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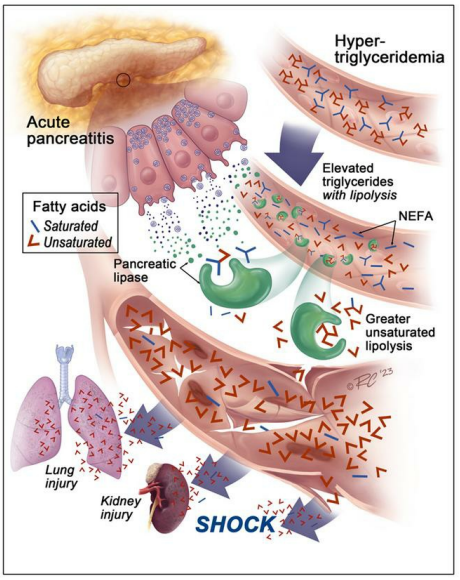
Graphical abstract



Prospective observational study and mechanistic evidence showing lipolysis of circulating triglycerides worsens hypertriglyceridemic acute pancreatitis

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Short Title: Fatty acids worsen hypertriglyceridemic pancreatitis.

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Author contributions
Acquisition of data was facilitated and carried out by PR, BK, MS, SK, AA, ME, MM, ANP, ST, BM, SJ. Analysis and interpretation were done by PR, ST, BK, BM, SK, MS, MM, YC, and VPS, who also helped in the critical evaluation of the manuscript. The manuscript was drafted by PR, VPS. Statistical analysis was done by PR, YC, and VPS. VPS and CS supervised the study and VPS designed and conceptualized the study.

Conflict of interest: The authors have declared that no conflict of interest exists.

Abstract:

Background: While most hypertriglyceridemia is asymptomatic, hypertriglyceridemia-associated acute pancreatitis (HTG-AP) can be more severe than other AP etiologies. The reasons underlying this are unclear. We thus studied whether lipolytic generation of non-esterified fatty acids (NEFA) from circulating triglycerides (TGs) could worsen clinical outcomes.

Methods: Admission serum TGs, NEFA compositions and concentrations were analyzed prospectively in 269 patients with AP. These and demographics, clinical outcomes were compared between HTGAP (TGs >500mg/dL; American Heart Association 2018 guidelines) and other AP etiologies. Serum NEFAs were correlated with the serum triglyceride fatty acids (TGFAs) alone, and with the product of TGFA x serum lipase (NEFA-TGFA x lipase). Studies in mice, rats were done to understand the role of HTG lipolysis in organ failure and to interpret the NEFA-TGFA correlations.

Results: HTG-AP patients had higher serum NEFAs and TGs and more severe AP (19% vs. 7% $p<0.03$) than other etiologies. Correlations of long-chain unsaturated NEFA with corresponding TGFAs increased with TG concentrations up to 500mg/dL and declined thereafter. However, NEFA-TGFA x lipase correlations got stronger with TGs >500mg/dL. AP, and intravenous lipase infusion in rodents caused lipolysis of circulating TGs to NEFA. This led to multi-system organ failure, which was prevented by pancreatic triglyceride lipase deletion, or lipase inhibition.

Conclusions: HTG-AP is made severe by the NEFAs generated form intravascular lipolysis of circulating TGs. Strategies that prevent TG lipolysis may be effective in improving clinical outcomes of HTG-AP.

Trial registration: Not applicable.

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Introduction: Acute pancreatitis (AP) is a common gastrointestinal disorder. The incidence of hypertriglyceridemia associated AP (HTG-AP) has been increasing with recent reports showing it to comprise up to 30% of AP (1, 2). Several metanalysis show HTG-AP increases the risk of persistent organ failure and severe AP(3-6). Even modest increases in triglycerides > 100-200mg/dL(2) increase the risk(2, 7) of severe AP. Several studies, including those by Nawaz et al (8), Pascula et al(2), and Wan et al (7) show severe HTG-AP may occur even when the hypertriglyceridemia is associated with a biliary or other AP etiology. Severe AP is requires life support(9), increases days of hospitalization(9), burden on healthcare(9), and increases mortality(10, 11). However, the mechanisms behind these observations are unclear.

