlt-1: 221 +/- 24.3 pg/mL) and moderate (Tie-2: 1,794 +/- 270 pg/mL; sFlt-1: 113 +/- 15.2 pg/mL) acute pancreatitis in comparison to mild (Tie-2: 1,316 +/- 139 pg/mL; sFlt-1: 55 +/- 4.5 pg/mL) acute pancreatitis. VEGF-A and bFGF were lower in severe acute pancreatitis (VEGF-A: 318 +/- 33.43 pg/mL; bFGF: 3.9 +/- 0.17 pg/mL) than mild (VEGF-A: 1,055 +/- 143 pg/mL; bFGF: 11.43 +/- 2.8 pg/mL).

The mole % free fatty acids are compared between the patients with acute pancreatitis and normal levels reported in the literature in Figure 4. The acute pancreatitis group has a significantly

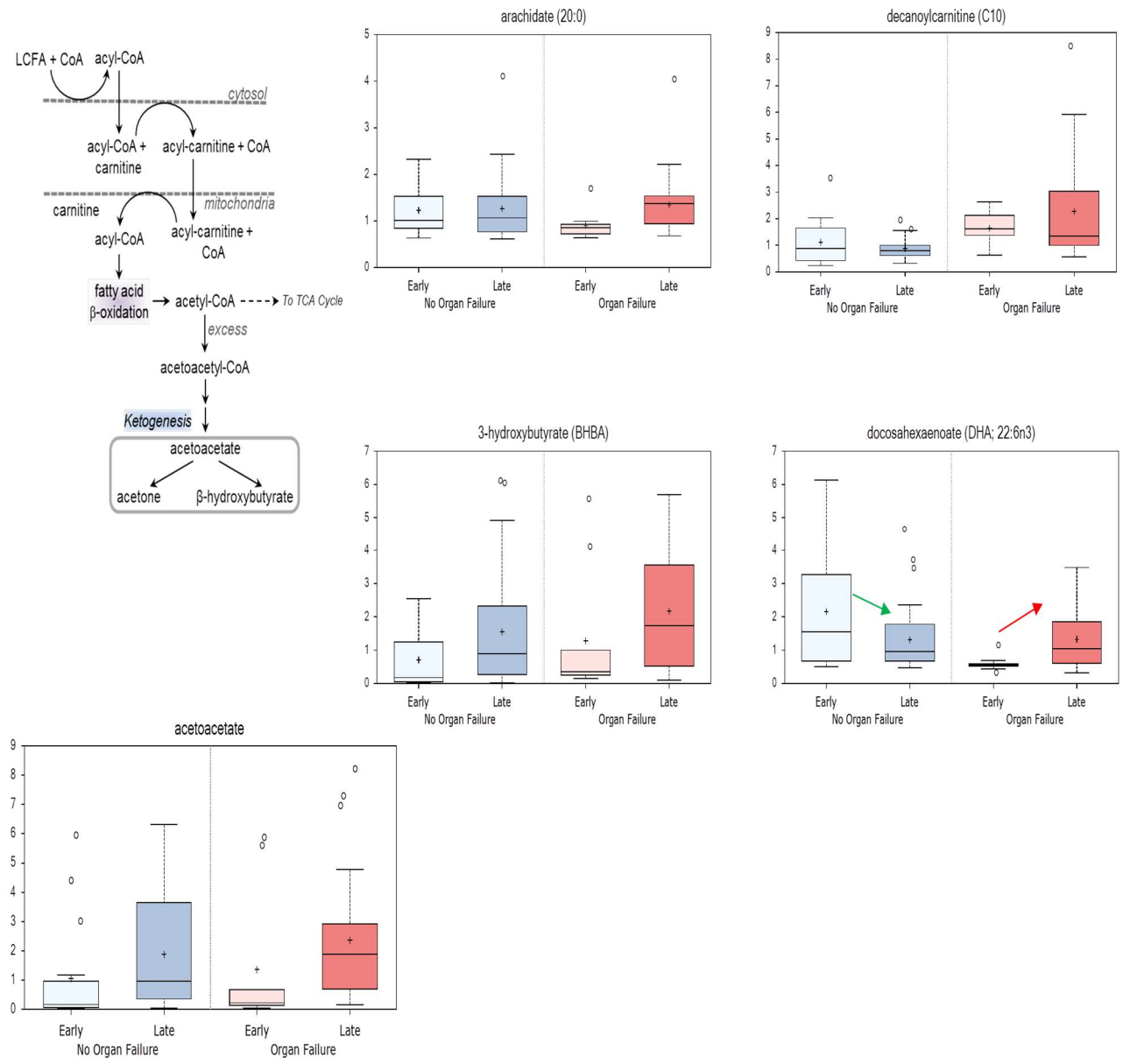


Figure 3

higher composition of palmitic, stearic, and oleic acids while the normal group has a higher composition of linoleic acid. It is interesting that the % composition of total saturated fatty acids (SFA) is higher in the acute pancreatitis group while the unsaturated fatty acids (UFA) are higher in the normal group. The mole % composition of the free fatty acids are compared between mild, moderate, and severe acute pancreatitis in Figure 5. There is no significant difference between the 3 groups for palmitic, palmitoleic, and oleic acids. Severe acute pancreatitis serum has a significantly lower % composition of stearic acid and significantly higher % composition of linoleic acid. The % composition of saturated fatty acids are higher in mild and moderate than severe acute pancreatitis and unsaturated fatty acids are higher in severe than mild and moderate acute pancreatitis.

