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TITLE: Acute Pancreatitis as a Model to Predict Transition of Systemic Inflammation to Organ Failure in Trauma and Critical Illness

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14. ABSTRACT
Trauma, extensive burns, bacterial infections, and acute pancreatitis (AP) are common conditions of tissue injury and immune system activation that can result in the systemic inflammatory response syndrome (SIRS). Surprisingly, about half of the patients with SIRS quickly recover, while the others develop a multiorgan dysfunction syndrome (MODS). SIRS and MODS do not occur immediately: SIRS evolves over a 4-12 hour period, while MODS evolves over 12-24 hours. Vascular leak syndrome (VLS) is a critical component of the transition from SIRS to MODS. Understanding the mechanism by which SIRS triggers VLS and progresses to MODS is critical to correctly model disease course thereby aiding in treatment of patients. We tested the effect of severe acute pancreatitis patient serum on the viability of human vascular endothelial cells grown in a monolayer and evaluated potential cell death mechanisms. Serum from enrolled subjects was also tested for potential biomarkers in order to better understand course from SIRS to organ failure (OF). Our data shows that main modes of cell death are necrosis and autophagy and that multiple mechanisms are involved in course to OF.

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

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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 acute pancreatitis, multiple trauma, severe burn, or sepsis responsible for the unpredictable progression of systemic inflammation to the vascular leak syndrome (VLS), which in turn leads to multi-organ dysfunction syndrome (MODS). Our experimental approach is designed to understand and predict progression from systemic inflammation to MODS. The primary observation is that serum or plasma from patients with severe acute pancreatitis (AP) or trauma with VLS is toxic to endothelial cells.

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

Pancreatitis, systemic, inflammation, vascular leak, multiple organ dysfunction, biomarkers, endothelium, 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. Define the clinical setting in which SIRS progresses, and fails to progress, to VLS and MODS using molecular and clinical measures. (months 4-36)

Aim 2. Determine the effect of serum from patients with SIRS ±VLS as well as Ang-2 and other target molecules (identified in Aim 3) on human organ-derived endothelial cells in terms of morphology, gene activation, and mode of cell death. (months 4-36)

Aim 3. Identify serum molecule(s) that best predict specific in vitro changes in endothelial cells (Aim 2) as well as which molecule(s) and endothelial cell changes best predict clinical progression to MODS (Aim 1). (months 6-36)

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.

Aim 1. Define the clinical setting in which SIRS progresses, and fails to progress, to VLS and MODS using molecular and clinical measures.

A total of 36 subjects were enrolled into this study. 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 36 subjects enrolled, 8 demonstrated severe, 12 moderate, and 16 mild acute pancreatitis (AP) according to the severity categories in Table 1. Of the 36 subjects, 12 exhibited organ failure - 4 transient and 8 persistent organ failure. Of the 12 subjects demonstrating organ failure, 4 exhibited (multi-organ dysfunction syndrome) MODS and 8 exhibited single organ failure. Primary organs that failed were respiratory, renal, and cardiovascular.

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)

Demographic data was collected for each subject and select variables are illustrated in Table 2. Sample collection was dependent on length of hospital stay and health of the subject. Samples for subjects with mild, moderate, and severe acute pancreatitis were collected an average of 5.35, 6.5, and 7 days, respectively. The subjects experiencing mild acute pancreatitis tended to have a shorter length of stay.

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. Cytokines well known to be involved in the SIRS early in AP were measured including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), Tumor Necrosis Factor-α (TNF-α). All of the cytokines analyzed except TNF-β and TNF-α were expressed at higher concentrations than normal levels reported in the literature. GM-CSF was significantly lower than normal levels. Results are in Table 3. Chemokines were also analyzed. Chemokines function by activating specific G protein-coupled receptors resulting in migration of inflammatory and non-inflammatory cells. The pro-inflammatory chemokines are responsible for migration of immune cells to the infection site, while homeostatic chemokines are responsible for recruiting cells for tissue maintenance and development. The chemokines measured included monocyte chemotactic protein-1 (MCP-1), IP-10, eotaxin, eotaxin-3, MIP-1β, TARC, MIP-1α, MDC, and MCP-4. All but MCP-4, eotaxin-3, and TARC were expressed at higher concentrations than normal levels (Table 4). Vascular growth factors, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) were also measured. These angiopoietin cytokines are involved in microvascular permeability, vasodilation, and vasoconstriction by signaling smooth muscle cells surrounding vessels. Ang-2 was expressed at very high levels in mild, moderate, and severe acute pancreatitis. The severe group had the highest amounts. Ang-1 was expressed at progressively lower levels in mild, moderate, and severe groups. Proteins indicative of vascular injury were also analyzed including C-reactive protein (CRP), soluble intercellular adhesion molecule-1 (ICAM-1), and soluble vascular cell adhesion molecule-1 (VCAM-1). All of these proteins were expressed at higher than normal concentrations (Table 5). In addition, activin was analyzed in acute pancreatitis and matching control samples in the lab of Dr. Barbara Jung at University of Illinois Chicago and in the Whitcomb Lab. Activin is a cytokine that is a member of the TGF-β family. In inflammation, activin has been reported to have both pro- and anti-inflammatory functions ex vivo, resulting in either up or down regulation of a number of key inflammatory cytokines, including IL-6, IL-1β, or IL-10 in various human and murine cell types. Overall, serum activin levels were increased in acute pancreatitis samples when compared to controls (0.965 ng/ml versus 0.462 ng/ml, $p<0.0001$) (Figure 1). When grouped by severity, we observed an increase in moderate and severe

AP, but not in mild disease ($p<0.0001$ for difference in between groups, $p<0.05$ for moderate versus controls, $p<0.0001$ for severe versus controls, mild versus controls n.s.) (Figure 1).

Table 2. Demographic data

DOD ID	Age	Sex	BMI	Etiology	Severity
DOD001	68	M	33.34	Hemorrhagic	Severe
DOD002	52	F	41.04	Idiopathic	Moderate
DOD003	39	M	35.29	HTG	Moderate
DOD004	68	M	36.08	Idiopathic	Moderate
DOD005	24	M	32.28	HTG	Mild
DOD006	48	M	23.76	Alcohol	Moderate
DOD007	79	F	33.33	Gallstone	Severe
DOD008	25	F	28.9	Gallstone	Mild
DOD009	37	M	23.52	Alcohol	Moderate
DOD010	66	M	32.49	Gallstone	Severe
DOD011	49	F	24.16	Idiopathic	Severe
DOD012	52	F	40.65	Gallstone	Moderate
DOD013	36	F	24.5	Idiopathic	Mild
DOD014	28	F	24.61	Post-ERCP	Mild
DOD015	41	M	31.83	HTG	Mild
DOD016	59	M	25.23	Gallstone	Moderate
DOD017	42	M	31.26	HTG	Severe
DOD018	53	M	44.40	Gallstone	Mild
DOD019	37	F	30.2	HTG	Moderate
DOD020	34	F	34.8	HTG	Mild
DOD021	34	F	18.1	Alcohol	Mild
DOD022	40	M	25.9	Gallstone	Moderate
DOD023	84	F	31.1	Periampullar diverticula	Mild
DOD024	37	F	28.7	HTG	Mild
DOD025	58	F	52.3	Gallstone	Mild
DOD026	43	M	25.2	Idiopathic	Mild
DOD027	75	F	20.9	Gallstone	Mild
DOD028	18	F	37.3	Gallstone	Moderate
DOD029	56	M	28.5	HTG	Moderate
DOD030	64	M	36.5	Gallstone	Severe
DOD031	45	F	29.9	HTG	Mild
DOD032	27	M	42.6	HTG	Mild
DOD033	82	F	45.3	Gallstone	Severe
DOD034	43	F	34.1	Alcohol	Mild
DOD035	45	M	26.8	HTG	Severe
DOD036	33	F	51.5	Gallstone	Moderate

Table 3: Cytokines

Cytokines	Mean Mild Concentration pg/mL	Mean Moderate Concentration pg/mL	Mean Severe Concentration pg/mL
GM-CSF	0.32	0.25	1.19
IL-12	478.93	302.89	274.11
IL-15	21.14	24.16	40.21
IL-16	1,363.74	1,320.07	2,183.21
IL-17	16.61	25.49	37.73
IL-1β	2.85	2.22	2.97
IL-5	7.26	4.21	1.10
IL-7	67.21	70.23	50.22
TNF-β	0.58	0.52	0.42
VEGF	950.96	820.17	461.88
TNF-α	6.02	5.10	12.37

Table 4: Chemokines

Chemokines	Mean Mild Concentration pg/mL	Mean Moderate Concentration pg/mL	Mean Severe Concentration pg/mL
IP-10	1,555.20	870.03	1,349.08
MDC	2,257.67	2,257.41	1,003.42
Eotaxin	125.20	104.67	110.03
Eotaxin-3	9.66	8.36	10.50
IL-8	385.08	442.40	898.07
MIP-1α	10.22	8.90	11.18
MIP-1β	126.30	103.59	112.27
MCP-1	1,177.85	708.80	1,005.18
MCP-4	87.22	81.24	77.83
TARC	166.15	103.83	111.76

Table 5: Vascular Injury

Analyte	Mean Mild Concentration mg/L	Mean Moderate Concentration mg/L	Mean Severe Concentration mg/L
CRP	232.37	251.02	282.60
sICAM-1	3.07	4.62	3.45
sVCAM-1	3.19	4.13	3.81
Angiopoietin-1	0.0168	0.0152	0.0073
Angiopoietin-2	0.0315	0.0120	0.1240

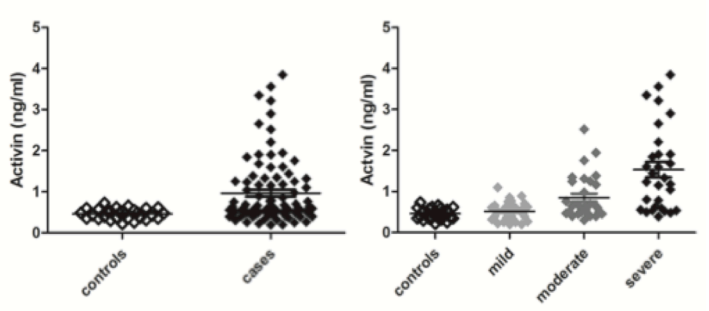

Figure 1: Activin is increased in moderate and severe AP and is correlated with worse prognosis: A) Activin is increased in samples from patients suffering from AP when compared to samples from healthy controls (left panel, $p<0.05$). B) Stratification by severity demonstrates that activin is specifically upregulated in moderate and severe AP, but not in mild AP (right panel).