Serum TGs of 100-200 mM have been noted in human HTG(12, 13), and an increase in viscosity is assumed to cause AP in HTG. However, hyperviscosity induced pancreatic ischemia seems an unlikely mechanism for AP severity (Figure 1A) since there is no evidence that hyperviscosity syndromes like Waldenstrom macroglobulinemia, and polycythemia vera cause AP(14), or worsen its severity. Additionally, while perfusion of an ex vivo-isolated pancreas with triglycerides(TGs) caused pancreatic necrosis, mineral oil infusion did not, despite its higher viscosity(15).

There is strong evidence to support the theory that unsaturated fatty acids cause severe AP. E.g. Sztefko et al. showed patients developing necrotizing AP had significantly higher serum unsaturated Non Esterified Fatty Acids(NEFAs) on admission compared to edematous AP(16). Similarly, Domschke et al. found necrotizing AP with complications had higher serum NEFAs on admission vs. those without complications(17), and Phillips et al. showed severe AP patients to have elevated serum unsaturated NEFAs(18). The unsaturated NEFA linoleic acid (C18:2) and oleic acid (C18:1) cause lung injury, shock, renal failure(19, 20), and infections(21) during AP. Such organ failure may result from unsaturated fatty acids being released from unsaturated TG(22) breakdown in visceral fat during AP(23). Saturation interferes with the hydrolysis of unsaturated TGs(23). This is due to unsaturated TG fatty acids (TGFAs) improving the “fit” of TGs into the catalytic pocket of pancreatic lipases that hydrolyze them, as detailed elsewhere(23, 24). However, whether unsaturated TGFAs in the circulation are more likely to be hydrolyzed and released as NEFA during HTG-AP is unknown.

TGs have three long chain (≥16 carbon length) fatty acids (i.e. TGFAs) covalently esterified to a glycerol backbone(23). The principal long chain NEFAs comprising the serum NEFA pool(25) are 1) stearic acid (S, C18:0), 2.) palmitic acid (P: C16:0), 3.) palmitoleic acid (PO; C16:1), 4.) oleic acid (O, C18:1), and 5.) linoleic acid (L: C18:2). Cumulatively these five NEFA comprise 90-95% of the circulating NEFA pool, of which unsaturated ones comprise 55-75%(25, 26). Serum TGs are normally in the < 2mM range (<150 mg/dL), and serum NEFA are normally 0.1-0.5 mM(27) ; increasing to the 0.6-2.0 mM range in AP(17, 21).

While previous studies have associated severe AP with unsaturated NEFA(16, 18) and higher total serum TGs; there is scarce data comparing the composition of circulating TGs with NEFA during AP. Under normal states there is a weak to moderate strength relationship between composition of NEFAs and TGs based on TGs being synthesized from NEFA; referred to as the Kennedy pathway(28). It is, however, unclear whether breakdown of TGs to NEFA can occur in the circulation due to AP. If so, this can explain increased severity during HTG-AP especially if the TGs, and NEFA are unsaturated.

Based on the above, and previous studies showing HTG-AP or elevated unsaturated NEFAs to be associated with severe AP(2, 7, 8, 10, 11), we aimed to study if HTG-AP had increased intravascular lipolysis of TGFAs to NEFA, whether this was associated with severe AP(19, 21) and the relationship of this to fatty acid unsaturation. Hence, we compared the association between serum NEFA and serum TGFA, and studied if this was strengthened by the serum lipase increase in HTG-AP. We chose serum TGs >500mg/dL for defining HTG-AP based on the definition of severe hypertriglyceridemia (29) as per the AHA 2018 guidelines.

