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TITLE: Severe Alcoholic Pancreatitis-Associated Acute Lung Injury in Veterans: Risks, Mechanisms, Prediction, and Therapeutic Relevance

PRINCIPAL INVESTIGATOR: Stacie A. F. Vela, MD

CONTRACTING ORGANIZATION: Carl T Hayden Medical Research Foundation, 650 E Indian School Rd, Phx, AZ 85012-1839

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650 E Indian School Rd,

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14. ABSTRACT

Background: Acute pancreatitis is a painful, potentially life-threatening condition of the pancreas with an unpredictable course. In this study we hope to identify and propose simple and reliable ways to predict and treat acute pancreatitis.

Hypothesis: Alcohol increases systemic bioavailability of unsaturated fatty acids(UFAs). This, along with the resulting hypocalcemia and hypoalbuminemia worsen cell injury. We propose to test a novel yet simple ratio as a reliable predictor and therapeutic target in the management of alcoholic AP.

Objective: To compare the [Serum free fatty acid/ (Serum calcium x albumin)] ratio as a predictor of severe alcoholic pancreatitis in veterans vs. other classical and proposed predictors.

Methods: Patients admitted with acute pancreatitis are enrolled and laboratory results are recorded. Total of 103 patients and controls were enrolled. Serum samples obtained and sent to Mayo Clinic, Site 1, for analysis of FFA and circulating dead inflammatory cells. Echocardiogram done within 24 hours of admission. Control groups include patients who abuse alcohol but do not have pancreatitis and healthy patients. We studied the strength of associations of various risk factors for severe acute pancreatitis in comparison to the [Serum free fatty acid/ (Serum calcium x albumin)] ratio.

Conclusion: The 20-50-fold increase in serum FAEEs during alcoholic pancreatitis greatly exceeds the 2-4-fold increase in parent NEFAs which FAEEs mirror. These large changes in FAEEs resulting from their release from a visceral pool, are sustained, and independent of ethanol. These characteristics may allow FAEEs to be tested as biomarkers in challenging clinical scenarios, including the diagnosis, prognosis, and resolution of alcoholic pancreatitis.

15. SUBJECT TERMS

Acute pancreatitis, Severe acute pancreatitis, serum free fatty acids, alcohol pancreatitis, hypocalcemia, hypoalbuminemia

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Severe Alcoholic Pancreatitis-Associated Acute Lung Injury in Veterans: Risks, Mechanisms, Prediction, and Therapeutic Relevance
Final Report

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1. **INTRODUCTION:** This Final Report details the accomplishments of the 4 years of CDMRP funding of the project PR151612P1 (Severe Alcoholic Pancreatitis-Associated Acute Lung Injury in Veterans: Risks, Mechanisms, Prediction, and Therapeutic Relevance) from the time period of 9/30/2016-9/30/2020. Acute pancreatitis is a painful, potentially life-threatening condition of the pancreas with a typically abrupt onset and an unpredictable course. In this study, we attempted to identify and propose simple and reliable ways to predict and treat acute pancreatitis. Our central hypothesis was that alcohol increases the systemic bioavailability of unsaturated fatty acids generated from visceral fat lipolysis. This, along with the resulting hypocalcemia and hypoalbuminemia, worsens cell injury, resulting in multisystem organ failure (MSOF) and converting AP to SAP. Our objective was to compare the [Serum free fatty acid/ (Serum calcium x albumin)] ratio as a predictor of severe alcoholic pancreatitis in veterans vs. other classical and proposed predictors, and test it as a therapeutic target.

2. **KEYWORDS:** Acute pancreatitis, Severe acute pancreatitis, serum free fatty acids, alcohol pancreatitis, hypocalcemia, hypoalbuminemia

3. ACCOMPLISHMENTS:

3.1. **Goals:** As stated in our statement of work (SOW), the third-year goals include finalizing recruitment of patients and controls and final analysis.

3.2. **What was accomplished:** Enrollment of patients was challenging the first 2 years. We increased enrollment through aggressive efforts for the last year, however the COVID-19 pandemic forced us to stop all recruitment. By the end of year three the goals were to have enrolled an additional 140 for a total of 280 subjects. This was to include an additional 30 controls, 30 patients with alcohol abuse, and 80 with pancreatitis. We finished below our recruitment goal but we were still able to obtain meaningful data. Patient table shown below.

3.3. **Paper submitted to GASTROENTEROLOGY:** Manuscript has been submitted to Gastroenterology titled: **Serum fatty acid ethyl esters mirror the fatty acid profile independent of alcohol levels during alcoholic pancreatitis**

3.4.

Parameter	Controls	Alcoholics	Alcoholic pancreatitis
Patient number	36	25	11
Age (Yr. ± SD)	50.5±12.9	48.2±14.0	43.5±13.5
Sex (M:F)	32:4	24:1	9:2
Race (W:NW)	20:16	19:6	7:4
BMI(mean ± SD)	27.9±4.2	27.7±4.9	28.3±3.3
BUN (mg/dL)	14.8±4.8	11.6±5.0	11.6±4.7
HCT (%)	42.8±3.7	44±4.9	44±6
Albumin (gm/dL)	4.2±0.2	4.3±0.5	4.2±0.3
AST (U/L)	22±11	58±43*	59±48*
ALT (U/L)	22±20	53±45*	49±41*
WBC (10^9/L)	6.7±2.4	6.8±2.0	12.5±6.7*

3.5.