Proteomic analysis was also performed on control, mild, moderate, and severe acute pancreatitis subject serum samples. The serum samples were first fractionated by gel filtration chromatography on a column packed with Superdex 200 (GE Healthcare) (1.0 cm x 15 cm) using a low pressure chromatography system (Biologic LP System, Bio-Rad), UV wavelength 280 nm. The peaks from the column were collected using a fraction collector and the eluted fractions dried using a Labconco CentriVap Centrifugal Concentrator. The fractions were then reconstituted in 1 ml of water and total protein in each fraction quantitated using the BCA Protein Assay (Thermo Fisher Scientific/Pierce). One μg of total protein per peak was then loaded and run on a 4-12% Bis-Tris SDS-PAGE mini-gel (Thermo Fisher Scientific) at 150V using an Invitrogen XCell SureLock Electrophoresis System to visualize the potential number of proteins in each peak. The gel was stained with SimplyBlue Safe Stain (Thermo Fisher Scientific) and specific bands of higher molecular weight excised from the gel. The samples were digested with trypsin and tryptic peptides analyzed by nano reverse phase HPLC interfaced with a mass spectrometer at the University of Pittsburgh Biomedical Mass Spectrometry Center. The tandem mass spectra (MS/MS) were analyzed by the MASCOT (Matrix Science) search engine and identified peptides and proteins further statistically validated with the Scaffold software.

DATABASE SEARCHING-- Tandem mass spectra (MS/MS) were extracted. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1). Mascot was set up to search the Uniprot_Human_BGAL_PhosB_20131212 database (unknown version, 88,505 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 1.4 Da. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine and acetyl of the n-terminus were specified in Mascot as variable modifications. CRITERIA FOR PROTEIN IDENTIFICATION-- Scaffold (version Scaffold 4.4.8, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability by the Peptide Prophet algorithm (Keller, A et al Anal. Chem. 2002;74(20):5383-92). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Al et al Anal. Chem. 2003;75(17):4646-58). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Some proteins identified that are unique to the SAP patient serum are alpha-1-antichymotrypsin, ceruloplasmin, desmoglein 1, hornerin, desmoplakin, carboxypeptidase N subunit, suprabasin. In another batch of serum samples, normal, mild AP, and severe AP serum samples were fractionated and analyzed by mass spectrometry. Unique proteins to the severe AP serum were dermcidin (MW 11KDa), thioredoxin (MW 12KDa), caspase-14 (28 KDa). Proteins not found in the normal serum include desmoplakin (332 KDa), hornerin (282 KDa), desmoglein (114 KDa), and annexin A2 (39 KDa). These are potential biomarkers that are associated with cellular junctions and inflammation that we will further explore.

Aim 2. Determine the effect of serum from patients with SIRS ±VLS as well as Ang-2 and other target molecules (identified in Aim 3) on human organ-derived endothelial cells in terms of morphology, gene activation, and mode of cell death.

The viability, morphology, and physiology of newly isolated vascular endothelial cells were assessed using the following assays: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) where MTT is converted to formazan in healthy cells via mitochondrial enzymes, and lactate dehydrogenase (LDH) release assay that measures the release of intracellular LDH from damaged plasma membrane (viability); F-actin stress fiber staining with phalloidin after treatment with or without activators (LPS); and autophagy, a conserved cellular process that mediates degradation of intracellular components including proteins and organelles via LC3B containing autophagosome formation and degradation in the autolysosome, measured with LCB3 immunostaining.

The serum samples collected from subjects were screened for toxicity to the HIMEC using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The HIMEC were treated with 10 to 20 % of the serum sample added to the basal medium (MCDB131) for 24 and 48-hour time points. Cell death mechanisms were also evaluated. The Molecular Probes Live/Dead Viability/Toxicity Kit and propidium iodide assay were used to assess necrosis and late phase apoptosis. Apoptosis was further evaluated with caspase 3/7 measurement. Autophagy upregulation was measured with LCB3 immunostaining. Many of the mild and moderate AP sera induced toxicity to the HIMEC under these conditions. Some of the serum from each category also led to cell death. The severe acute pancreatitis samples induced cell death. The primary modes of endothelial cell death appeared to be via necrosis and autophagy.

Several of the serum samples collected that showed significant toxicity to the endothelial cells were analyzed for serum free fatty acids (FFA) in order to evaluate the different levels and potentially correlate them to the level of toxicity, and possibly other factors that are being monitored from the clinical data. Both saturated fatty acids (SFA) i.e. palmitic (16:0) and stearic (18:0) and unsaturated fatty acids (UFA); monounsaturated, i.e. palmitoleic (16:1), oleic (18:1), and polyunsaturated, i.e. linoleic (18:2) were measured. The FFA were analyzed using an Agilent Technologies 6890N Network gas chromatography (GC) System with Flame Ionizaton Detector. The serum samples were extracted with isopropanol-heptane-hydrochloric acid. Heptane and water were added and the tubes vortexed. Tubes were then centrifuged and the upper phase (heptane) transferred into clean screw top tubes and dried in a SpeedVac centrifugal concentrator. FFA were then derivatized with dimethylamine and diisopropylethylamine using the Deoxo-Fluor reagent (Sigma Aldrich). The results indicate severe subject DOD001 had very high FFA with average palmitic acid of 431.2 μM, palmitoleic acid of 31.1 μM, stearic acid of 133.4 μM, oleic acid of 344 μM, and linoleic acid of 258.6 μM. The other 2 subjects with severe pancreatitis analyzed (DOD002, DOD004, DOD005) had averages of 253, 22.7, 83.5, 166, 61.5 μM palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid respectively. Subject DOD003 with moderate pancreatitis had 295, 19.4, 105, 180, 74.7 μM palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid respectively. A subpopulation of subjects with mild pancreatitis had an average of 174, 13.6, 58.2, 150, 97.8 μM palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid respectively. Control subjects without pancreatitis had 106, 7.7, 40.8, 62.0, 45.3 μM palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid respectively. There is a decrease in FFA between the severe/moderate and mild control subjects.

Aim 3. Identify serum molecule(s) that best predict specific in vitro changes in endothelial cells (Aim 2) as well as which molecule(s) and endothelial cell changes best predict clinical progression to MODS (Aim 1).

In Aim 1, very high mean concentrations of Ang-2 and lower than normal Ang-1 levels were found in many of the serum samples. Cells were treated with 50,000 to 100,000 pg/mL of Ang-2 added to complete cell growth medium for 24 and 48 hours. The high levels of the Ang-2 were not toxic to the cells.

Ang-1 also had no effect on the HIMEC at concentrations of 8,000 and 15,000 pg/mL. Treatments with both Ang-1 and Ang-2 were slightly toxic to the HIMEC.

Hypertriglyceridemic (HTG) acute pancreatitis is often severe, with up to 30% mortality. While HTG itself is rather benign, release of pancreatic lipase(s) into the circulation during an episode of AP facilitates massive hydrolysis of TG to free fatty acids (FFA). Release of unsaturated FFAs (uFFA) causes direct toxicity in animal models. HIMEC were treated with the fatty acids linoleic, oleic, and stearic acid. The fatty acids were toxic as measured by MTT in Figure 2. HIMECs treated with increasing concentrations of SA, OA, and LA showed corresponding increases in cell toxicity. Specifically, significant increases in toxicity measured by MTT were seen with 0.3 mM, 0.6mM and 1.2 mM SA, OA, and LA ($p<0.05$). When grouped by AP severity, serum studies revealed mean Stearic Acid (µM) levels of: Controls 41.1 ± 15.2, Mild 79.1 ± 45.2, Moderate 93.9 ± 50.9, Severe 99.9 ± 60.5 ($p=0.0004$). Mean levels of Oleic Acid were (µM): Controls 61.6 ± 39.0, Mild 174.9 ± 66.5, Moderate 265.2 ± 137, Severe 260.6 ± 130.4 ($p=0.00$). Mean levels of Linoleic Acid were (µM): Controls 41.9 ± 28.0, Mild 102.8 ± 63.2, Moderate 175.8 ± 123.6, Severe 144.4 ± 77.7 ($p=0.00$). Mean levels of unsaturated fatty acid levels (OA and LA) were higher than saturated (SA) (Figure 3).