Results:

Patients with HTG-AP have high serum triglycerides, serum NEFA and worse clinical outcomes:

Between August 2019 and November 2021, 488 patients presented to Emergency department of Mayo Clinic Arizona with lipase levels greater than 3 times the upper limit of normal. Of these, 201 patients were excluded as they did not fulfil the diagnostic criteria of AP. A flowchart showing selection of study population is shown in Figure 1B. Of the 287 AP patients, 18 patients were excluded as they lacked admission TG measurements or were duplicate serum samples. Based on chart review, 27 patients had HTG-AP and 242 patients had non-HTG-AP. As shown in Table 1, age, sex distribution, BMI, diabetes (data not shown), race, median duration of abdominal pain, and duration from sample collection to processing were similar between HTG and non-HTG-AP patients. The HTG-AP group had fewer alcoholic AP (3), and biliary AP (1) than non-HTG-AP patients (54 and 48 respectively; $p<0.001$). Eight patients with HTG-AP had documented hyperlipidemia before AP admission. Six of these were on a statin, among whom 4 were also on fenofibrate, and one was on gemfibrozil. Only one patient with HTG-AP was suspected to have a genetic cause, which however was not identified.

We first compared the serum TGs and NEFAs among the patients. As shown in table 1, HTG-AP patients had significantly elevated median serum TGs [730 mg/dL (IQR: 561-1064) or 8.5mM (6.6 – 12.5) vs. 161 mg/dL (IQR: 111-238) or 1.9 mM (1.3-2.8) in non-HTG-AP patients; $p<0.0001$]. Patients with HTG-AP had significantly higher admission serum NEFAs of 0.95mM (0.6-1.5mM) compared to non-HTG-AP patients with median NEFAs 0.61mM (0.37-0.87mM; $p<0.0001$). All the principal long chain NEFAs were increased in HTG-AP. These included palmitic acid (P), its unsaturated product palmitoleic acid (PO), and also stearic acid(S), oleic acid (O) and linoleic acid (L).

On comparing clinical outcomes, patients with HTG-AP had significantly higher organ failure rate (30% vs 10%; *p*:0.02) and severe AP (19% vs 7%; *p*:0.03) compared to non-HTG-AP patients. There was no difference in early severity [i.e., within the first week(30)] between the two groups (3/5 in HTG-AP vs. 8/16 in the non-HTG-AP group, *p*=1.0). 15/153 (9.8%) males, and 6/116 females (5.1%) developed severe AP (*p*=0.18). Patients with HTG-AP also had higher ICU admission rate (26% vs 8%; p:0.003), median length of stay (5 vs 3 days;

p:0.0002) compared to non-HTG-AP patients. 2/27 of HTG-AP patients had pancreatic necrosis, compared to 13/242 non-HTG AP patients (*p*=0.63).

We thus went on to look for evidence supporting lipolytic generation of NEFAs during HTG-AP.

Lipolysis of circulating triglycerides by pancreatic lipase generates injurious unsaturated NEFA:

To determine the role of lipolytic NEFA generation on HTG-AP severity we studied whether organ failure [which defines pancreatitis severity in humans(30)] could be induced in experimental models by intravascular lipolysis during hypertriglyceridemia. For this we first induced HTG ("H" in Figure 2A-G) in the UFA fed C57bl/6 mice (wild type) by administering them poloxamer-407 on day -1 (as described in methods). We then induced pancreatitis (HTG+AP) using interleukin (IL)12,18 in some of these (red symbols Figure 2A-G), while others were followed with only HTG (blue symbols). We also induced similar HTG-AP mice in UFA fed mice with genetic deletion of pancreatic triglyceride lipase (*PNLIP*) gene; herein described as PTL KO mice (green symbols in Figures 2A-G). Mice with HTG alone (blue symbols in Figures 2A-G) were used as controls. Mice in all groups increased serum TG from < 100 mg/dL at baseline to the 15,000- 25,000 mg/dL range within a day of inducing HTG. Serum triglycerides ere similar in all groups before AP induction (Day 0 in Figure 2B). These were 18667±2402 mg/dL in controls, 21588± 4245 mg/dL in C57bl/6 mice, and 19597±4450 om PTL KO mice (*p*=0.83) . Mice with AP (induced on day 0 after blood collection) had a significant increase in serum lipase by day 1 (Figure 2A, B). AP caused a rapid reduction in serum TGs (red dots in pink oval, Figures 2B) but not in mice with HTG alone (blue, Figure 2B) or in PTL KO mice (green dots, Figure 2B). The reduction in TGs resulted in a corresponding significant increase in serum NEFA (red dots in pink oval, Figure 2C) on day 1 of AP. Mice with HTG alone had no change in serum TGs or NEFAs on day one (Blue dots in blue ovals in Figure 2B, C). Similarly, PTL KO mice had no change in serum NEFA on day 1 of AP (Green dots figure 2C). Between day 1 and 2, mice with AP started appearing moribund requiring euthanasia. Wild type (WT) mice with HTG-AP had a higher serum creatinine at euthanasia (Figure 3D), and a pre-terminal reduction in pulse distention consistent with hypotension(19, 31) (Figure 3E). WT mice also developed generalized hypothermia (Figure 3F), along with an 80% reduction in survival by day 3 (Figure 3G). These findings are consistent with HTG-AP inducing multi-system organ failure. WT mice had 4.7±5.3% pancreatic parenchymal necrosis seen as pale