3.6. **Opportunities for training and professional development:** Dr. Vela attended Digestive Disease Week 2018, 2019 which allowed for knowledge expansion in the field of pancreatology. Due to COVID19, no conferences were attended for the last year.

3.7. **How were the results disseminated to communities of interest?** Manuscript submitted.

3.8. **Plan for the next reporting period to accomplish goals?** n/a

4. IMPACT

4.1. **Impact on the development of the principal discipline of the project:**

4.2. **What is already known about this subject?**

- Alcohol intake is associated with an increase in serum fatty acid ethyl esters (FAEE)
- The concentrations of serum FAEEs parallel ethanol concentrations.
- FAEE levels in the serum increase for 1-2 hours after alcohol intake and then return to near normal by 8 hours

What are the new findings?

- FAEEs are increased >20 fold over controls in the sera of alcoholic pancreatitis patients.
- The FAEE increase in alcoholic pancreatitis is independent of alcohol blood levels.
- Concentrations of the various FAEEs in the serum during alcoholic pancreatitis mirror those of the circulating non-esterified fatty acids (NEFA)
- Circulating FAEEs in alcoholic intoxication are unrelated to the circulating NEFA.
- Concentrations of circulating FAEEs are 0-1% of those of circulating NEFA.

How might it impact on clinical practice in the foreseeable future?

- Serum FAEEs could help in the diagnosis of alcoholic pancreatitis, especially when the history of alcohol intake is not reliable, and alcohol levels are undetectable.
- The large increases in FAEE levels over baseline could be studied as a maker of ongoing alcoholic pancreatitis.

4.3. Impact on technology transfer: Nothing to report.

4.4. Impact on society beyond science and technology: Nothing to report.

5. CHANGES/PROBLEMS:

5.1. Changes in approach and reasons for change:

5.1.1. The etiology of our low enrollment overall was primarily patient presentation. When we planned the study, the number of patients who could potentially qualify for the study was overestimated. Patients were easily identified with elevated lipase or acute pancreatitis but it seemed that they would often fall into exclusion. One of the largest groups that did not qualify were those with prior history of pancreatitis. We have the exclusion criteria of prior pancreatitis because patients with recurrent acute pancreatitis or chronic pancreatitis have more scarring and more injury to the pancreas which may decrease the acuity of their illness in subsequent flares. We maintained the goal to enroll patients with first time episodes of acute pancreatitis only throughout the study to keep the integrity of the initial project. We also had limitation with echocardiograms getting completed in a timely manner but this was overcome with the addition of echo staff.

5.2. **Actual or anticipated problems or delays and actions or plans to resolve them:** COVID 19 stopped all recruitment as we limited personnel having contact with patients unless absolutely necessary in care.

5.3. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**: Nothing to report

5.4. **Significant changes in use or care of human subjects:** Nothing to report

5.5. **Significant changes in use or care of vertebrate animals:** Nothing to report

5.6. **Significant changes in use of biohazards and/or select agents:** Nothing to report

6. PRODUCTS

6.1. **Publications, conference papers, and presentations:** Paper submitted to Gastroenterology below:

Serum fatty acid ethyl esters mirror the fatty acid profile independent of alcohol levels during alcoholic pancreatitis

Short title: FAEEs levels increase by ≈20 fold in alcoholic pancreatitis but are about 0.1% of NEFAs

Stacie Vela[1], Andre Guerra[2*], Gail Farrel[1], Shubham Trivedi[2], Christopher Rood[3], Ravinder Singh[4], Sergiy Kostenko[2], Biswajit Khatua[2*], Vijay P. Singh[2,5]

[1]Gastroenterology Section, Carl T. Hayden Veterans' Administration Medical Center, Phoenix, AZ. [2]Department of Medicine, Mayo Clinic, Scottsdale, AZ. [3]School of Medicine, Saint Louis University, MO. [4]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN. [5]Department of Biochemistry and Molecular Biology, Mayo Clinic Arizona.

** Contributed equally to the study*

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Abbreviations used in this paper: ALT, alanine aminotransferase; AP, acute pancreatitis; AST, aspartyl transaminase; FAEE, fatty acid ethyl esters; LDH, lactate dehydrogenase; OAEE, oleic caid ethyl ester; PAEE, palmitic acid ethyl ester; SAEE, stearic acid ethyl ester; TEER, trans-endothelial electrical resistance.

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Disclosures: No conflicts of interest exist.
Author contributions: VPS, SV designed and conceptualized the study. Acquisition of data was carried out by AG, GF, ST, SK, BK and VPS. Statistical analysis was done and manuscript was drafted and revised for important intellectual content by SV, AG, GF, BK, and VPS. Funding was obtained by SV and VPS and the entire study was supervised by SV and VPS.