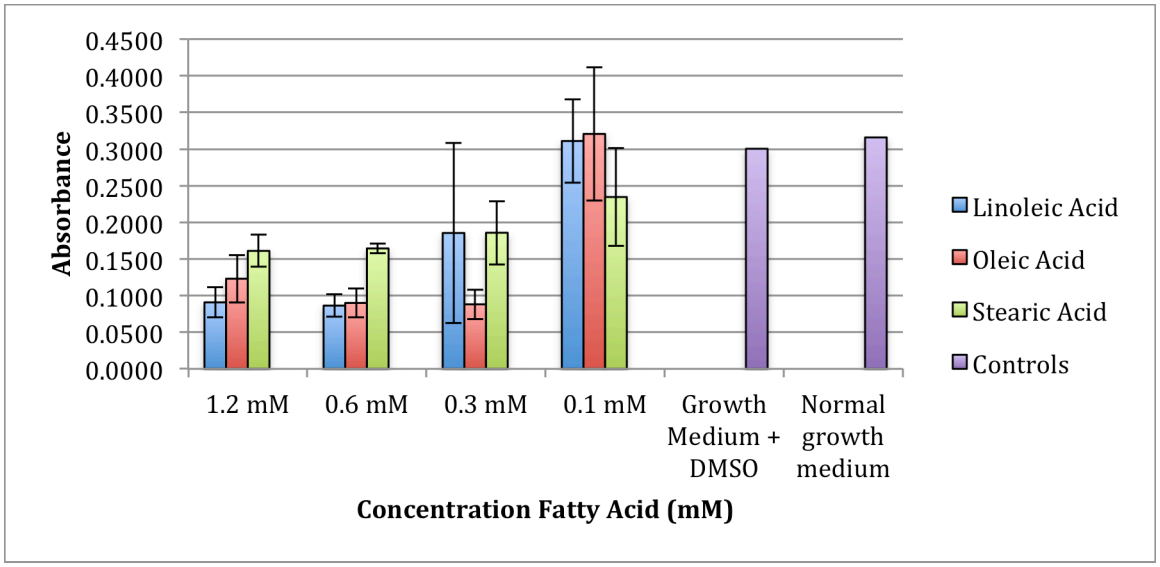

Figure 2. Fatty acid toxicity as measured by MTT.

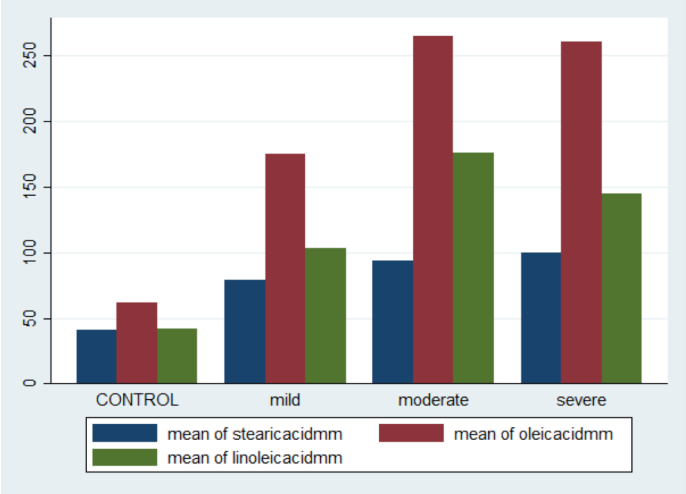

Figure 3. Mean serum fatty acids levels according to severity of acute pancreatitis.

The cell viability was also assessed with the Molecular Probes Live/Dead Viability/Toxicity Assay to focus on potential cell death. The HIMEC were treat with 0.6 mM of oleic, linoleic, and stearic acids. Figure 4 shows that the toxicity effects are more significant with the oleic and linoleic acid treatments.

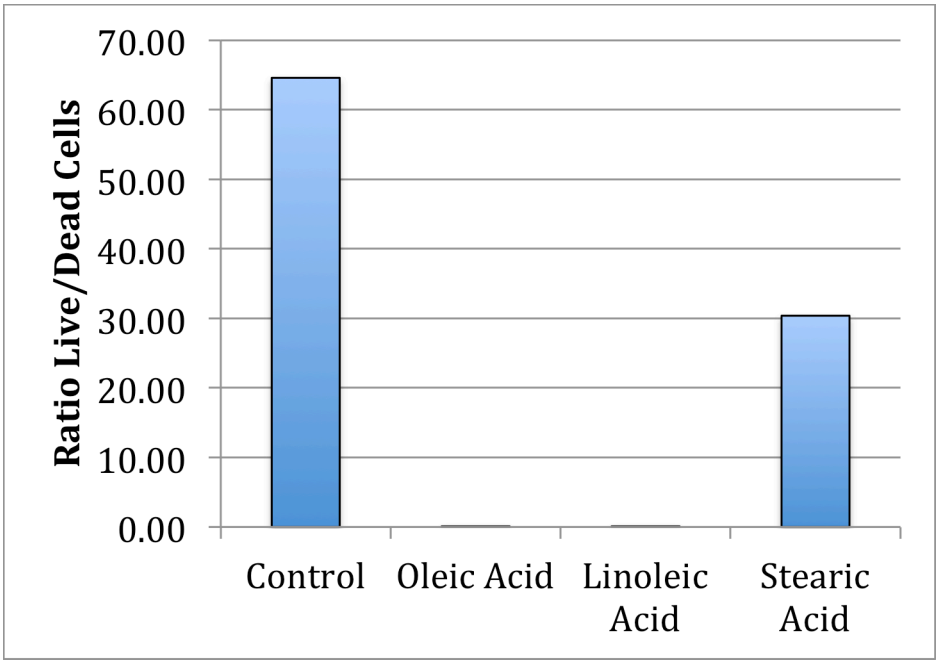

Figure 4. Cell death assay with Molecular Probes Live/Dead Viability/Toxicity Assay with 0.6 mM fatty acid treatments.

Metabolomics were performed on the serum samples. The small molecular weight factors of the serum were assessed by LC-MS/MS. There is a large data set from which we are still deciphering information. At this time, a mixed model ANOVA was used to identify amino acid metabolome that differed significantly between the experimental groups. Experimental groups divided into AP with or without toxicity to HIMEC, and AP with or without organ failure. Correlation of toxicity and organ failure was evaluated using Chi-Square test. There was a significant correlation between toxicity and organ failure ($P<0.001$). 15 sub-pathways of amino acids, including 187 amino acids and derivatives were detected with 91 amino acids and metabolites increasing > 2 fold in organ failure. 6 amino acids and associated metabolites were in toxicity group (> 2 fold increase, $P<0.001$), including 1-methylhistidine (2.90 fold), 4-hydroxyphenylacetate (6.81 fold), 3-methoxytyramine sulfate (7.89 fold), vanillic alcohol sulfate (5.99 fold), lanthionine (4.11 fold), and acetycitrulline (6.86 fold) Figure 4. Based on metabolism maps, 1-methylhistidine and 4-hydroxyphenylacetate may be involved in the anabolism of tyramine, a metabolite of tyrosine. Previous research confirmed that 1-methylhistidine, tyramine, lanthionine, and N-acetylcitrulline may play a role in cytotoxicity. Lanthionine is a sulfur metabolite that may be a uremic toxin. Acute pancreatitis and other conditions that transition from SIRS to organ failure are associated with widespread derangement of metabolism.

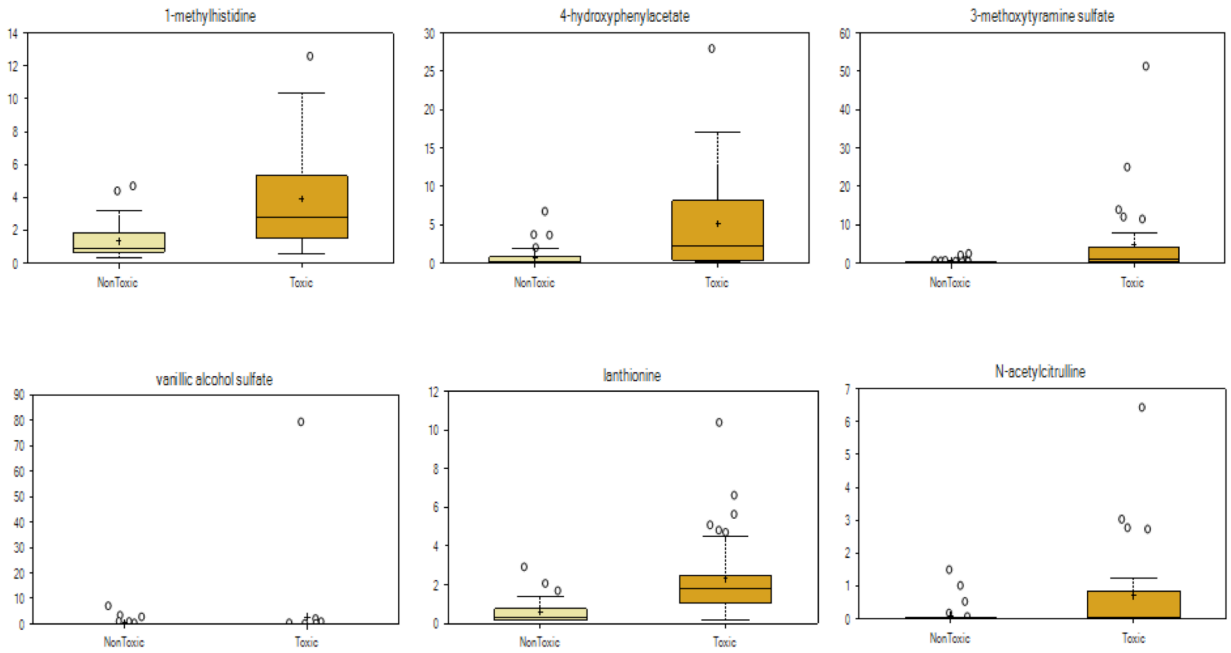

Figure 5. Comparison of 1-methylhistidine, 4-hydroxyphenylacetate, 3-methoxytyramine sulfate, vanillic alcohol sulfate, lanthionine and N-acetylcitrulline between AP with or without toxicity

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.