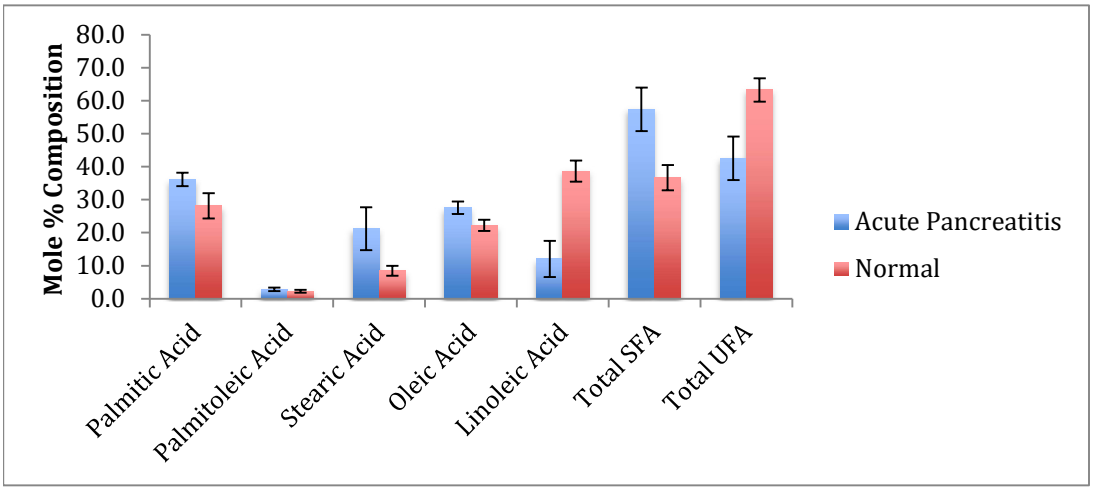


Figure 4

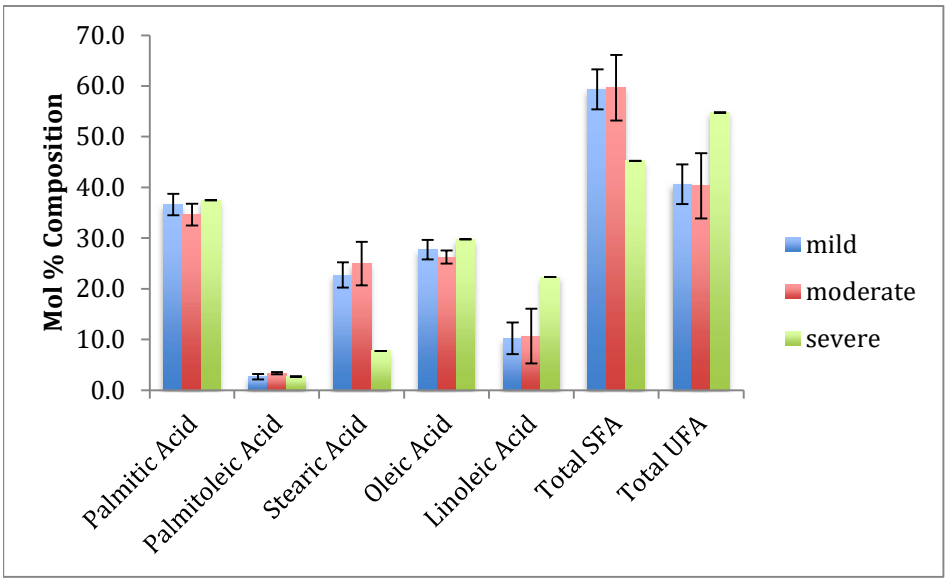


Figure 5

The serum samples were analyzed for candidate toxic factors to determine if they correlate with severity. Protein measurements were performed using the Meso Scale Discovery (MSD) technology. MSD technology enables measurement of biomarker levels using electrochemiluminescense detection. **Angiopoietin 1** and **angiopoietin 2** were measured. Very high mean concentrations of Ang-2 and lower than normal control Ang-1 levels were found in many of the serum samples and appear to correlate to severity (Figures 6 and 7).

Adiponectin, a metabolic biomarker, was also measured to determine if there is any correlation with severity of acute pancreatitis. The level of adiponectin has been reported as an average of 16,700 ng/mL in normal controls. Levels have been shown to decrease with obesity. Interestingly, the average amount of adiponectin is significantly higher in participants with severe acute pancreatitis and not related to BMI as indicated below (Figures 8 and 9).