Corresponding Author:
Vijay P. Singh, MD,
Division of Gastroenterology and Hepatology
Mayo Clinic,
Scottsdale, AZ 85259,
Phone: 480-301-4286
Fax: 480-301-7017
Email: singh.vijay@mayo.edu

ABSTRACT:

Objective: The diagnosis of alcoholic pancreatitis can be sometimes difficult without a reliable history or specific marker. While fatty acid ethyl esters (FAEEs); the products of ethanol and non-esterified fatty acids (NEFA), are elevated during acute alcoholic intoxication, their levels in alcoholic pancreatitis are unknown. We prospectively compared FAEE levels to their precursors in alcohol intoxication and alcoholic pancreatitis. **Design:** This was a prospective blinded study comparing blood levels of FAEEs, NEFAs and

ethanol in patients with alcoholic pancreatitis on the first day of admission to levels during alcohol intoxication and normal controls. Kinetics of FAEE increase was studied in mice administered alcohol or the ethyl ester of oleic acid (OAEE). **Results:** Median FAEEs were similarly elevated during alcohol intoxication (205nM; CI 71.8-515nM, p<0.001) and alcoholic pancreatitis (78.6nM; CI 3-1027nM, P<0.001) vs. controls (2.8nM; CI 0.02-1.4nM). In alcoholic pancreatitis, individual FAEEs strongly correlated with serum NEFA concentrations and type, in the absence of significantly elevated ethanol levels. However, during alcohol intoxication FAEE levels correlated with ethanol levels, but not serum NEFA. In both scenarios, FAEE concentrations were 0-1% of NEFA. In mice, alcohol caused a transient peak in FAEEs at 2 hours, while OAEE levels remained elevated 24 hours after intraperitoneal administration. **Conclusions:** During alcoholic pancreatitis, serum FAEEs remain elevated in the absence of detectable ethanol, and their profile parallels the circulating NEFAs. While their concentrations are too low to cause injury, the utility of serum FAEEs as a biomarker of alcoholic pancreatitis can be explored in future studies.

SUMMARY BOX:

What is already known about this subject?

- Alcohol intake is associated with an increase in serum fatty acid ethyl esters (FAEE)
- The concentrations of serum FAEEs parallel ethanol concentrations.
- FAEE levels in the serum increase for 1-2 hours after alcohol intake and then return to near normal by 8 hours

What are the new findings?

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- Concentrations of circulating FAEEs are 0-1% of those of circulating NEFA.

How might it impact on clinical practice in the foreseeable future?

- Serum FAEEs could help in the diagnosis of alcoholic pancreatitis, especially when the history of alcohol intake is not reliable, and alcohol levels are undetectable.
- The large increases in FAEE levels over baseline could be studied as a maker of ongoing alcoholic pancreatitis.

INTRODUCTION: Alcohol intake results in a rapid peak in blood levels within 1-2 hours, followed by elimination in about 8 hours[1]. Excess alcohol consumption can cause numerous diseases[2 3], however chronic frequent alcohol use may be undetected due to this turnover, coupled with an often inaccurate history of alcohol consumption[4]. These factors can confound the diagnosis of diseases like alcoholic pancreatitis whose risk increases with the amount consumed[5 6]. Overall, these considerations may affect clinical management.

Fatty acid ethyl esters (FAEE) are non-oxidative alcohol metabolites, which were first reported to be elevated in the pancreas and visceral fat of alcoholics dying in motor vehicle accidents[7]. The most

abundant FAEEs are ethyl esters of long chain fatty acids palmitic acid (PAEE), oleic acid (OAEE), and stearic acid (SAEE) [7]. Studies in healthy individuals given alcohol show blood FAEE levels follow alcohol by 30 minutes [1]. FAEEs may be rapidly degraded or excreted, resulting in a half-life of 58 seconds [8].

While several studies have examined the role of FAEEs in animal[9-11] and cellular models[11-14] of AP, the significance of FAEEs in human alcoholic AP is unclear. For example, it is unknown if FAEEs are elevated in human alcoholic AP, and if so how these levels compare to those during alcohol intoxication. Similarly the relationship of FAEE to ethanol concentrations, and those of the parent fatty acids remains unexplored during human alcoholic AP. Knowing the levels and behavior of FAEEs during alcoholic AP is important. A situation demonstrating the potential clinical relevance of this is if the pancreatic enzymes that leak into visceral adipose tissue and cause necrosis during AP [15-17] were to release the FAEEs present in the adipose of alcoholics [7] along with the non-esterified fatty acids (NEFA) [18-23]; FAEEs could perhaps then used as biomarkers of alcoholic pancreatitis. Similarly, if the FAEE levels in the circulation were in the range that cause cell injury [11-14], these could then be studied in more depth and compared to parent NEFA which worsen AP [18 24 25]. Such studies would help identify whether FAEEs should be a therapeutic target to reduce AP severity. We therefore aimed to characterize the profile and behavior of FAEEs in human alcoholic AP in comparison to alcohol intoxication, and used animal and cell models to understand these patterns.