This study proved to be a tremendous opportunity to train health care professionals in translational research. The trainees are listed with collaborators.

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.

The results were presented to scientific community at conferences. The international conferences were Digestive Disease Week and American College of Gastroenterology. 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?
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.

Nothing to Report.

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).

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 the patient and multiple time points for detailed analysis. We discovered that people with MOF have something highly toxic in their blood, and possibly more than one thing. We found that some fatty acids are highly toxic and levels seen in some of the patients. We also found massive digestion of body proteins, likely from pancreatic digestive enzymes released into the blood, and that processing of the amino acids in the wrong way leads to additional toxins. Finally, we found that several very large blood molecules are also toxic, and directly kill cells without triggering the safer, self-destruction process called apoptosis. Together, these findings provide insights into why some patients have very bad outcomes, including MOF, while others do not. Knowledge of these pathways may lead to early detection and diagnosis of patient who will go into MOF, strategies for avoiding or minimizing MOF, and potential identifying patients at high risk of MOF so that they could be kept out of harms way.

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.

See above.

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.*

The importance of diet and 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 who have entered our study.

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.

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

There was a delay in getting the IRB approval from the University of Pittsburgh IRB Committee as a "less than minimal risk" classification. Approval was received February 4, 2015. This was followed by the US Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protections Office (HRPO) approval on February 28, 2015. There was also a delay in hiring a Research Nurse Coordinator for this study. All of these delays resulted in a slow start with enrollment. With the NCE of this grant, we were able to reach our recruitment goal of a total of 36 subjects.

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).*

  Staudacher JJ, Yazici C, Carroll T, Bauer J, Pang J, Krett N, Xia Y, Wilson A, Papachristou G, Dirmeier A, Kunst C, Whitcomb DC, Fantuzzi G, Jung B. Activin in acute pancreatitis: Potential risk-stratifying marker and novel therapeutic target. Sci Rep. 2017 Oct 6;7(1):12786. doi: 10.1038/s41598-017-13000-3: PMID: 28986573

  We are currently developing additional manuscripts that based on this research that will be submitted to professional journals in early 2019.

  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.*

Abstracts for presentations at meetings:

Digestive Disease Week Sunday May 21, 2016: Is Endothelial Cell Injury the Link between Systemic Inflammatory Response Syndrome and Multiorgan Dysfunction Syndrome? Annette Wilson, Weiping DeBlasio, William Rivers, Efstratios Koutroumpakis, Georgios Papachristou, Stephen O'Keefe, David G Binion, David C Whitcomb; Poster

Digestive Disease Week Sunday May 21, 2016: Increased Serum Levels of Unsaturated Free Fatty Acids Are Associated With Disease Severity in Human Acute Pancreatitis Anna C. Evans, James P. DeLany, Kimberly Stello, Stephen J. O'Keefe, Georgios I. Papachristou, David C. Whitcomb; Poster

Digestive Disease Week Tuesday May 9, 2017: Effects of unsaturated free fatty acids (uFFA) on human endothelial cells: Is there a threshold uFFA level causing cell toxicity and death in acute pancreatitis? Anna C Evans, Annette S Wilson, Georgios I Papachristou, David C Whitcomb; Poster

Digestive Disease Week Tuesday May 9, 2017: Activin in acute pancreatitis: potential risk-stratifying marker and novel therapeutic target
Jonas Staudacher, Cemal Yazici, Timothy Carroll, Nancy Krett, Jessica Bauer, Yinglin Xia, Jingbo Pang, Annette Wilson, Georgios Papachristou, David Whitcomb, Giamila Fantuzzi, Barbara Jung; Poster

American College of Gastroenterology Tuesday, October 18, 2016: A Model to Predict Transition of Systemic Inflammation to Organ Failure in Acute Pancreatitis; Annette Wilson PhD, Weiping DeBlasio RN, William Rivers BS, Efstratios Koutroumpakis MD, Georgios Papachristou MD PhD, Stephen O'Keefe MD MSc, David G Binion MD, David C Whitcomb MD, PhD; Poster

American College of Gastroenterology Tuesday, October 15, 2017: Severe Acute Pancreatitis and Shock: Are High Angiopoetin-2 Levels causing endothelial cell dysfunction and a Vascular Leak Syndrome? Annette Wilson PhD, Anna Evans Phillips MD, Kelley Woods, Kimberly Stello, David Binion MD and David C. Whitcomb MD, PhD; Poster

- **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?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: David C. Whitcomb, MD
Project Role: PI
Contribution to Project: Dr. Whitcomb oversaw all research in this project. Weekly research meetings were held to disseminate progress.

Name: David G. Binion, MD
Project Role: Co-Investigator
Contribution to Project: Dr. Binion provided assistance with experiments in this project and participates in research meetings.

Name: Annette S. Wilson, PhD
Project Role: Laboratory Manager
Contribution to Project: Dr. Wilson coordinated the experiments and performed imaging and data analysis. She participated in the weekly research meetings.

Name: Weiping DeBlasio, RN
Project Role: Research Nurse Coordinator
Contribution to Project: Mrs. DeBlasio consented patients currently in the study. She transported the blood samples to the research lab and assisted in processing, aliquotting, and storing samples. She attended weekly research meetings.

Name: William M. Rivers
Project Role: Research Technician
Contribution to Project Mr. Rivers was responsible for endothelial cell isolation, cell maintenance, and set up of experiments.

Name: Kelley Woods, RN
Project Role: Research Nurse Coordinator
Contribution to Project: Mrs. Woods replaced Mrs. DeBlasio as nurse coordinator.

Name: Shari Reynolds
Project Role: Clinical Research Coordinator
Contribution to Project: Mrs. Reynolds replaced Mrs. Woods as clinical coordinator.

Name: Juan Castaneda
Project Role: Research Technician
Contribution to Project Mr. Castaneda replaced Mr. Rivers as Research Technician.

Trainees:

Efstratios Koutroumpakis MD was an international research scholar who worked with the severe acute pancreatitis team during it's inception. He helped with establishment of the final IRB protocol and consenting, helped in patient ascertainment and he learned sample processing. Based on this experience he helped develop the international APPRENTICE study of acute pancreatitis. He obtained an internal medicine residency position in Albany, NY and is now doing a fellowship in Houston, TX. He continues to participate in translational research projects.

Amir Gougol MD was an international research scholar who joined the pancreas research team following Dr Koutroumpakis. He learned all of the protocols and assisted the clinical research coordinators in patient identification, consenting, ascertainment and data collection. He also participated in the initial data cleaning and data analysis. He also obtained an internal medicine residency position and recently matched to be a GI Fellow at the University of Pittsburgh, where he plans to continue his training.

Pedram Paragomi MD is an international research scholar who worked with, and replaced Dr Gougol. He has taken an leadership role in the data analysis, including writing the initial drafts of clinical pancreatitis papers. He plans to continue as a postdoctoral fellow for the next year to complete his role on the project.

Xiping Tang MD is a recent addition to the translational team. She is a visiting scholar who is working on the analysis and reporting of the metabolomics data, which assesses differentially expressed small molecules that are associated with organ failure. She has exciting findings, an has submitted an abstract to a national meeting while completing the manuscripts on the key findings.

Anna Evans Phillips MD Ms is a physician-scientists who worked extensively on the study as a research fellow through an NIH T32 program led by Dr. Whitcomb. She participated in all aspects of the study, including clinical ascertainment, data organization and analysis, cell culture and toxicity work and analyte measures. Her major focus has been on the role of fatty acids in epithelial cell toxicity.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

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.

Appendices

SCIENTIFIC REPORTS

OPEN

Activin in acute pancreatitis: Potential risk-stratifying marker and novel therapeutic target

Received: 19 April 2017
Accepted: 12 September 2017
Published online: 06 October 2017

Jonas J. Staudacher[1], Cemal Yazici[1], Timothy Carroll[1], Jessica Bauer[1], Jingbo Pang[2], Nancy Krett[1], Yinglin Xia[1], Annette Wilson[3], Georgios Papachristou[3,4], Andrea Dirmeier[5], Claudia Kunst[5], David C. Whitcomb[3], Giamila Fantuzzi[2] & Barbara Jung[1]

Acute Pancreatitis is a substantial health care challenge with increasing incidence. Patients who develop severe disease have considerable mortality. Currently, no reliable predictive marker to identify patients at risk for severe disease exists. Treatment is limited to rehydration and supporting care suggesting an urgent need to develop novel approaches to improve standard care. Activin is a critical modulator of inflammatory responses, but has not been assessed in pancreatitis. Here, we demonstrate that serum activin is elevated and strongly correlates with disease severity in two established murine models of acute pancreatitis induced by either cerulein or IL-12 + IL-18. Furthermore, in mice, inhibition of activin conveys survival benefits in pancreatitis. In addition, serum activin levels were measured from a retrospective clinical cohort of pancreatitis patients and high activin levels in patients at admission are predictive of worse outcomes, indicated by longer overall hospital and intensive care unit stays. Taken together, activin is a novel candidate as a clinical marker to identify those acute pancreatitis patients with severe disease who would benefit from aggressive treatment and activin may be a therapeutic target in severe acute pancreatitis.