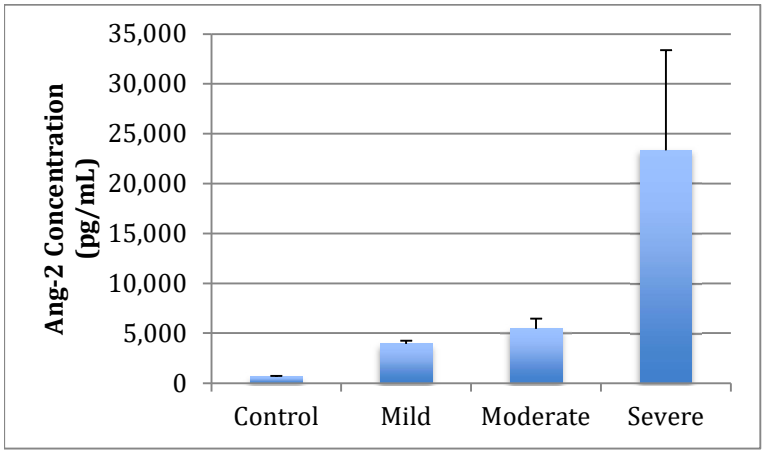


Figure 6

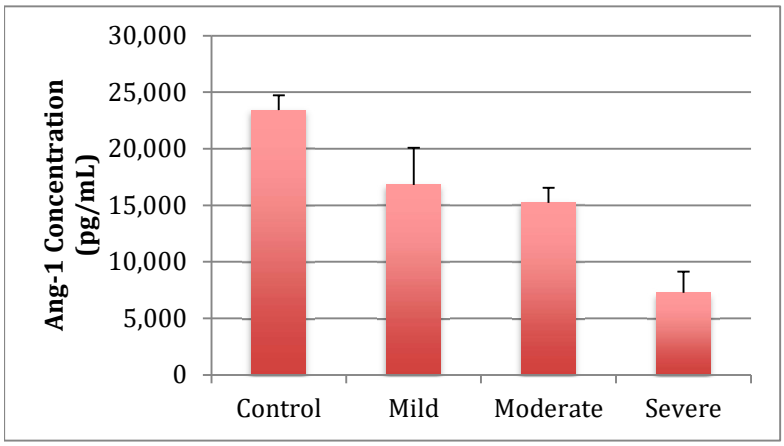


Figure 7

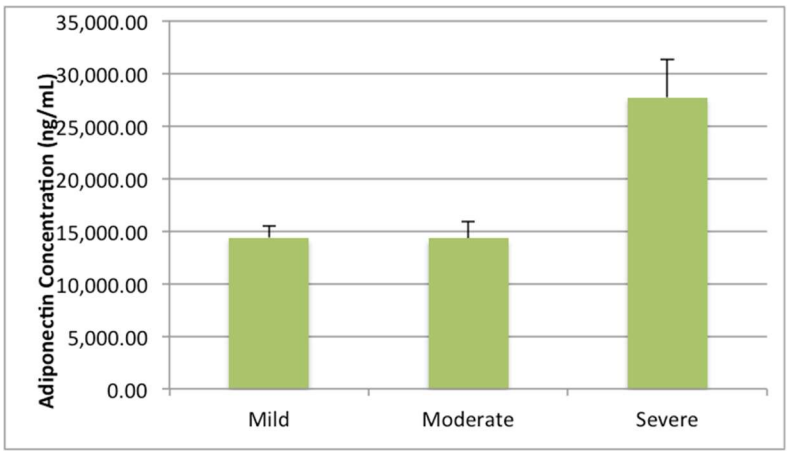


Figure 8

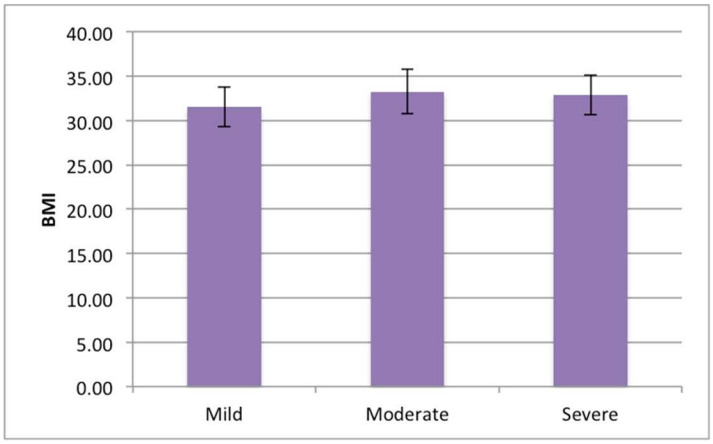


Figure 9

The serum samples were also analyzed for candidate toxic factors to determine if they correlate with severity. **Lactate dehydrogenase (LDH)**, an indicator of tissue damage, was measured by ELISA assay (MyBiosource). Normal adult value range is **140 to 280 IU/L**. **High mobility group protein B1 (HMGB1)**, a nonhistone nucleoprotein and an extracellular inflammatory cytokine, was measured by ELISA (Biomatik). Under conditions of infection, injury and sterile inflammation, HMGB1 can be passively released from damaged cells or actively secreted from activated immune cells. Levels in normal healthy controls has a median of **1.17 ng/mL** (Peltz ED, Moore EE, Eckels PC, Damle SS, Tsuruta Y, Johnson JL, Sauaia A, Silliman CC, Banerjee A, Abraham E. HMGB1 is markedly elevated within 6 hours of mechanical trauma in humans. Shock. 2009 Jul;32(1):17-22. doi: 10.1097/shk.0b013e3181997173. PMID: 19533845; PMCID: PMC4097145). **Citrullinated histone H3 (citH3)** was measured by ELISA (Cayman Chemical). Microbial infection stimulates neutrophil/macrophage/monocyte extracellular trap formation, which leads to the release of CitH3 catalyzed by peptidylarginine deiminase (PAD) 2 and 4. Levels in healthy volunteers has a median of **8 ng/mL** (Thålin C, Lundström S, Seignez C, Daleskog M, Lundström A, Henriksson P, et al. (2018) Citrullinated histone H3 as a novel prognostic blood marker in patients with advanced cancer. PLoS ONE 13(1): e0191231. https://doi.org/10.1371/journal.pone.0191231).

Table 3. Toxic Factors

Biomarker	Median Mild Concentration (IQR)	Median Moderate Concentration (IQR)	Median Severe Concentration (IQR)	P value
LDH (IU/L)	128.2 (128.7)	210.5 (60.8)	126.7 (61.7)	0.100
HMGB1 (ng/mL)	5.12 (3.833)	6.69 (3.915)	6.86 (6.920)	0.284
citH3 (ng/mL)	15.5 (21.85)	22.1 (36.36)	18.80 (31.73)	0.284

IQR, interquartile

The Kruskal-Wallis test was used to evaluate significance of the group medians across severity of acute pancreatitis. There was no significance for any of the toxic factors. However, while the LDH levels fell within normal levels, HMGB1 and citH3 levels were higher in patients with acute pancreatitis than normal healthy controls, 6-fold and almost 3-fold respectively (Table 3).

Chemokines and cytokines were also analyzed. IL-8 levels were significantly higher in mild, moderate and severe acute pancreatitis (1,559 +/- 835, 2,146 +/- 1,514, and 4,172 +/- 1,043 pg/ml) vs normal controls (145 +/- 109 pg/ml). IL-15, IL-17, and IL-5 levels were also higher in acute pancreatitis (27 +/- 16, 33 +/- 51, 4.63 +/- 10.5 pg/ml) vs control (9.9 +/- 1.98, 7.36 +/- 4.69, 1.13 +/- 1.08 pg/ml).

3. Analysis of Cell Toxicity and Death

Additional specific objectives under **Aims 1 and 2** were to study the mechanisms of cell dysfunction in both endothelial and epithelial cells. Both human intestinal vascular endothelial cells and human dermal microvascular endothelial cells were utilized in experiments along with colonic epithelial cell lines Caco-2 and HT-29.

Serum samples from patients with severe pancreatitis, etiology of hypertriglyceridemia, in comparison to healthy human control serum were applied to monolayers of human dermal microvascular endothelial cells and colonic epithelial cell line HT-29 at varying concentrations of

5, 10, and 20% serum added to basal cell culture medium (MCDB 131). The cells were seeded from freshly thawed vials onto collagen coated wells. The cell monolayers were treated with the serum samples for 24, 48, and 72 hours incubated at 37˚C, 5% CO_2. Three different assays were used to assess cell viability – (1) Molecular Probes Live/Dead Viability/Toxicity Kit, (2) Image-it DEAD Green Viability assay (Molecular Probes), and (3) Molecular Probes Propidium Iodide Assay. These assays measure necrotic cell death.