METHODS:

Patient recruitment: This was done at the Carl T. Hayden Veterans' Administration Medical Center, Phoenix, AZ. All protocols were reviewed and approved by the institutional review board (IRB; Project #1123) and that of the Mayo Clinic Foundation (16-002800). The studies were conducted between May 2017 and March 2020. All consecutive patients were approached to be included in the study unless they met exclusion criteria. Normal controls were recruited by research study flyers posted throughout the hospital and through quarterly Research Department outreach tables. Patients presenting with acute alcoholic intoxication to the emergency room, or alcoholic pancreatitis within 24 hours admission were

approached after chart review of the emergency room electronic medical records along with review of the high alcohol or lipase alerts generated by the laboratory staff and sent to research staff. Diagnosis of pancreatitis was made based on the presence of at least 2 out of 3 criteria[26] as per the American Pancreatic Association/ International Association of Pancreatology Guidelines. Etiology of pancreatitis was determined based on physician evaluation of patient history. Consent: Subjects were either consented in the hospital or in an exam room located within the research department. They were presented with the informed consent and after reading it, any questions or concerns were addressed before the subject signed the form. Exclusion Criteria: Acute pancreatitis, and acute alcoholic intoxication patients: History of chronic pancreatitis, pancreatic cancer or pancreatic surgery, Congestive heart failure with ejection fraction <35%, history of myocardial infarction, history of Stage 4 renal failure and/or on dialysis, women of child bearing potential, and patients enrolled in another research study, or if patient could not be approached within 24 hours of presentation. Controls: All the above, and body mass index >35, Chronic renal insufficiency, home oxygen use, diabetes with complications including retinopathy, neuropathy. Overall 99 patients were recruited in this manner with 55 controls, 25 alcohol intoxicated patients, 11 alcoholic pancreatitis patients and 13 nonalcoholic pancreatitis patients. Since the study was focused on alcohol; thirty six age, sex, and race matched controls were chosen.

Sample collection and transport: Samples from the controls and acute alcoholic intoxication were collected without their being admitted to the hospital in an exam room or the emergency room respectively . Samples from alcoholic pancreatitis patients were collected within 24 hours of presentation, which could be the same day as presenting to the ER, or the next morning, in case a patient presented after regular work hours. The blood samples were collected in a serum container, de-identified, and labeled with code number and the time of collection. Samples were placed on ice and packed in a styrofoam container prior to being sent by courier service to the Research Team at the Mayo Clinic,

Scottsdale, AZ, where the samples reached within 3-5 hours from collection. Serums were separated immediately, aliquoted and stored at -80C until further use.

Routine hematology and biochemistry values: Laboratory values including WBC count, hematocrit (HCT), and serum triglycerides, ethanol, Aspartate aminotransferase (AST), Alanine amino transferase (ALT), albumin, BUN of the human subjects were retrieved from the electronic medical record, and cataloged in a secure excel sheet by the staff at the Carl T. Hayden Veterans' Administration Medical Center, Phoenix, AZ. Staff at the Mayo Clinic were blinded to the values till after all lipidomic analyses (described below) were done.

Animal studies: All studies were approved by the institutional animal care and use committee (IACUC) of the Mayo Clinic (protocol number A00001961), and the Animal Care and Use Review Office of the Department of Army. 8-12 week CD-1 were from Charles River Labs (Wilmington, MA) were used. All animals were housed with a 12 hour light/dark cycle at room temperature, fed normal laboratory chow (Purina 5053 diet, LabDiet, Fortworth, TX), were allowed to drink ad libitum, and were acclimatized for at least 2 days prior to use. Ethanol was administered intraperitoneally (2.2 ml/Kg as a 50% solution in saline) as a single dose, which is representative of the blood levels noted in acute intoxication (Fig 1B). In separate studies oleic acid ethyl ester (Sigma-Aldrich, Saint Louis, MS) was injected intraperitoneally (4 gm/Kg). This dose achieves blood levels of FAEEs in the range noted in alcoholics and alcoholic pancreatitis (Fig 1D). 15-30 minutes after either agent, the mice were given 1.5 ml saline subcutaneously. Animal were euthanized at the time points indicated (4-6/group) and blood was collected by cardiac puncture, and allowed to clot for chemical analysis on the serum. Serum was immediately separated, and stored at -80°C till further analysis.

Harvest and use of pancreatic acini: the pancreas was excised from normal CD-1 mice and acini were harvested as described previously [18 25]. These were incubated in HEPES buffer containing 0.01% albumin

at 37°C for 4 hours to study the ethanol induced cell injury. Injury was calculated by measuring lactate dehydrogenase (LDH) activity in the medium as a percentage of total activity measured upon lysis by 1% tritone as described previously [18 25].

Cell culture and use: Human embryonic kidney 293 (HEK293) cells were used as described previously[25 27] for studying LDH leakage. Established human umbilical vein endothelial cell (HUV-EC) monolayers were cultured and used as described previously for measurement of ethanol's effect on trans-endothelial electrical resistance (TEER)[24].