Acute Pancreatitis (AP) is the sterile inflammation of the pancreas in response to various insults. The incidence of acute pancreatitis is rising in the developed world[1]. With an incidence of 58 cases per 100,000 inhabitants, AP is the most common gastroenterological cause of hospitalization in the United States[2]. In most cases, AP is self-limiting and resolves in the first week after symptom onset. However, a substantial subset of patients develops local complications or organ failure. Severe pancreatitis, defined by current guidelines[3,4] as persistent organ failure for more than 48 hours, is associated with high mortality rates in the range of 15–20%[5], partly due to limited therapeutic options[3].

Activin, a TGF-β superfamily member, is a cytokine with multiple context specific functions. After ligand binding to its type II receptors, activin type I receptors are activated through dimerization and phosphorylation[6], leading to the activation of canonical SMAD-dependent[7] and non-canonical SMAD-independent pathways[7,8]. Two major physiologic inhibitors are known, the competitive antagonist inhibin[9] and the specific ligand trap follistatin[10,11]. First described as a reproductive hormone upstream of follicle-stimulating hormone (FSH)[12], subsequent studies showed substantial roles in such diverse contexts as embryogenesis[13], cancer[8,14,15], and inflammation[16].

In inflammation, activin has been reported to have both pro- and anti-inflammatory functions *ex vivo*, resulting in either up-or down-regulation of a number of key inflammatory cytokines, such as IL-6, IL-1-β or IL10 in a spectrum of human and murine cell types[17–20]. *In vivo*, activin's reported actions are primarily pro-inflammatory. Systemic levels increase very early in the inflammatory response to LPS even before tumor necrosis factor (TNF)[21]. Furthermore, activin plays a central role in such diverse inflammatory conditions as an experimental model of inflammatory bowel disease (IBD)[22], asthma[23] and viral infections[24]. Given the substantial role activin

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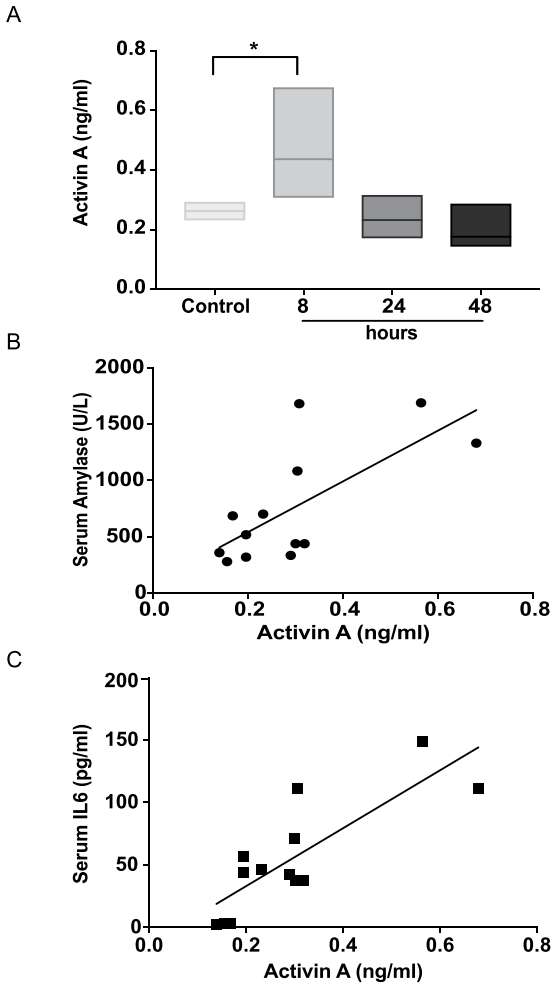

Figure 1. Activin levels are increased in a cerulein-induced model of acute pancreatitis. Panel A: Time course of cerulein treatment (hours) with activin serum levels obtained by ELISA and compared to vehicle treated controls as described in Methods. $n = 4$ for all groups except for 24 hours timepoint where $n = 5$, $p = 0.026$ per ANOVA with Dunnet's post-test. Panel B: ELISA indicating serum amlyase (U/L) compared to activin levels for each cerulein-treated mouse. $n = 13$; $r = 0.69$, $p < 0.05$ per Pearson product-moment correlation coefficient. Panel C: ELISA of serum IL6 (pg/ml) compared to activin levels for each cerulein-treated mouse. $n = 13$, $r = 0.818$, $p < 0.001$ per Pearson product-moment correlation coefficient.

plays in these conditions, we hypothesized that activin may be upregulated in AP, and constitute a potential marker of disease severity or a novel therapeutic target.

Treatment of severe AP is a clinical challenge and currently limited to supportive care with pain control and aggressive hydration therapy[3]. Despite studies demonstrating a possible clinical benefit when started early[25], the use of broad spectrum antibiotics remains controversial and is not recommended in the US[3]. Despite the identification of several candidate biomarkers purported to associate with severe acute pancreatitis[26,27], risk stratification of pancreatitis patients has proven difficult, and a simple and reliable method to identify patients at risk for developing severe AP is lacking[28]. Such a tool would have the potential to reduce overall health care cost through reduction of hospitalization and increase survival through early aggressive treatment in patient populations at risk while allowing for early discharge of patients with mild disease.

Here, we present the first study assessing activin as a potential marker and/or therapeutic target in AP.

Results

Activin is increased in mild AP *in vivo* and correlates with markers of disease severity. To investigate activin's role in AP, we first investigated systemic activin levels in a standard, well-characterized murine model of AP, in which intraperitoneal (IP) cerulein peptide injections induce a mild edematous form of acute pancreatitis observed by changes in pancreatic histology (Supplementary Figure 1). We observed an approximately 2-fold increase in circulating activin ligand at 8 hours (average 0.5655 ng/ml versus 0.26 ng/ml; $p = 0.026$) when compared to the control group, but no change at 24 or 48 hours (Fig. 1A). Activin levels in animals with AP correlated strongly with circulating amylase ($r = 0.69$, $p < 0.05$), a marker for pancreatic tissue damage, and

very strongly with IL-6 ($r = 0.818$, $p < 0.001$), a key component of the inflammatory response (Fig. 1B and C), supporting our initial hypothesis of a role for activin in AP.

Circulating activin distinguishes severe from mild AP in a non-invasive model of severe AP. As morbidity and mortality in AP primarily occurs in severe disease, we investigated activin in a murine model of severe necrotic pancreatic disease with mortality, mimicking severe AP in humans. Since activin is increased in animals after sham-operations[29,30], we used a non-invasive induced model of AP, in which IP injections of IL-12 + IL-18 on subsequent days lead to aggressive necrotizing AP in *ob*/*ob* mice and a mild, edematous pancreatitis in wild-type animals. To verify the validity of the model, we histologically scored the pancreas for edema, lymphocytic infiltrate, acinar necrosis, and fat necrosis. As published previously[31], the *ob*/*ob* animals displayed a more aggressive pancreatitis phenotype after IL-12 + IL-18 stimulation compared to wild-type animals (median histologic score 11/12 versus 6/12, $p < 0.001$). We then measured an array of cytokines critical in the inflammatory response in AP and observed cytokine patterns similar to those reported in human disease with marked increases in IL-6, IL-10, interferon-gamma and TNF confirming the applicability of our model. As anticipated[31], cytokines were higher in severe disease when compared to mild disease (Supplementary Figure 2). Mice with severe disease also displayed a higher level of activin when compared to mild disease (Fig. 2A). We observed no change in systemic activin levels in mild AP compared to animals treated with vehicle control at any time point. However, as early as 4 hours (Fig. 2B), we observed marked and highly statistically significant increases in activin levels in severe AP. Activin levels also strongly correlated with macroscopic necrosis ($r = 0.903$, $p < 0.0001$) (Fig. 2D) and histologic severity score ($r = 0.5722$, $p < 0.001$) (Fig. 2E). Interestingly, of all histologic parameters scored, the correlation was strongest with histologic fat necrosis ($r = 0.686$, $p < 0.001$).

Next, we performed Receiver Operator Characteristic (ROC) analysis to determine whether the level of circulating activin may distinguish between mild and severe pancreatitis. Comparing all animals with mild and severe disease, respectively, activin proved to be an excellent marker for severe disease with an area under the curve (AUC) of 0.928. In summary, activin *in vivo* strongly correlates with disease severity and is an excellent marker to distinguish animals with mild and severe pancreatitis, respectively.