pink areas with loss of cellular detail (Arrows Figure 2H). This necrosis was reduced to 0.6±0.7% ($P<0.01$) in PTL KO mice (Figure 2I, J) Pancreatic edema averaged 2/4 in both groups of mice (Figure 2K).

To determine the specific roles of unsaturated NEFA, we compared organ failure parameters in mice given the saturated NEFA palmitic acid vs. the unsaturated NEFA linoleic acid. Both these NEFA are increased in HTG-AP patients (Table 1). Only linoleic acid increased blood urea nitrogen (BUN), lowered carotid pulse distention, which is consistent with hypotension(19, 31), worsened hypothermia (Figure 2L-N), and increased apoptotic cells in the lung, consistent with acute lung injury (Supplementary Figure 1) resulting in a moribund appearance requiring euthanasia by 3 days. Therefore, lipolytic release of unsaturated NEFA like linoleic acid may worsen AP outcomes.

Severe AP includes respiratory and renal failure. We thus studied if intravascular lipolysis of TGs worsens HTG-AP in a distinct and direct intravascular lipolysis model of HTG in rats. For this we intravenously infused TGs (GTO; Glyceryl trioleate; triglyceride composed of 3 oleic acid chains) into pre-cannulated rats alone or with porcine pancreatic lipase (PPL) and the lipase inhibitor orlistat (orli). GTO infusion increased serum TGs from 94±29 mg/dL to 233±120 mg/dL (p=0.0004; Supplementary figure 2A). As shown in figure 3, PPL infusion increase lipase (Fig 3A), which was inhibited by orlistat. As shown in Figure 3B, PPL + GTO group had the highest NEFA consistent with lipolysis of TGs, which was prevented in PPL + GTO + Orlistat group. PPL+ GTO group also had significantly higher serum BUN (Figure 3C) and lower ionized calcium (Figure 3D), which were prevented by orlistat. PPL+GTO only induced edema in the pancreas but no necrosis or inflammation while other groups remained similar to controls(Fig 3E, Supplementary figure 2B). While infusion of PPL or GTO did not cause any changes in oxygen saturation, infusion of PPL + GTO together caused a significant decrease in % oxygen saturation (Ox Sat %; Figure 3F) compared to baseline, requiring euthanasia. This was associated with an increase in lactate dehydrogenase (LDH) levels and protein concentrations in the bronchoalveolar lavage (BAL) fluid supporting lung injury (Figure 3G, H). Histologically the PPL+GTO group had fluid in the alveoli that was seen as diffuse pink staining (* in figure 3I), along with damage to the alveolar walls (arrows in Figure 3I; PPL+GTO panel). However, infusing orlistat in addition of PPL and GTO (PPL + GTO + Orlistat) prevented the drop in oxygen saturation, the LDH and protein increase, and histological changes noted in the

PPL+GTO group. Thus, intravascular lipolysis of circulating triglycerides can cause lung injury and organ failure with minimal pancreatic injury during severe AP.