FAEE analysis: Methods for extraction and measurement of FAEEs were derived from Kulig et al. [28]. For this 375 µl of patient plasma was added to 3 mL of cold acetone containing 55 µM ethyl heptadecanoate as an internal standard. Samples were briefly vortexed and centrifuged at 1400 g for 10 minutes. Lipids were extracted in two subsequent additions of 3mL hexane, then were combined and dried under vacuum. Samples were reconstituted in 75 µl hexane containing 10ppm caffeine and 1µl was injected via Agilent 7693 Automatic Liquid Sampler into an inlet maintained at 260°C. Gas chromatography was performed on an Agilent 7890B gas chromatograph and HP-5ms (30m x 0.25 mm ID) (5%-phenyl)-methylpolysiloxane ultra inert column. Helium carrier gas was maintained at 1mL/min flow rate. Samples were introduced onto the column with the GC oven at 80°C, upon injection the temperature increased at 30°/min to 150°C and held there for 2 min, then slowly raised to 250°C at 4°/min, and finally increased to 300°C at 20°/min and held for 2 min. The GC-MS interface was maintained at 280°C and samples were quantified using an Agilent 5977A mass spectrometer. FAEEs were identified by retention time and quantified using Selected Ion Monitoring with the following m/z: Lauric acid ethyl ester- 88, 101, 183; Myristic acid ethyl ester- 88, 101, 183; Palmitoleic acid ethyl ester- 88, 101, 194; PAEE- 88, 101, 241; LAEE- 81, 95, 263; OAEE- 88, 101, 202, 265; SAEE- 88, 101, 269; Arachidonic acid ethyl ester - 91, 105, 203. The low limit of detection for PAEE and SAEE were 1, 3 nM. The rest were all detectable at levels ≥ 10nM. Since free SA in high concentrations co-elutes with and obstructs the base ion ratio for OAEE; samples were

again dried and reconstituted in 200µl hexane and free fatty acids were selectively esterified in a 'soft' derivatization reaction to form dimethylamides, previously described by Kangani et al[29]. This shifts the retention time of stearate, enabling detection of OAEE.

Assays: Non-esterified fatty acid (NEFA) levels were measured using gas chromatography at the Hormone Assay and Analytical Services Core (Vanderbilt University Medical Center) as described previously [30]. Alcohol was measured using the colorimetric method by Pointe Scientific (Canton, OH).

Statistical analysis and graphical representation: Independent variables are shown in Box plots with mean as "+", median (solid line), boxes (interquartile range), error bars (range) and points or circles (individual values). line graphs were used for continuous variables (e.g. time courses or dose responses or correlations), with line graphs depicting mean ± standard error of mean (SEM). Spearman correlation was studied, and correlation was depicted as "R". Significance was determined at a $p<0.05$. Data for multiple groups were compared by 1-way ANOVA versus controls and values significantly different from controls were shown as (*) or with the p-value mentioned above the corresponding conditions. When comparing a time course or dose response, the P value was determined using a Student's *t*-test versus the control group. Graphing was done using SigmaPlot 12.5 (Systat Software, Inc, San Jose, CA) or using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

RESULTS:

Patients with alcoholic pancreatitis have elevated FAEEs in the absence of significantly elevated blood alcohol levels.

As seen in table 1, all groups had similar age, body mass index (BMI), and there was no significant difference in sex distribution, or race. While blood urea nitrogen (BUN), hematocrit (HCT), and serum albumin were similar in all groups at presentation, all alcoholics had a significant AST, ALT elevation vs. controls, and alcoholic AP patients had a significant elevation in WBC counts.

Patients with alcoholic AP (Alc AP), had higher serum lipase (428±294 U/L) vs. controls (37±27U/L, p<0.001), unlike alcoholics without AP (Alc) whose levels were 39±28 U/L (Fig 1A). Conversely, while alcoholics without pancreatitis had elevated serum alcohol levels (213±84 mg/dL), only 2 of 11 patients with alcoholic AP had detectable alcohol levels (Fig 1B), which were not significantly elevated compared to controls. Serum triglycerides in alcoholic pancreatitis were 424± 534 mg/dL vs. 172±104 mg/dL, in controls (p=0.056), and were 206±370 mg/dL in patients with alcohol intoxication. Interestingly, FAEEs (mean±SD) were similarly elevated in both the alcoholic intoxication (581±1234 nM, p<0.001) and alcoholic AP groups (1497±4385nM, P<0.001) vs. controls (3.2±3.9nM). Patients with non-alcoholic AP had similar FAEEs controls (1.45±2.7nM) and no detectable alcohol. Noting this large elevation of FAEEs in the alcoholic groups, we went on to study the relation of FAEEs to the levels of their precursor lipids and alcohol.

FAEE concentrations correlate with blood alcohol but not serum triglyceride or NEFA levels in alcoholics without pancreatitis.