Increased activin levels in severe pancreatitis are independent of mouse genotype and correlate with reported mortality rates. *Ob*/*ob* animals are leptin deficient due to homozygous mutation in the *ob* gene[32]. To confirm that increased activin levels in pancreatitis are independent of leptin deficiency, we used a complementary IL-12 + IL-18 induced model of necrotizing AP based on a high fat diet leading to diet-induced obesity (DIO). Animals with DIO develop severe pancreatitis, even though the phenotype is somewhat milder when compared to AP induced in *ob*/*ob* animals[33]. Induction of circulating activin was observed only in animals with DIO (Supplementary Figure 3), but not in control animals on normal chow. The increase in systemic activin levels correlated well with the mortality rates in our models of severe AP, which was reported to be approximately twice as high in the *ob*/*ob* mice (80–90%) when compared to the DIO animals (30–50%) and confirmed that the increase in activin levels in *ob*/*ob* mice with pancreatitis is independent of leptin.

Continuing Activin inhibition conveys survival benefit in severe AP. Next, we explored whether inhibition of activin may lead to an improved outcome in severe AP. As the clinical challenge in treating AP is to reduce mortality in severe disease, we monitored mortality in a model of severe AP as an endpoint. Follistatin is a physiologic and specific antagonist that inhibits activin through trapping the ligand with high affinity[10,11]. Follistatin has previously been used to inhibit activin in murine models of IBD[34] and endotoxemia[16]. One limitation to this approach is the short *in vivo* half-life (below 15 minutes[35]) of follistatin limiting bioavailability and the compound cost which limits the use of repeated dosage. Given these constraints, we assessed whether early administration may have any effect on ensuing mortality. Consistent with a causative role of activin in severe AP, we observed a biologically relevant and statistical significant, albeit modest, reduction of mortality on the first day of treatment (23% versus 0%, $p < 0.05$) with an overall favorable hazard ratio of 0.579, which did not reach statistical significance. ($p = 0.3888$, 95% CI 0.176 to 1.908) (Supplemental Fig. 4).

In a second approach, we used a neutralizing antibody against activin which has been reported to block activin *in vivo*[36]. The serum half-life of monoclonal IgG antibodies in adult mice is estimated to be 6 days[37] which obviates the short half-life issue of the follistatin approach. In addition, preliminary experiments in pancreatic cancer tissue culture cell lines demonstrate that the activin neutralizing antibody blocks activin functions as measured by a decrease in SMAD2 phosphorylation (data not shown). Acute severe pancreatitis was induced by IL-12 + IL-18 injections in *ob*/*ob* mice as detailed above. The mice were divided into two arms, one receiving the activin neutralizing antibody (anti-activin) and the second arm receiving a non-specific IgG (IgG). The mice were monitored for mortality for 7 days. As shown in Fig. 3, the mortality in the anti-activin group was 1/10 while the mortality in the non-specific IgG group was 6/9 (Mantel-Cox $p = 0.0189$ and Hazard ration of 0.1426 (95% CI 0.028 to 0.7251) reducing the mortality from 66% to 10%.

Serum activin is increased in AP, strongly correlates with severe pancreatitis, and is predictive of worse prognosis in patients with pancreatitis. After these promising results in our animal models, we proceeded to investigate activin levels in a human cohort of AP, consisting of a total of 30 cases with 10 cases of mild, moderate and severe pancreatitis (as per revised Atlanta criteria[4]) respectively and 30 controls. Serum was collected as close to hospital admission as possible, and on each of two subsequent days. Overall, serum activin levels were increased in acute pancreatitis samples when compared to controls (0.965 ng/ml versus 0.462 ng/ml, $p < 0.0001$) (Fig. 4A). When grouped by severity, activin was specifically increased in moderate and severe AP, but not in mild disease ($p < 0.0001$ for difference in between groups, $p < 0.05$ for moderate versus controls, $p < 0.0001$ for severe versus controls, mild versus controls n.s.) (Fig. 4). This effect was seen both in samples at admission

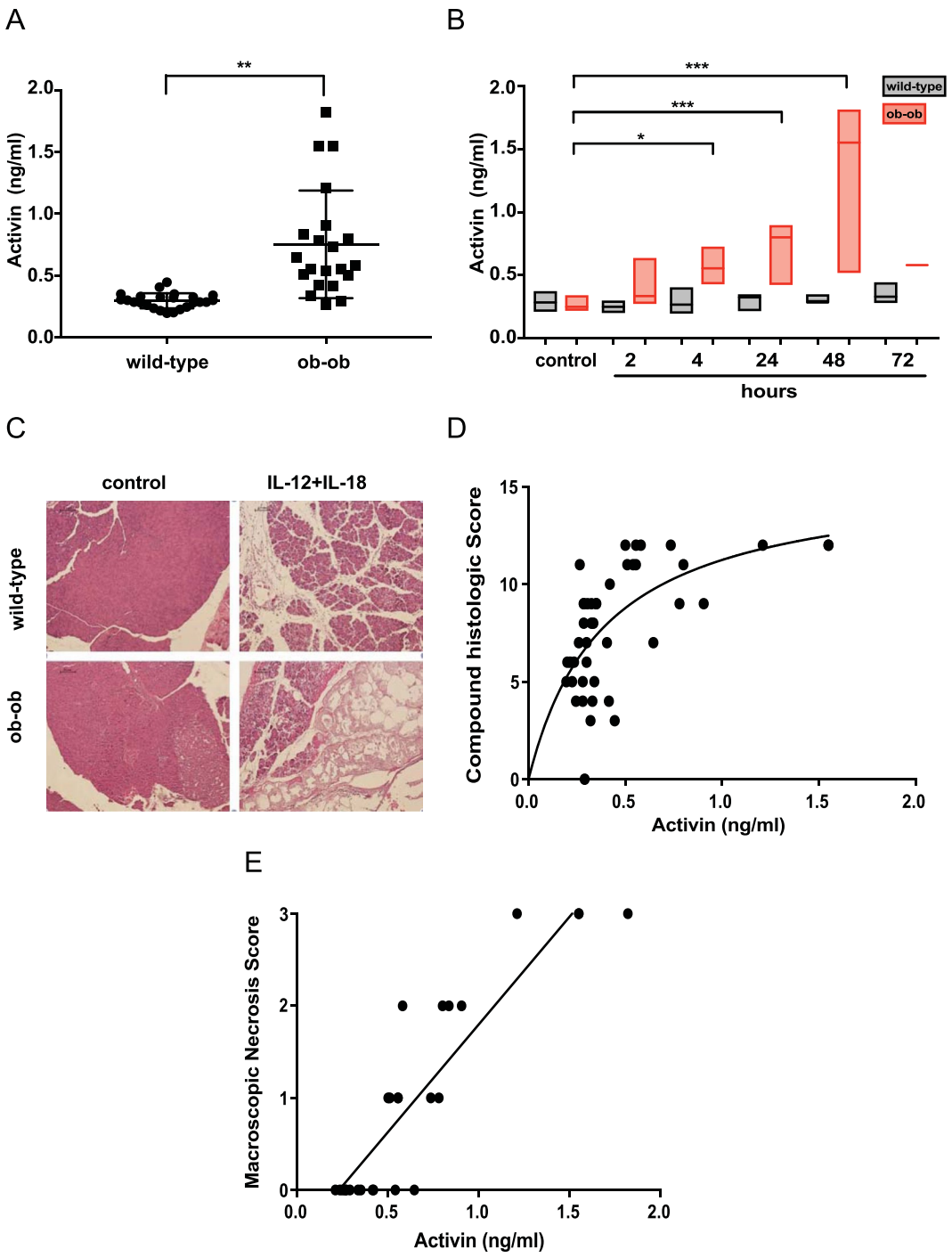

Figure 2. Activin levels are strongly increased in a model of severe necrotizing pancreatitis. Wild type and *ob*/*ob* animals were treated with IP injections of IL-12 + IL-18 on subsequent days as described in Methods, which leads to severe necrotizing pancreatitis in the *ob*/*ob* mice and mild pancreatitis in the control mice. Panel A: Serum activin levels measured by ELISA in wild-type compared to *ob*/*ob* mice ($p < 0.01$). $n = 25$ for wildtype and $n = 21$ for *ob*/*ob* animals, $p < 0.05$ per t-test Panel B: Serum activin levels in wild-type (gray bars) and *ob*/*ob* (red bars) at increasing time points. $n = 5$ per group except 72 hours in the *ob*/*ob* group where $n = 1$ due to mortality. ANOVA plus Dunnet's post-test for statistical testing. Panel C: H/E stain of wild-type and *ob*/*ob* pancreas after 24 hours of IL-12 + IL-18 treatment. Panel D: Macroscopic necrosis score compared to activin serum levels. $n = 46$; $r = 0.903$, $p < 0.0001$ per Pearson product-moment correlation coefficient. Panel E: Compound histologic score compared to activin serum levels. $n = 46$; $r = 0.5722$, $p < 0.001$ per Pearson product-moment correlation coefficient.