(1) **Live/Dead Viability/Toxicity Assay** – green cells are viable cells that have converted calcein AM green with esterase activity; red cells are non-viable cells that have compromised membranes allowing ethidium homodimer-1 (EthD-1) to enter cells and bind with nucleic acids producing red fluorescence.
(2) **DEAD Green Viability Assay** – green cells are injured cells, the cell membranes are compromised. Healthy cells are impermeable to the green dye.
(3) **Propidium iodide** – red dye that is impermeable to healthy cells.

The greatest cell death is observed using the Live/Dead Viability/Toxicity and Propidium Iodide assays with both disease samples 1 and 2 at 10 and 20% concentrations, 48 and 72 hours relative to the normal serum treatments in both endothelial and epithelial cells. Disease sample 2 shows greater toxicity to endothelial cells than disease sample 1 at both 48 and 72 hour treatments with Live/Dead assay and at 72 hours with propidium iodide. Both disease samples show toxicity to epithelial cells at all time points at 10 and 20% concentrations (Figure 10).

MTT and Lactate Dehydrogenase (LDH) Release Cellular Viability Assays were performed (Figure 11). The viability assays were performed following exposure of human intestinal microvascular endothelial cells (HIMEC) to) serum from patients with mild, moderate and severe acute pancreatitis for 24 hours. These assays were performed in triplicate for each serum sample. Error Bars = Standard Error of the Mean. Number of serum samples – Severe n=12, Moderate n=12, Mild n=7. Kruskal-Wallis Test was performed across severity. Pairwise testing by Mann Whitney with Bonferroni correction was then performed. *Significant differences were demonstrated by severe vs mild % viability MTT (p=0.012) and moderate vs mild % viability MTT (p=0.029). Significant differences were also demonstrated by severe vs moderate (p=0.033) and severe vs mild (p=<0.001) % cytotoxicity LDH Release Assay.*

The XTT Cell Viability assay (Biotum) 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 Image-it DEAD Green Viability assay (Molecular Probes) was also 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 DEAD Green assay measures cytotoxicity by the uptake of the green dye that is otherwise impermeable to cells. The blue DAPI stain is taken up by all cells in the field. The figures below show that both endothelial and epithelial cells treated with severe acute pancreatitis (SAP) serum have greater toxicity leading to cell death in comparison to treatments with normal control serum (Figure 12).

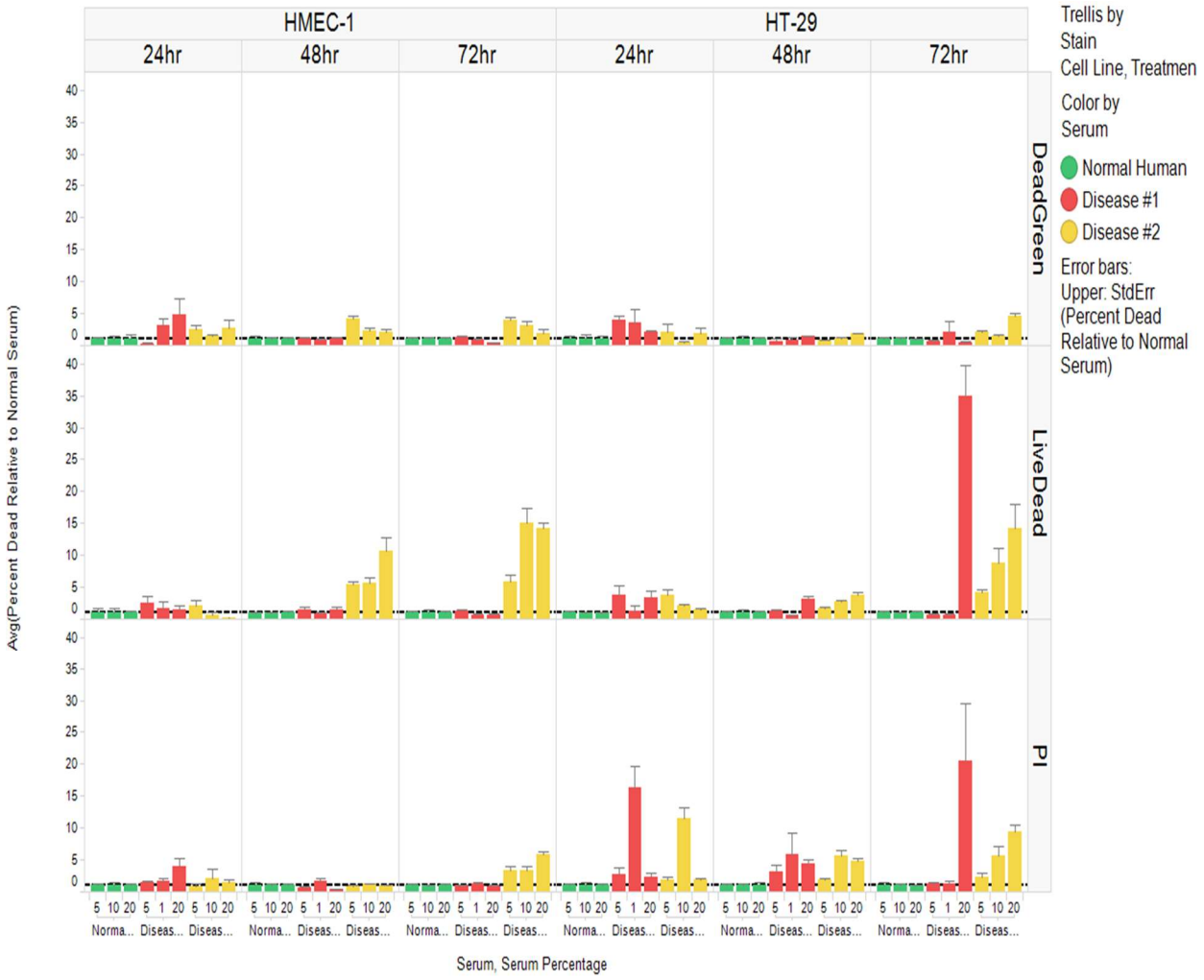

HMEC-1
HT-29
24hr
48hr
72hr
24hr
48hr
72hr
DeadGreen
LiveDead
PI
Avg(Percent Dead Relative to Normal Serum)
Serum, Serum Percentage
Trellis by
Stain
Cell Line, Treatmen
Color by
Serum
Normal Human
Disease #1
Disease #2
Error bars:
Upper: StdErr
(Percent Dead
Relative to Normal
Serum)