While serum FAEEs in alcoholics without pancreatitis increased with alcohol concentrations (R=0.67, p=0.002; Fig 2A), FAEE concentrations did not correlate with the serum triglyceride or non-esterified fatty acid (NEFA) levels (Fig 2B, C). Similarly, concentrations of individual FAEEs, i.e. PAEE, SAEE, and OAEE did not correlate with the corresponding fatty acid concentration (Figs 2C-F). These suggest that serum FAEE in alcoholics are related to alcohol blood levels, but not the fatty acid levels.

FAEE concentrations increase with serum triglyceride and NEFA but not blood alcohol concentrations in alcoholic pancreatitis.

We then studied the FAEE concentrations on patients with alcoholic AP. In this group FAEE concentrations were unrelated to the blood alcohol levels (Fig 3A), but increased with the serum triglyceride and, NEFA concentrations (Fig 3B, C). Concentrations of individual FAEEs, PAEE, SAEE and OAEE (Figs 3D-F) also

increased with their corresponding serum NEFA concentrations. Thus FAEE concentrations in alcoholic pancreatitis increase with the concentrations of the parent fatty acids and precursor triglycerides independent of blood alcohol levels.

Intraperitoneal FAEEs cause a sustained increase in serum levels, while alcohol causes a transient FAEE increase, and a dose dependent cell injury.

We then compared the relative contributions of acute alcohol intoxication to serum FAEE levels, vs. release of FAEE from visceral stores, as may happen in acute pancreatitis. For this we studied the kinetics of FAEE elevation in mice. Administration of OAEE intraperitoneally (dashed blue line, Fig 4) did not affect PAEE, or SAEE levels (Fig 4 A, B), but caused a gradual and sustained increase in serum OAEE (Fig 4C.) These levels peaked at 8 hours and remained elevated up to 24 hours, equivalent to the highest quartile of FAEEs noted in humans (Fig 1D). However, alcohol induced FAEE increase had a different pattern. Mice given a single dose of alcohol (2.2ml/kg), which is in the range of blood levels found in alcoholics (Fig 2A; up to 4.5 gm/Kg), increased blood alcohol to 104±30 mg/dL at 2 hours, similar to that noted during alcoholic intoxication in humans (Fig 1B). This caused a peak of all 3 principal ethyl esters at 2 hours (black line Fig 4) which were again in the highest quartile of FAEEs noted in alcoholics (Fig 1D). While these approached baseline 8 hours later, PAEE and SAEE remained significantly elevated, consistent with the slower elimination of saturated FAEEs described previously[31]. In vitro studies showed ethanol at concentrations ≥ 500 mg/dL increased LDH leakage from HEK293 cells, pancreatic acini (Fig 5A, B) and caused a drop in trans-endothelial electrical resistance (TEER) of transformed human umbilical vein endothelial cells (HUV-EC) monolayers (Fig 5C). Thus ethanol at high concentrations can cause cell injury, and transiently increase serum FAEE during alcohol intoxication, which contrasts the sustained increase in serum FAEEs released from a visceral pool, which we noted in case of OAEE.

Circulating FAEEs concentrations are small compared to those of circulating NEFA.

Since FAEEs and NEFA are biologically active at ≥ 10 micromolar concentrations [14 32], we went on to compare their amounts in the circulation. Consistent with the median FAEE concentrations being 205 nM (CI: 72-515nM) in alcoholic intoxication, and 79 nM (CI: 3-1027 nM) during alcoholic pancreatitis vs. 2.8 nM (CI 0.02-1.4nM) in controls. (Fig 1D), these averaged at ≈ 0.15% of serum NEFA concentrations irrespective of the type of FAEE, or group of alcoholic patients(Figure 6). In contrast, serum NEFA concentrations were 475μM (CI: 349-673 μM) in alcoholics, and 812 μM (CI: 374-1318 μM) during Alcoholic pancreatitis. Thus even though FAEEs increase >20 fold over controls, their molar amounts are typically <1% of the circulating NEFA.

DISCUSSION:

In these studies, we note demographically similar individuals who consume significant amounts of alcohol have a large increase in serum FAEE levels during acute intoxication and alcoholic pancreatitis. FAEE levels in acute alcoholic intoxication correlate with the blood alcohol levels. However during acute alcoholic pancreatitis, FAEEs correlate with the serum NEFA concentrations both in amount and type of fatty acids, irrespective of alcohol blood levels.

While both alcoholic groups had evidence of mild liver injury (Table 1A), the group with alcoholic pancreatitis had elevated serum lipase (Fig 1A), along with abdominal pain and/or radiographic evidence of pancreatitis, along with elevated WBC counts (Table 1), which are consistent with ongoing inflammation from acute pancreatitis[33].