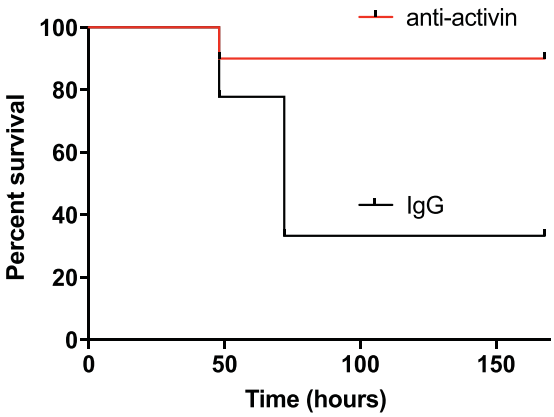

Figure 3. Activin inhibition through activin neutralizing antibody treatment is protective against mortality in acute pancreatitis. *Ob*/*ob* animals where pretreated for 30 minutes with either anti-activin antibody (red line, n = 10) or non-specific IgG (black line, n = 9) before administration of IL12 + IL18 and 24 hours after the last IL12 + IL-18 administration. Live animals were recorded on each day for one week. Hazard ratio 0.146 (0.028 to 0.7251) for anti-activin antibody versus non-specific IgG, p = 0.0189 per Mantel-Cox test.

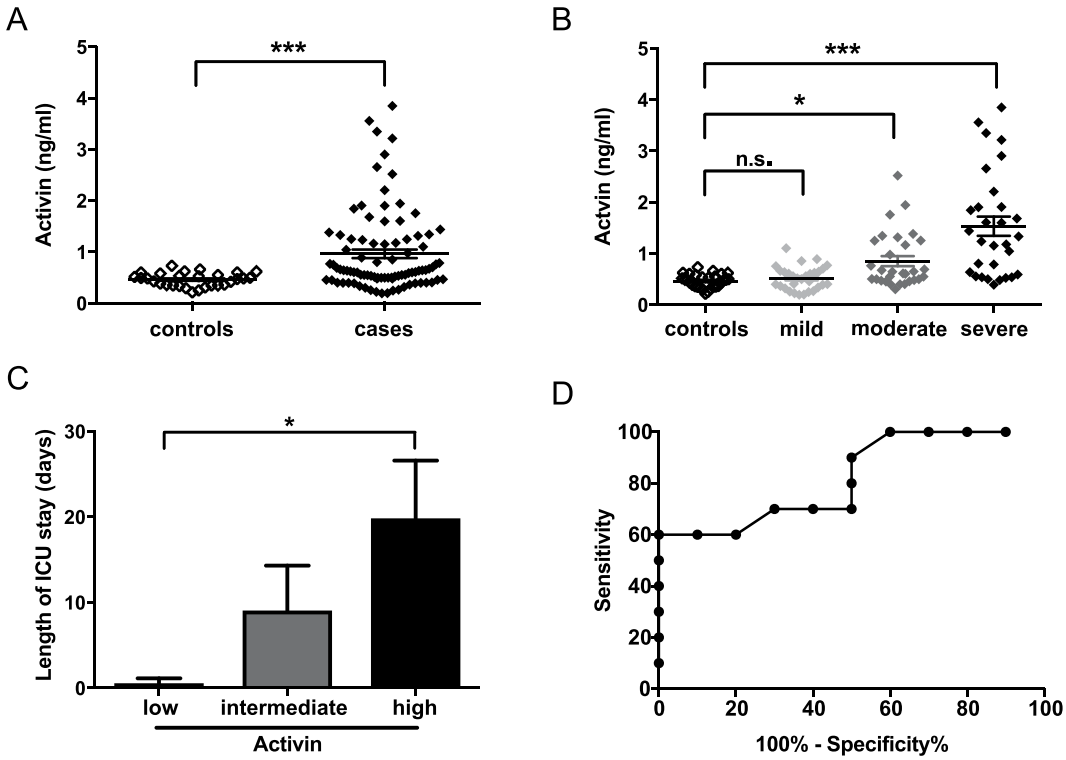

Figure 4. In patients with acute pancreatitis, activin is increased in moderate and severe disease and increased activin correlates with worse prognosis. Panel A: Serum collected from admission and two subsequent days was collected from each pancreatitis case in the cohort and serum activin measured by ELISA. Similarly, samples from one time point from healthy controls were collected and serum activin compared to pancreatitis cases. n = 30 for controls and n = 90 for cases, $p < 0.001$ per t-test. Panel B: Pancreatitis samples are grouped by severity as per revised Atlanta criteria (controls are open diamonds, mild are light gray diamonds, moderate are gray diamonds and severe pancreatitis are black diamonds). n = 30 per group, ANOVA plus Dunnet's post-test used for statistical testing. Panel C: Cases were grouped by activin levels at admission with cut-offs at 25% and 75% percentile. Average length of ICU stay is shown for each group. n = 16 for intermediate and n = 7 for activin high and low groups. ANOVA plus Dunnet's post-test used for statistical analysis. Panel D: ROC analysis for distinguishing mild versus severe pancreatitis using admission activin levels n = 20, AUC 0.820.

and comparing all samples from AP cases. Activin levels from subsequent blood draws were not statistically different from first activin measurements (Supplementary Figure 5). Importantly, high activin levels at admission were predictive of a longer hospital stay when compared to intermediate or low activin levels (median 26 versus 8 versus 5 days, $p < 0.05$, Supplementary Figure 6) and predicted a longer stay in the intensive care unit (ICU) (median 23 versus 0 versus 0 days, $p < 0.05$, Fig. 3). Also, activin levels at admission distinguished between mild and severe disease with an AUC of 0.8200 (Fig. 4), with an even higher predictive power (AUC of 0.8900) at the time of the second blood draw. As a control experiment to put our findings of activin in AP into context, we assessed circulating activin levels in a human IBD cohort. In contrast to the findings in our AP cohort, activin levels were unchanged from control in a spectrum of patients with IBD (Supplementary Figure 7, p = n.s. for difference between groups), with no correlation of activin serum levels with clinical inflammation activity. This clearly indicates that activin levels are not elevated in all inflammatory diseases, and that there may be a specific role of activin in acute pancreatitis.

Discussion

This is the first study investigating activin levels in AP, in which we demonstrate that activin, a TGF-β superfamily member and key modulator of the inflammatory response, is upregulated in two distinct animal models and a human cohort of AP. It should be noted that activin ligand exists in various isoforms. There is a limited understanding of the potential differences in the function of these isoforms in the context of inflammation. Our study focused on activin A, the most common and best studied isoform in the context of inflammation[16,38] and is referred to as activin throughout the manuscript.

To our knowledge, all preclinical model of AP have limitations[39]. To minimize those, we used two very distinct animal models of AP – a cerulein-induced standard model of mild AP, and an IL-12 + IL-18 induced model that leads to severe or mild AP depending on whether it is used in *ob*/*ob* or wild-type animals. The IL-12 + IL-18 induced model, extensively characterized in previous studies by our group[31,33], is a clinically applicable, reproducible, non-invasive model of necrotizing AP. Even though neither model mimics AP physiology with regards to etiology, the reproducibility of our animal data in our human cohort supports the use of the models we chose. It should also be noted that we used animals from a balb/c background in our cerulein experiments and C57BL6 animals in our IL-12 + IL-18 driven models, which decreases the chance that our observations are background-dependent, albeit limiting direct comparison across models.

Our clinical cohort enabled us to investigate activin for the first time in AP to include outcome, yet limitations should be kept in mind. We analyzed this cohort retrospectively and biliary or post ERCP etiologies of AP are overrepresented when compared to other published cohorts, while patients with ETOH-induced pancreatitis are not included. These will need to be addressed in future prospective studies following our pilot study reported here.

Clinically, identifying patients that will transition to more severe disease is challenging[40]. Clearly, a treatment that minimizes the deleterious effects of severe inflammation without interfering with the normal healing process is needed, as it benefits patients directly and could markedly reduce health care costs. Existing predictive tools are limited and complicated by either a complex algorithm or the need for a second time-point, or both. A simple early predictive marker especially allowing stratification regarding hospital admission or need for ICU care would have a substantial impact on the management, outcomes of AP as well as the cost-effectiveness of its care.

In this pilot study, we observed a significant correlation of elevated activin ligand levels at admission with clinical severity in AP. These observations are very promising for stratifying AP patients, especially as high activin ligand was also predictive for longer hospital stay and higher rates of ICU admission. Whether activin can be combined with existing clinical markers to increase predictive power of current diagnostic criterion and if high activin levels are predictive for increased mortality will be addressed in future research directions.

In addition, activin showed promise as a potential therapeutic target in AP. Our experiments demonstrated that the activin inhibitor follistatin reduced mortality at an early time point in our model of severe AP. The observed overall hazard ratio for follistatin of 0.579 is encouraging and biologically relevant. Serum half-life of follistatin is less than fifteen minutes *in vivo*[35], which limits its efficacy over time as a single dose treatment. In a second approach we utilized an antibody which neutralizes activin to inhibit activin prior to onset of severe AP in the *ob*/*ob* mouse model. We observed a robust decrease in mortality compared to non-specific IgG control animals indicating that activin has a functional role in AP-induced mortality. Future experiments will address if blocking activin after the initiation of AP can lead to a decrease in mortality providing a potential therapeutic application.

Increased knowledge of the mechanism of activin action in inflammation in general and AP specifically and better pharmacologic inhibitors will help us to fully elucidate the potential of activin inhibition in severe AP. Given the well tolerated safety profile of activin inhibition in clinical phase II studies of cancer associated anemia and the lack of current therapeutic options in AP[41], activin inhibition holds great promise as a therapeutic intervention in AP.