Figure 10

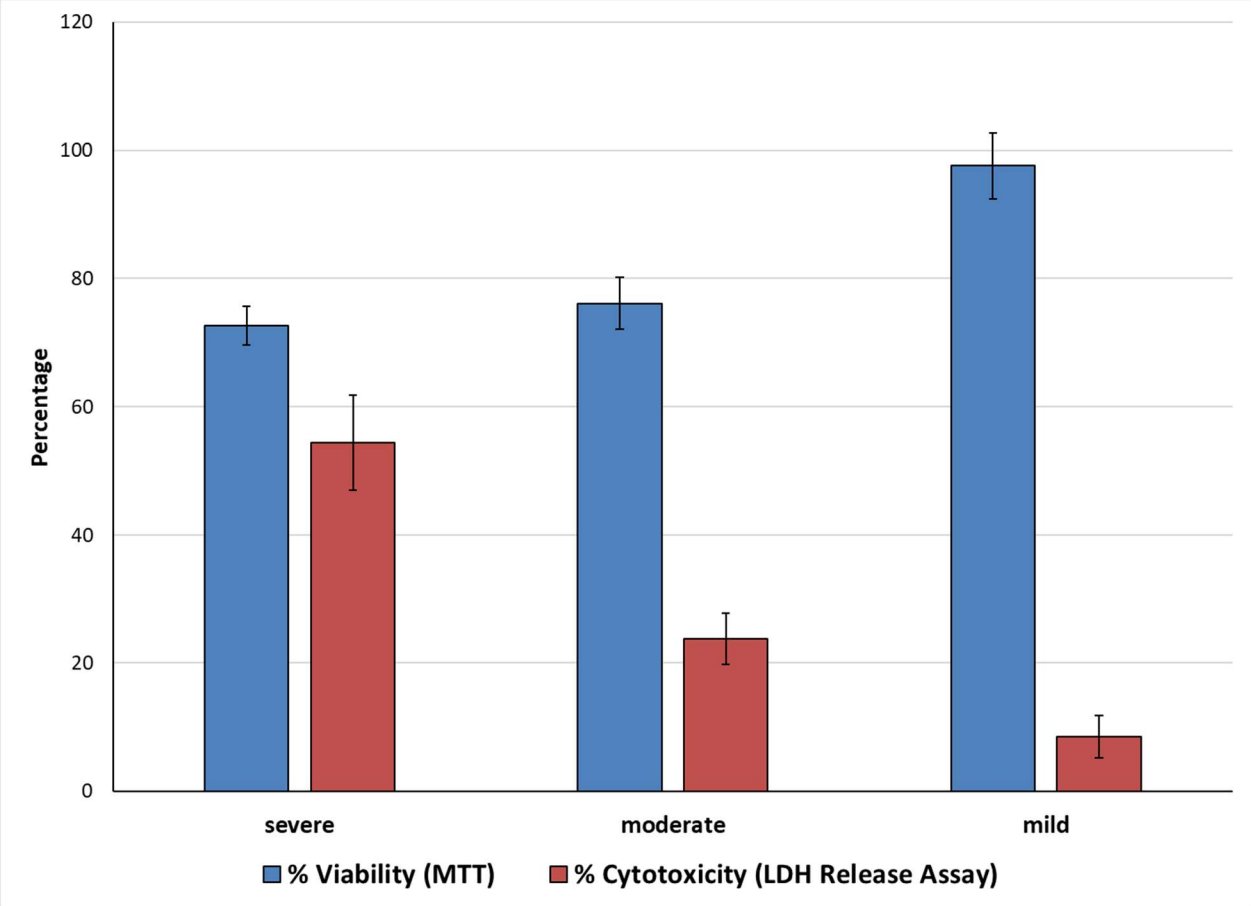
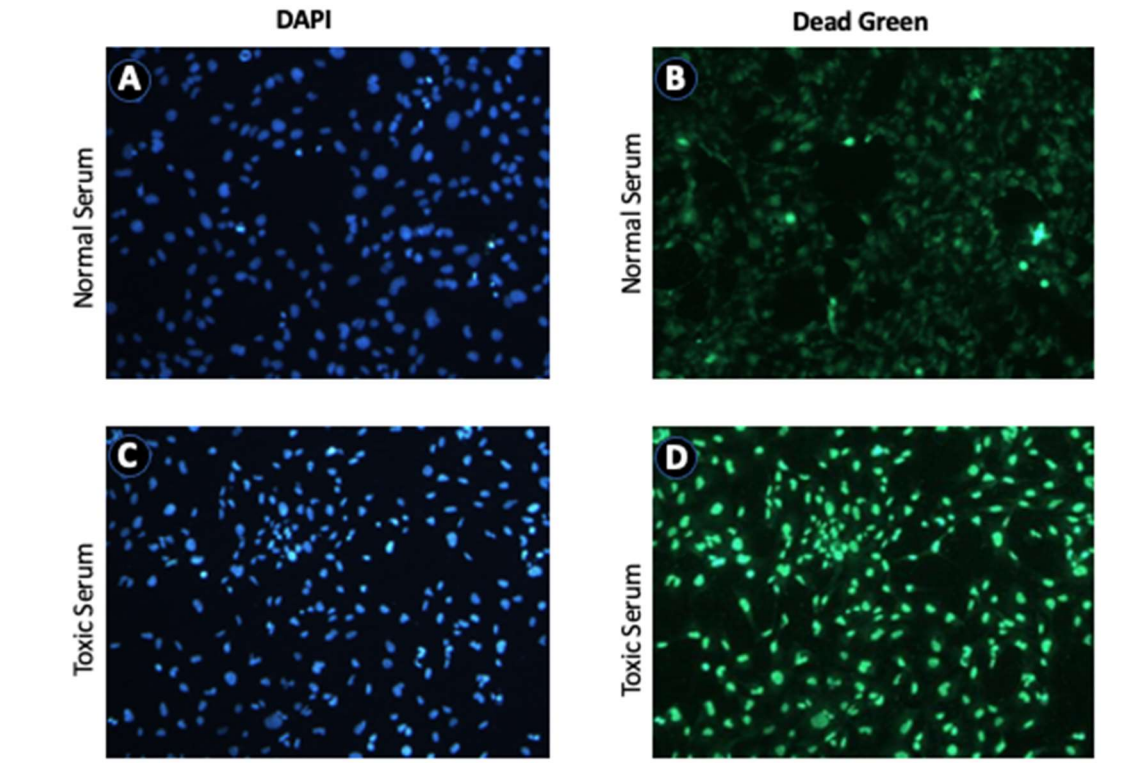