The similar levels of FAEEs in the two alcoholic groups (Fig 1C), along with the large differences in blood alcohol levels (Fig 1B) pose an interesting question about the distinct sources, and the clinical relevance of the FAEE elevation. The correlation of FAEE concentrations with alcohol blood levels in humans (Fig 2A) and the transient 2 hour peak of FAEEs we note in mice given ethanol (Fig 4) suggest rapid synthesis of

the FAEEs during acute alcohol intoxication. This transient increase correlates well with the 90-120 minute peak in ethanol and FAEEs previously reported in normal human subjects given ethanol[1]. Similarly, the return of FAEE to near baseline by 8 hours in mice is similar to the blood alcohol and FAEE kinetics noted in normal humans given alcohol[1]. The lack of correlation between FAEE types and concentrations with the corresponding serum NEFA during acute alcohol intoxication (Fig 2B-F) suggests an alternate source of the precursor lipids. While we do not identify and exact source, we do note that clinically relevant alcohol concentrations (Fig 1B) damage cell membranes, and impair function in multiple cell types (Fig 5). Such alcohol induced cell membrane damage could result in the release of preformed FAEEs from cells, or provide the lipid precursors, and enzymes[1] for generating FAEEs in the presence of alcohol[34]. The low FAEE concentrations (median <0.1% NEFA, Figure 6) relative to the total circulating NEFA concentrations support this.

In contrast, the strong correlation of FAEE concentrations and composition to that of circulating NEFAs during alcoholic pancreatitis (Figure 3) suggests a different dynamic, especially since this in independent of blood alcohol levels. This can be explained by the sustained release of FAEE from a preformed visceral pool, which in mice is seen as elevated serum OAEE levels, up to 24 hours after administering a single dose of the OAEE intraperitoneally (Fig 4C). This slower, sustained, but detectable release of intraperitoneal FAEEs in mice (Fig 4), mimics their behavior in alcoholic pancreatitis, i.e. elevated serum FAEE levels in the absence of alcohol (Fig 1B, D). The lack of PAEE or SAEE increase after administering OAEE to mice implies that the FAEEs in serum are representative of those present intraperitoneally (Fig 4B, C). Clinically this translates to the release of FAEEs from visceral fat necrosis (Fig 1D, 3B) being associated with the increase in serum triglycerides (Fig 1C, 3B) and NEFA (Fig 3C-F) during alcoholic AP. Indeed visceral fat of alcoholics has the highest FAEE concentrations [7], which would form intracellularly by the action of carboxylesterase on fatty acyl-CoA in the presence of alcohol[35] over the duration alcohol is consumed. This would also explain the similarity of FAEE composition to that of circulating NEFAs, since both would

be released from fat necrosis during pancreatitis[18-20 36]. Moreover, the elevation of serum triglycerides we note in alcoholic pancreatitis (Fig 1C), is supported by previous reports that show hypertriglyceridemia can occur secondary to pancreatitis[37]. Thus, the parallel increase in NEFA and FAEEs during alcoholic pancreatitis (Fig 3) is likely due to release from visceral adipose tissue damage around the pancreas, which commonly reported as "peripancreatic stranding" on radiologic studies[38], and is present early in acute pancreatitis[39].

We note FAEEs are present in much lower amounts (0-1%) compared to circulating NEFA (Fig 6), with typical FAEE concentrations in our patients being in the 0.01-1μM range (Fig 2C, 3C) compared to the NEFA concentrations being in the 300-1300 μM range. However the FAEE levels in alcoholic pancreatitis, being ≈20 fold over controls, provide a larger signal to noise ratio than the ≥1.5-3.0 fold increase in NEFA during acute pancreatitis[22 23]. The presence of FAEEs in the absence of alcohol (Fig 1B, D) during alcoholic AP make fat necrosis a plausible source of their release, since adipose tissue is their main reservoir [7]. Therefore, FAEEs could be studied as a biomarker for alcoholic pancreatitis. However, 1) the relatively small nanomole amounts of FAEEs vs. NEFA(Fig 3), 2) their equivalent concentrations being present in alcoholics without pancreatitis (Fig 1B, D), and 3) their lower toxicity in comparison to the parent NEFA[40], make FAEEs unlikely mediators of the disease. Thus future studies could be designed to see if FAEEs can improve the diagnostic accuracy of alcoholic pancreatitis, when a reliable history cannot be obtained or alcohol levels are undetectable.

Our study has some limitations, including the small number of alcoholic AP patients (n=11), two of whom did not have serum triglycerides available (Fig 3B). However the strong correlation between FAEE and their adipose source is supported by the relationship between FAEEs and NEFA (Fig 3C) and reinforced by the strong correlation between serum palmitate, stearate, oleate and their ethyl esters (Fig 3D-F). The timing of the blood draws from the patients with alcoholic AP was within 24 hours of admission, with the

first clinical sample being used for lipase and other admission labs to make a diagnosis of AP. While this may have been delayed compared to blood samples tested for FAEEs in acute alcohol intoxication, making the interval from the last drink >24 hours in AP patients ; the presence of FAEEs without detectable levels of alcohol in 9 of the 11 AP cases supports our conclusions that FAEEs during alcoholic AP are unrelated to alcohol levels. This is further supported by the studies of Soderberg et al on acute alcohol intoxication, where FAEEs were undetectable by the end of the first day[1]. Lastly we do not identify the exact source of FAEES in acute alcoholic intoxication. However, we do provide a couple of plausible explanations which are consistent with the alcohol blood levels of our patients (Fig 1B), and how these may correlate with FAEE levels (Fig 2A) irrespective of circulating NEFA. Whether FAEEs are released from a preformed intracellular pool by alcohol mediated cell injury (Fig 5), or by rapid synthesis from lipid precursors in proportion to the high alcohol concentrations [35], or something else, remains to be determined.