Activin levels have been described to be increased in other inflammatory conditions including sepsis[42,43], cystic fibrosis[44] and allergic airway disease[45]. The data obtained in our IBD cohort indicates that activin does not play a central role in all human inflammatory conditions. A close connection between activin and neutrophils has been proposed before[46]. Neutrophils play a major role in severe acute pancreatitis[47], however, IBD is thought to be a process driven mainly by T-lymphocytes[48]. Our findings might hint towards a dominant role of activin in neutrophil-driven inflammation. Additional mechanistic studies will be needed to fully understand activin's action on neutrophils. Moreover, the predominant underlying cause of mortality from AP is organ failure, which is caused by an uncontrolled self-sustained cytokine response. As serum activin is specifically increased in severe cases of AP, but unchanged in mild AP, we propose that activin plays a role in perpetuating the overshooting

cytokine response, and may not directly be connected to AP etiology per se. This is consistent with our observation that activin levels remain at control levels in the IL-12 + IL-18 mild form of AP in wild type mice, while activin levels increase as early as 4 hours in the IL-12 + IL-18 severe form of AP in ob/ob mice. This notion would make activin a prime therapeutic target in a number of conditions such as multi-organ failure in sepsis.

In conclusion, serum activin is a novel marker for the prediction of severity and hospital course of AP, as well as a potential new therapeutic target in this poorly understood and highly morbid condition.

Methods

In vivo models of acute pancreatitis. All animals used in our study were obtained from The Jackson Laboratory.

Cerulein model of pancreatitis. For the cerulein model, female Balb/c mice, 8 to 10 weeks old, received 7 hourly injections of cerulein (R&D systems, Minneapolis MN, USA) at 50 micrograms/kg, while controls mice received PBS injections[49].

IL-12 + IL-18 model of pancreatitis. For the IL-12 + IL-18 model, male C57BL6 mice were fed either regular chow or high-fat diet (diet induced obesity (DIO), 60% Kcal/fat from Research Diets, New Brunswick NJ) for 6 weeks and the experiments performed at 20 weeks of age. The mice on diet as well as the C57Bl6 female WT and *ob*/*ob* mice received two IP injections of a combination of IL-12 (150 ng/mouse) and IL-18 (750 ng/mouse) (R&D systems) 24 h apart, whereas control mice received PBS[31,33]. Cage randomization was used to assign animals to either treatment groups or time groups. Mice were euthanized at time points indicated in the figures after the end of treatments; blood and tissues were collected for analyses. To assess the effects of activin inhibition on survival, female *ob*/*ob* mice received an IP injection of human recombinant follistatin 288 (R&D systems) (10 micrograms/mouse) 30 minutes prior to each injection of IL-12 + IL-18. As a control, the mice received an equivalent injection of phosphate buffered saline alone. Survival was monitored for up to 8 days post induction of AP. As a second approach, female *ob*/*ob* mice (n = 10) received 5 mg/kg of an activin neutralizing antibody (AF388, R&D Systems) 30 minutes prior to the first IL-12 + IL-16 injection and 24 hours after the last IL-12 + IL-18 injection. This antibody was previously reported to effective in blocking activin *in vivo*[36]. As a control, female *ob*/*ob* mice (n = 9) received injections of a similar concentration of a non-specific IgG. Mortality was monitored as described above. All samples and histologic assessment of AP were blinded and coded by sample identifier. Blinding was revealed after assessment for statistical analysis.

Sample processing and histologic assessment of AP. Murine serum samples were allowed to clot at room temperature for 30 minutes, centrifuged and aliquoted. Samples were subsequently stored at −80 degrees. Pancreas, liver, and lung tissue was fixed in 10% formalin, paraffin-embedded, and sectioned at 4mm interval as previously described[50]. Macroscopic necrosis was scored as previously described. In short, severity of necrosis was scored as 0 (absent), 1 (few pinhead-sized necrotic areas without retropancreatic necrosis), 2 (moderately extended necrotic areas with moderate/extensive retropancreatic necrosis), and 3 (extensive areas of necrosis with extensive retropancreatic necrosis)[51]. Slides of the pancreas where then scored blindly for edema, inflammatory infiltrate as well as acinar and fat necrosis using a previously reported score[31].

Quantification of cytokine serum levels. Activin from human and murine samples was measured utilizing the activin A Quantikine ELISA (R&D Systems) following the manufacturer's instructions. All samples were run in duplicates after a 1:4 dilution in PBS. A custom multiplex assay for interferon-gamma, TNF, IL-6 and IL-10 was purchased from R&D Systems and run following the manufacturer's instructions. All samples were run in duplicates.

Human pancreatitis cohort. Our retrospective cohort consisted of a total of 30 cases with 10 cases of mild, moderate and severe pancreatitis respectively (as per revised Atlanta criteria[4]) and 30 controls. Under IRB approved protocols at the University of Pittsburgh, serum was collected as close to hospital admission as possible, and on each of two subsequent days. Throughout the subject's hospital course, blood was collected daily on day 1 and, while the patient remained in the hospital and sampling was possible daily through day 7, and then weekly (starting on day 14 + /− 1 day), through day 28 or until discharge whichever comes first, for noting the trend in inflammatory markers and in enzymes of pancreatic injury.

Clinical information required for severity assessment using the Revised Atlanta Classification criteria[4] and for determination of the etiology of acute pancreatitis was obtained from patient's medical records and by following the hospital course. The necessary information includes clinical parameters, routine laboratory tests, imaging results, treatment interventions and other outcome variables. The clinical cohort was established previously using clinical images reviewed for the research study by UPMC radiologist co-investigators to assess for presence of any remote organ complications important in defining complications of acute pancreatitis. Data elements were collected using standardized case report forms and entered into a secure database on initial admission and on subsequent days of hospitalization. Subjects were divided into mild, moderate and severe acute pancreatitis following the Revised Atlanta Classification system[4].

For each subject recruited, approximately one unrelated family member who did not have a history of pancreatitis was recruited as a control. In cases where there was no such control available or willing to participate, we recruited the control from general medicine outpatient clinic when he/she needed to have the blood sample drawn for other reasons or was willing to provide the blood sample for the research purpose. This provides for an unrelated control population and a balanced estimate of allele frequencies[52]. We matched the subjects and controls for ethnicity and sex.

Human Inflammatory Bowel Cohort. We received serum from a subset of the Regensburg IBD cohort consisting of 45 patients with Crohn's Disease (CD) and 46 patients with ulcerative colitis (UC). Details of this cohort have been published previously[53–56]. Both the CD and UC arms of the cohort were sub-divided into 3 categories of 15 patients each based on the state of the disease at the time of collection; namely active, chronic active or in remission according to the Vienna Classification and CD activity index (CDAI) for CD and the Truelove-Witts index for UC.

Statistical analysis. Data are expressed as mean ± SD for continuous variables, median and range for categorical variables, respectively. Statistical significance level of $\alpha = 0.05$ was set before experiments. All statistical tests were two-sided if not noted otherwise. For correlation analysis, a Pearson product-moment correlation coefficient was used. For comparison of three or more groups, one-way analysis of variance (ANOVA) test with Dunnet's post-test was used to test differences among groups and adjust multiple comparisons of each experiment group with a single control. Considering the two samples under testing have unequal variances and unequal sample sizes, for comparison of two groups, a two sided Welch's unequal variances *t*-test was utilized. The modifiers strong and very strong with regards to correlations as referred by the statistical significance of calculated R values is per published methodology[57].

To determine predictive power of activin, we performed receiver operator characteristic analysis and calculated the respective area under the curves (AUC). For overall survival and mortality at day one analysis, Mantel-Cox and one sided Barnard's test were utilized, respectively. For comparison of the rate of ICU admission in our clinical cohort, we used Fishers exact test with Freeman-Halton extension. Differences in activin levels at the different time points was investigated using a repeated measure ANOVA. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA).

Study approval. All experiments involving animals were approved by the Animal Care and Use Committee (ACUC) of the University of Illinois at Chicago and all methods were performed in accordance with the guidelines and regulations of the ACUC. Acute pancreatitis and normal control subjects were recruited into this retrospective study at University of Pittsburgh Medical Center (UPMC) sites and at the Veterans Administration (VA) Pittsburgh under protocols approved under the University of Pittsburgh Institutional Review Board (IRB). Informed consent was received for all participants prior to their inclusion in the study. The subject serum was provided to the University of Illinois Chicago as coded by number and de-identified to remove all private health information. The serum was analyzed at the University of Illinois at Chicago after determination of non-human subject research status by Institutional Review Board. Inflammatory bowel human cohort subjects were recruited under institutional approval at University of Regensburg, Regensburg, Germany and the serum analyzed at the University of Illinois at Chicago as detailed above. All human studies were performed in accordance with the guidelines and regulations of their respective institutions.

Data Availability. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions

Hypothesis was created by J.J.S. and B.J. Study was designed by J.J.S., C.Y., N.K. and B.J. Animal studies were designed and executed by J.J.S., G.F. and J.P. ELISA experiment were planned and executed by J.T. and T.C. Clinical studies (APTITUDE, SNAP and PROOF) were designed by D.C.W. and G.I.P. and managed by D.C.W., G.I.P. and A.S.W. Patient serum samples from the Regensburg I.B.D. cohort were provided by A.D. and C.K. Statistical analyses were performed by J.J.S. and X.Y. Manuscript was drafted and written by J.J.S., N.K., D.C.W., G.F. and B.J. and critically reviewed and edited by J.J.S., C.Y., J.B., N.K., A.S.W., G.I.P., G.F., and B.J. Figures were created by C.Y. and J.B.

Additional Information

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