120
100
80
60
40
20
0
Percentage
severe
moderate
mild
% Viability (MTT)
% Cytotoxicity (LDH Release Assay)

Figure 11

Endothelial cells



Epithelial cells

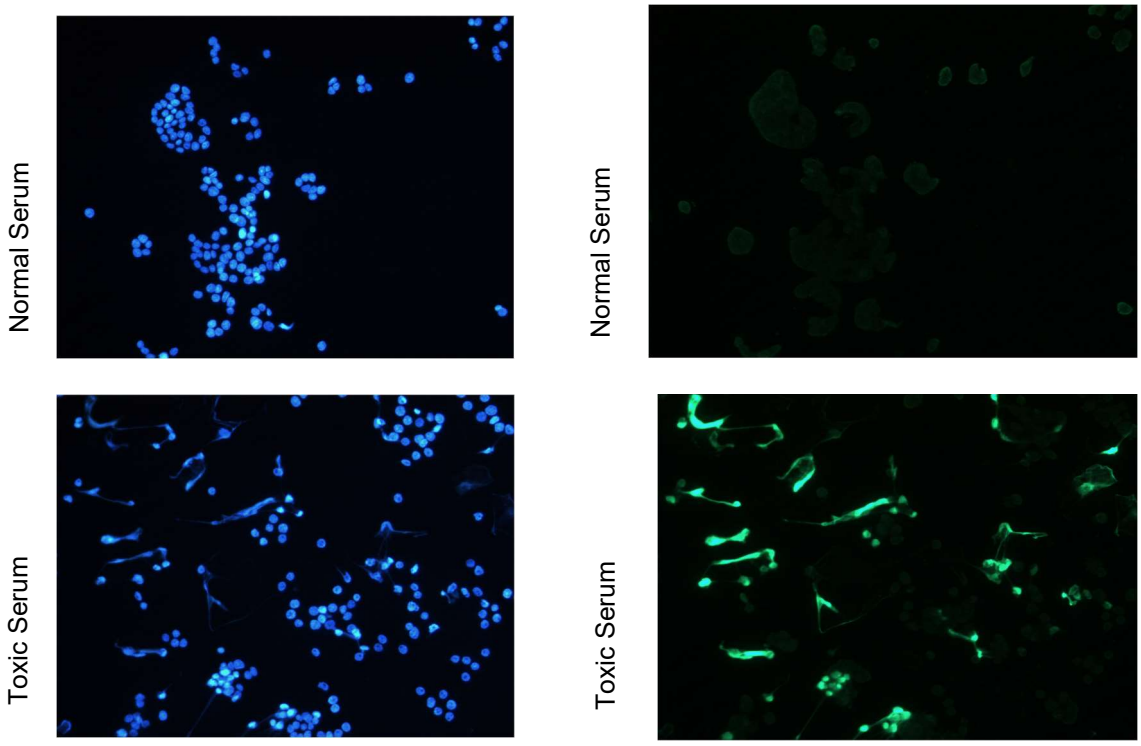


Figure 12

4. High Content High Throughput Screening

To dissect the mechanisms of toxicity we employed a chemogenomics approach utilizing high throughput (HTS), high content analysis. Cell death was measured using a 384-well Live/Dead Viability/Toxicity Assay explained above. Such a measure is required for the unbiased assessment of cytotoxic mechanisms. Experiments were performed in triplicate. Cell number, serum concentration and incubation time was optimized, along with DMSO effects being characterized. After demonstrating the robustness and reproducibility of the assay we screened for compounds with known mechanisms that protect the cells from SAP serum induced cell death. *Toxicity was primarily seen at the 20% serum concentration and at both 48 and 72 hours.*

We used the LOPAC library of 1,280 compounds at a single concentration and chose compounds that reduced the SAP serum induced cell death compared to the serum controls. These picked compounds were then tested in a concentration response assay to confirm their activity as well as determining their potency and efficacy in protecting the cells.

Compound treatment. Briefly, endothelial cells were plated at 3,000 cells per well in 384-well plates and allowed to attach in complete medium at 37º C and 5% CO_2 for 24 hr. Compounds (10μM) in complete medium were then added to cells using a Bravo multichannel automated liquid handler. Cells were incubated at 37º C and 5% CO_2 for 72 hours. Cells were treated with 20% of the SAP and normal serum and cell death was measured at 48 and 72 hours.

Imaging and Analysis. Images were collected on a ImageXpress Ultra (IXU) (Molecular Devices LLC, Sunnyvale, CA) a point-scanning confocal HCS platform. The IXU is used to sequentially acquire two fluorescence channels, calcein (Ch1) and EthD-1 (Ch2), for 1 field per well using a 10x Plan Fluor objective. Image analysis was carried out using the Multiwavelength Cell Scoring application in the MetaExpress software (Molecular Devices LLC, Sunnyvale, CA). The normalized calcein signal was used to assess cell viability. The normalized cell number was also used to assess cell toxicity in the confirmation experiment.

$$Normalized\ Calcein = \frac{Median\ compound\ calcein\ signal\ - Median\ diseased\ serum\ calcein\ signal}{Median\ normal\ serum\ calcein\ signal\ - Median\ diseased\ serum\ calcein\ signal}$$

Median calcein signal per well = median of the cytoplasmic calcein intensity for all cells with a well