Correlation of serum lipase with NEFAs increases by factoring in the corresponding TG fatty acids:

We next aimed to understand the relation between circulating NEFA and triglyceride, i.e. whether TG lipolysis led to NEFA formation in AP or if the fatty acids in TGs (i.e.. Triglyceride fatty acids; TGFA) merely correlated with their precursor NEFAs. For this we first, we correlated specific NEFAs to their TGFA concentrations for all patients, which would support synthesis via the Kennedy pathway (Figure 4A). To study if TGFA hydrolysis by lipase generates NEFA as would happened during AP, we factored in the serum lipase while correlating individual NEFAs to the corresponding TGFAs, i.e. we correlated NEFA to [TGFA x lipase]. We also correlated NEFA concentrations to the corresponding serum lipase activity for all patients.

On initial analysis, abnormal distributions were noted for individual NEFAs (Supplementary Figure 3), and also for serum lipase (i.e., enzyme), TGFAs (the substrate), and the multiplicative product of [TGFA x lipase] (Supplementary Figure 4). While serum lipase did not correlate (Spearman) with any TGFA concentration (Figure 4B), serum lipase in AP did have a weak but significant Spearman correlation with each of the major NEFAs measured (dark grey middle row figure 4C). We then correlated the concentrations of specific NEFAs to their corresponding TGFAs for all AP patients. NEFA concentrations of PA (C16:0) and palmitoleic acid i.e. POA (C16:1) correlated more strongly with corresponding TGFAs (upper row figure 4C) than with serum lipase. This NEFA vs. TGFA correlation for PA, POA was not improved by multiplying their TGFA concentrations by lipase (lowest row figure 4C) despite the later remaining stronger than the correlation with lipase. Therefore, PA and POA in the NEFA and TGFA fractions correlated independent of lipolysis, consistent with the Kennedy pathway contributing to their relationship in all AP patients. However, for total NEFAs and for specifically linoleic acid's (C18:2) NEFA, the correlation improved significantly after multiplying their TGFA concentrations by serum lipase ($p=0.03$ lower row figure 4C).

To determine the relevance of these correlations to HTG-AP we stratified the correlations by serum TG concentrations in mg/dL (Figure 4D). For this serum TGs <150 (n=107; or controls), 151-300 (n=101), 301-500 (n=35), > 500 mg/dL (n=27) were arranged on the x-axis with Spearman correlations on the y-axis. The mean,

median and other descriptors of these groups are shown in supplementary Figure 5. The black bars in Fig 4D depict NEFA-to- TGFA correlations (relevant to the Kennedy pathway). The red bars show NEFA correlations to lipase x TGFAs (supporting lipolysis). While NEFA-to-TGFA correlations of unsaturated palmitoleate, Oleate and Linoleate increased up to TGs<500 mg/dL (black * in Figure 4D), these correlations weakened at TG values >500mg/dL. Interestingly, at TG >500mg/dL, factoring in the lipase activity (red bars) further strengthened the NEFAs-to-lipase x TGFAs correlations of POA (r=0.8), LA (r=0.62) and OA (r=0.52) vs. corresponding correlations at TG <150 mg/dL (all with p<0.05, red *). Therefore, during AP, lipolysis of linoleate, palmitoleate and Oleate from their TGs increases with lipase activity at TGs >500 mg/dL. The weaker TGFA-NEFA correlations at TG>500mg/dL support this. Interestingly, neither correlation increased with TG concentrations for the saturated fatty acids SA and PA. These findings supporting lipolytic generation of unsaturated NEFA from TGs during AP and the established injurious roles of lipolytically generated unsaturated NEFAs(21, 24, 32) are consistent with HTG-AP being more severe(2, 7, 8, 14, 33-35).