In summary, we note that FAEEs are elevated in the blood of acutely intoxicated alcoholics, and also during alcoholic pancreatitis. FAEE elevation during intoxication depends of the concurrent presence of alcohol, while in alcoholic AP, serum FAEEs parallel serum triglyceride, NEFA concentrations, and type of NEFA, irrespective of alcohol concentrations. While FAEE levels are small compared to their parent NEFA which worsen AP, FAEEs could potentially be studied as biomarkers of alcoholic pancreatitis.

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LEGENDS:

Table 1: Comparison of biometric and clinical laboratory parameters of control, alcoholic intoxication (Alcoholics) and alcoholic pancreatitis patients. Each box shows data for the category listed at the top of the column with the mean ± standard deviation (SD) of the parameter compared in the row. The "*" indicates a *p* value < 0.05 vs. controls on ANOVA.

Figure 1: Box plots of serum parameters in controls (Ctrl) , acute alcoholic intoxication patients (Alc) and or alcoholic pancreatitis (Alc. AP) patients. Serum lipase **(A)**, Ethanol **(B),** triglycerides **(**TG; **C)**, and fatty acid ethyl esters **(**FAEE; **D)** for each individual patient are shown as dots, the median as the horizontal line, mean as "+", the boxes show the interquartile range and error bars show the range. The "*" indicates a p value < 0.05 vs. controls on ANOVA.

Figure 2: Graphs showing Spearman correlation between total and individual fatty acid ethyl ester concentrations and serum parameters in alcohol intoxicated patients without pancreatitis. The parameters, shown on the x-axis are **A:** Serum ethanol (Etoh) **B:** triglycerides (TG) **C:** Total non-esterified fatty acid (NEFA) **D:** Palmitic acid (PA) **E:** Stearic acid (SA) **F:** Oleic acid (OA).

Figure 3: Graphs showing Spearman correlation between total and individual fatty acid ethyl ester concentrations and serum parameters during alcoholic pancreatitis. The parameters, shown on the x-axis are **A:** Serum ethanol (Etoh) **B:** triglycerides (TG) **C:** Total non-esterified fatty acid (NEFA) **D:** Palmitic acid (PA) **E:** Stearic acid (SA) **F:** Oleic acid (OA).

Figure 4: time course of FAEE elevation in mice given 3gm/Kg ethanol (Etoh; black line) or 4.0 gm/Kg oleic acid ethyl ester (OAEE; dashed blue line). Shown are A**:** Serum PAEE, **B:** Serum SAEE , **C:** Serum OAEE. The "*" indicates a *p* value < 0.05 vs. values in control mice (time zero) using a Mann-Whitney or Student's *t*-test depending on normality of distribution. Data are from 4-6 mice per group. Error bars are standard error of mean

Figure 5: Dose response of injury and dysfunction induced by ethanol in different cell types: Necrosis, as marked by lactate dehydrogenase (LDH) leakage into the medium (shown as percentage of total) after 4 hours of incubation of different concentrations of ethanol in the kidney cell line HEK293 (**A**), and mouse pancreatic acini (**B**). C: Reduction in trans-endothelial electrical resistance (TEER) of transformed human umbilical vein endothelial cells (HUV-EC). The "*" indicates a *p* value < 0.05 vs. controls with no ethanol. Data are from 3 or more separate experiments.

Figure 6: Concentrations of the various fatty acid ethyl esters in the serum shown as a percentage of NEFAs present in in the same sample. Data is shown as box plots. The boxes show the interquartile range

and error bars show the range. Values for each individual patient are shown as circles. Alc; Alcohol intoxication, Alc AP; Alcoholic acute pancreatitis.

6.2. **Journal publications:** Nothing to report.

6.3. **Books or other non-periodical, one-time publications:** Nothing to report.

6.4. **Other publications, conference papers, and presentations.** Nothing to report.

6.5. **Website(s) or other Internet site(s):** Nothing to report.

6.6. **Technologies or techniques:** Nothing to report.

6.7. **Inventions, patent applications, and/or licenses:** Nothing to report.

6.8. **Other Products:** Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7.1. **Individuals who have worked on the project:**

7.1.1. PI: Dr. Stacie A. F. Vela: no change

7.1.2. Research Coordinator: Gail Farrell: no change

7.1.3. Research Coordinator added 8/2019: Bryan Remuto, removed 7/2020

7.2. **Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:** Nothing to report.

7.3. **Other organizations were involved as partners?**

7.3.1. SITE 1, Mayo clinic Arizona. Co-PI Dr. Vijay Singh: no change

8. SPECIAL REPORTING REQUIREMENTS

8.1. **COLLABORATIVE AWARDS:** Independent report sent by Dr. Singh

9. APPENDICES: Nothing to report