From the 1,280 compounds tested in the LOPAC library, 40 showed a degree of protective activity above all of the other compounds after 72 hours of treatment (Table 4). Thirty-seven of these compounds were available for additional testing in the concentration response assay to confirm their activity. Of these 37 compounds, **3** showed a concentration response for increasing cell viability calcein levels towards the normal serum levels (Dihydroouabain, Gisadenafil besylate, Sildenafil citrate) but did not increase cell number. **Four** of these compounds may be active in increasing calcein levels at concentrations below the range tested. **Six** compounds (1,10-Phenanthroline monohydrate, Benserazide hydrochloride, CyPPA, NF 023, NSC 95397, TMPH hydrochloride) showed a concentration response for protection from cell loss due to SAP serum treatment (note that protection from cell loss did not necessarily correspond with increased cell viability calcein levels). To understand the mechanism of protection by compounds that show enhanced efficacy or potency we mapped the canonical targets of active compounds to KEGG pathways (homo sapiens, http://www.kegg.jp, version 07, 2016) and analyzed shared pathways of each active compound combination pair. Convergence of pathways will inform us on the likely mechanisms of protection. Both sildenafil and gisadenafil selectively target and inhibit cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5), thereby inhibiting the PDE5-mediated degradation of cGMP found in smooth muscle and increasing cGMP

Table 4. Forty Compounds

Compound Name	Retested	Compound Name	Retested
(+)-Chlorpheniramine maleate	Y	Gisadenafil besylate salt	Y
(+)-Quisqualic acid	Y	GR 55562 dihydrobromide	N
(±)-Taxifolin	Y	Idazoxan hydrochloride	Y
(S)-3,5-Dihydroxyphenylglycine	N	Imidazole-4-acetic acid hydrochloride	Y
1,10-Phenanthroline monohydrate	Y	KRM-III	Y
2,3-Butanedione	Y	Kynurenic acid	Y
5-Hydroxyindolacetic acid	Y	Lometrexol hydrate	Y
ABT-418 hydrochloride	Y	Nedocromil	Y
Arvanil	Y	NF 023	Y
Benserazide hydrochloride	Y	NSC 95397	Y
CGS-12066A maleate	Y	Oxolinic acid	Y
cis(+/-)-8-OH-PBZI hydrobromide	Y	Pentylenetetrazole	Y
CyPPA	Y	PK 11195	Y
DAPH	Y	Sildenafil citrate salt	Y
Dihydroouabain	Y	Spironolactone	Y
Disopyramide	N	SU 5416	Y
DL-threo-beta-hydroxyaspartic acid	Y	Tamoxifen	Y
DMH4	Y	TMPH hydrochloride	Y
Domperidone	Y	Tyrphostin 51	Y
Eprosartan mesylate	Y	U-69593	Y

Table 5. Confirmed Concentration-Response Compounds

Compound Name	Pathway
Increase cell viability	
Dihydroouabain	Na(+)/K(+)-transporting ATPase inhibitor (cardiac muscle contraction)
Gisadenafil besylate salt	Purine metabolism, phosphodiesterase V inhibitor (vasodilator)
Sildenafil citrate salt	cGMP-PKG signaling pathway, Phosphodiesterase V inhibitor (vasodilator)
Protect against cell loss	
1,10-Phenanthroline monohydrate	Pyroglutamyl-peptidase I (protease) inhibitor and Hexokinase (glycolysis) inhibitor
Benserazide hydrochloride	Serotonergic synapse, Dopaminergic synapse, decarboxylase inhibitor
CyPPA	Positive modulator of Ca(2+) activated K(+) channels (SK2 and SK3)
NF 023	Methyltransferase inhibitor, competitive and reversible P2X1 receptor antagonist
NSC 95397	Cdc25 dual specificity phosphatase inhibitor; inhibits mitogen-activated protein kinase phosphatase-1 (MKP-1) and suppresses proliferation and induces apoptosis in colon cancer cells through MKP-1 and ERK1/2 pathway.
TMPH hydrochloride	Non-competitive antagonist of neuronal nicotinic ACh receptors (nAChRs)

availability (Figure 13).

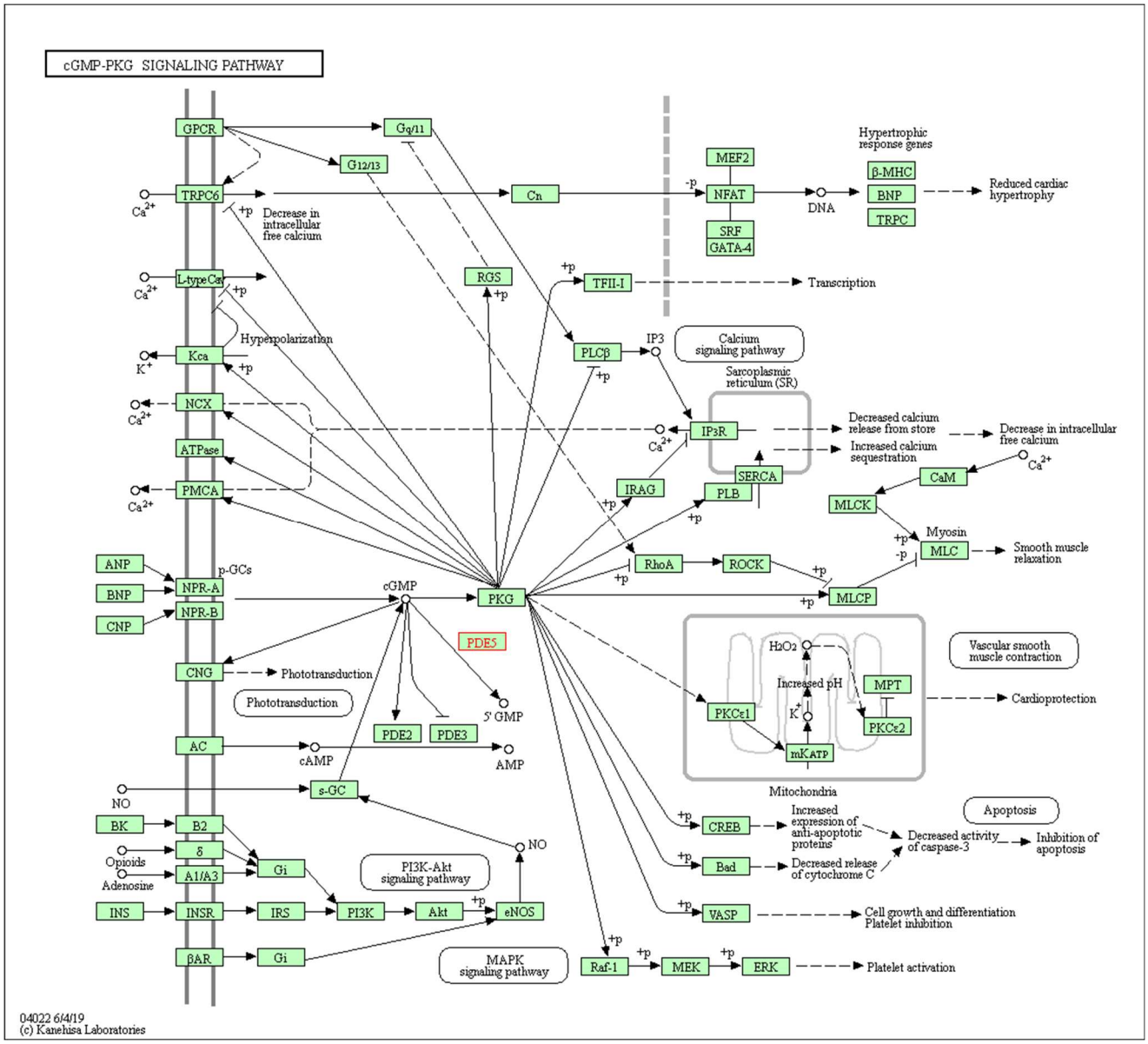


Figure 13

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

The results were presented to the scientific community at conferences such as Digestive Disease Week. Additionally, results were presented at the annual University of Pittsburgh Research Day.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Acute pancreatitis, multiple trauma, severe burns, massive hemorrhage and sepsis all result in activation of the entire body's immune system – a condition called "systemic inflammation", or SIRS. In some people, but not others, triggering the immune system leads to severe injury of the blood vessels, leakage of blood fluids into the tissues, pulmonary edema (fluid in the lungs) and cardiovascular shock – with multi-organ failure (MOF). We studied patients with acute pancreatitis as a well-defined example of SIRS and MOF, collecting detailed clinical information and biological samples from patients at multiple time points for detailed analysis. We discovered that people with severe acute pancreatitis have something highly toxic to both endothelial and epithelial cells in their blood, and possibly more than one compound. We found that some fatty acids are highly toxic and that high levels were seen in some of the patient serum samples. We also found high levels of cytokines in the serum of patients with acute pancreatitis. Angiopoietin 2 levels increased with severity and Angiopoietin 1 levels decreased with severity of acute pancreatitis. Together, these findings provide insights into why some patients have very bad outcomes, including MOF, while others do not. We explored compounds to block the toxicity caused by the AP serum. Several pathways were discovered with a couple of the compounds overlapping. Knowledge of these pathways may lead to early detection and diagnosis of patients who will go into MOF, strategies for avoiding or minimizing MOF, and potential identification of patients at high risk of MOF.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

The importance of diet and lifestyle/behavior impacts the likelihood of occurrences of acute pancreatitis. A high fat diet can lead to these attacks as can alcohol abuse. By making changes in diet and alcohol consumption, these attacks can be less frequent or eliminated. Modifications are stressed to the patients

5. CHANGES/PROBLEMS:

Nothing to report.

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays 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 in order to complete the project by the end of 2021.

Changes that had a significant impact on expenditures

Nothing to report.

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

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:

- **Publications, conference papers, and presentations**
  Journal publications.

Published:
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. (yes)

Manuscripts in Process:
1. X. Tang, J.C. Castaneda, P. Paragomi, A.E. Phillips, K. Hall, S. McCulloch, E.D. Karoly, P. Fogle, S.J. O'Keefe, G.I. Papachristou, D.G. Binion, A.S. Wilson and D.C Whitcomb. Severe Acute Pancreatitis: Multi-organ failure is Associated Unregulated Proteolysis and Endothelial Cell Toxicity
2. A.E. Phillips, A.S. Wilson, P.J. Greer, A Hinton, P. Paragomi, I. Pothoulakis, V. Singh, D.C. Whitcomb, G.I. Papachristou. Relationship of Circulating Levels of Saturated and Unsaturated Long-Chain Fatty Acids to Persistent Organ Failure in Acute Pancreatitis
3. D.C Whitcomb, P.J. Greer, A.S Wilson, et.al.. Hypertriglyceridemic in Acute Pancreatitis: Genetic Analysis of an Acute Pancreatitis Cohort
4. A.E. Phillips, A.S. Wilson, J.C. Castaneda, D.G. Binion and D.C. Whitcomb. Unsaturated Fatty Acids are Toxic to Microvascular Endothelial Cells and May Contribute to Multi-organ Failure in Acute Pancreatitis

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Presentations:
*DDW 2019: High levels of amino acids and their metabolites are associated with severe acute pancreatitis, and 6 amino acids form a signature in toxic serum during multi-organ failure; Xiping Tang, Pedram Paragomi, Annette Wilson, Anna Phillips, Juan Castaneda, Stephen O'Keefe, Georgios Papachristou, and David Whitcomb

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)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

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 (years 1-4)
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 (years 1-3)
Contribution to Project: Dr. Binion provided assistance with experiments in this project and participates in research meetings.

Name: Georgios Papachristou, MD
Project Role: Co-Investigator
Nearest person month(s) worked: 1 month (years 1-2)
Contribution to Project: Dr. Papachristou provided assistance in recruitment of subjects for this project.

Name: Lansing Taylor, PhD
Project Role: Co-Investigator
Nearest person month(s) worked: 1 month (years 1-4)
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 (years 1-4)
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 (years 1-4)
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 (years 1-4)
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 (years 1-4)
Contribution to Project: Oversees database at Drug Discovery Institute.

Name: Kelley Woods, RN
Project Role: Research Nurse Coordinator

Nearest person month(s) worked: 6 months (Years 1-2)
Contribution to Project: Mrs. Woods has consented all 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: Shari Reynolds
Project Role: Clinical Research Coordinator
Nearest person month(s) worked: 6 months (years 2-3)
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 (years 1-4)
Contribution to Project: Mrs. Comsa was responsible for cell culture propagation.

Name: Juan Castaneda
Project Role: Research Technician
Nearest person month(s) worked: 3 months (years 1-2)
Contribution to Project: Mr. Castaneda was responsible for performing experiments and data compilation.

Name: Kristen Hall
Project Role: Research Technician
Nearest person month(s) worked: 12 months (years 3-4)
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:
   QUAD CHARTS:

9. **APPENDICES:**