We also correlated the proportions of individual NEFAs vs. TGFAs in relation to serum TG levels, double bond number (i.e. unsaturation) and fatty acid chain length (Supplementary figure 6). When comparing the correlations (cocor) among fatty acids based on double bond number using R (alpha 0.05, confidence interval 0.95) (36, 37), we noted Pearson coefficients to be significantly higher for linoleic acid than oleic acid or stearic acid (which have 18 carbon atoms; p<0.001). Among 16 carbon fatty acids, cocor for palmitoleic acid was stronger than palmitic acid (p<0.001; Supplementary Figure 6A-F). These findings are consistent with previous studies showing unsaturation improving the fit of TG in pancreatic lipases(23) . All fatty acids except stearic acid had stronger correlations at TG >200mg/dl (n=111 patients, Supplementary Figures 6G-L). Equivalent results were obtained when the cut off was >150mg/dL (n=162, data not shown). Overall, these findings agree with breakdown of TGFAs contributing to the unsaturated NEFA increased during HTG-AP.

Lastly, to experimentally verify the clinical observations, we used TGs containing Linoleic (L), Oleic (O), Palmitic (P) and palmitoleic acid (PO) in various combinations and exposed them to lipases released by acinar cells in suspension. As shown in Supplementary figure 7A lipolysis of the triglyceride of palmitic acid (C16:0), i.e. tripalmitin (PPP) was negligible, and increased significantly by replacing two acyl chains in the TG with palmitoleic acid (C16:1; PO) in PO(2)-P. Similarly, addition of the saturated palmitate (C16:0) to the

triglycerides of linoleate or oleate (i.e., Using LLP or OOP in place of LLL or OOO) also reduced lipolysis of their triglycerides by 50-80% (Supplementary figure 7B). Moreover, replacement of one L in LLP with an O, i.e., LOP further reduced triglyceride lipolysis by >90%. Therefore, while L (C18:2) has the highest fidelity of lipolysis by pancreatic lipases, adding O (C18:1) and P (C16:0) incrementally decrease this fidelity. These results support the concept that double bonds (i.e., unsaturation) in long chain fatty acids increase their lipolysis from a TG, and saturation interferes with TG lipolysis(23). Overall our results show excessive generation of unsaturated NEFA from circulating triglyceride lipolysis may cause organ failure, and worsen HTG-AP severity.

Discussion:

Here we examined the reasons underlying greater severity reported in patients with HTG associated AP (HTG-AP) (2, 7, 8). These patients had higher serum NEFAs (Table 1) that cause organ failure and severe AP (Figure 2,3). NEFAs' role in worsening clinical AP is well known(16-18), and is supported by numerous mechanistic studies(19, 21, 22, 38, 39). Here, we note that during HTG-AP serum NEFAs correlate stronger with the product of multiplying unsaturated TG fatty acid (TGFA) concentrations with serum lipase activity, than with either lipase activity or TGFAs alone (Figure 4D) . Figure 5 summarizes these findings. It shows that lipolysis during HTG-AP, preferentially generates unsaturated NEFAs, which injure cells and cause organ failure as previously shown (19) (22, 23). These findings also agree with previous studies showing that unsaturation increases TG hydrolysis to NEFA by the pancreatic lipases released in AP(23), and that HTG-AP is detrimental irrespective of AP etiology(2, 7, 8). Overall, this study shows that worse outcomes in HTG-AP are due to elevated NEFAs generated from intravascular lipolysis of the circulating TGs.

The study highlights the implications of using heparin, which releases lipoprotein lipase and may generate NEFA when used alone or during plasmapheresis to treat HTG-AP, since several studies show no improvement in outcomes in such scenarios despite a reduction in serum TGs(40-43). The study also explains worse outcomes reported in pancreatitis patients receiving parenteral nutrition(44-46), since intravenous lipid emulsions contain triglycerides that can be hydrolyzed to NEFA by the circulating lipases. It also brings forth the relevance of a lipase inhibitor (RABI-767) in phase-2 clinical trials (NCT06080789) for preventing severe AP.

There is no association reported between severe AP and hyperviscosity syndromes(14) . Moreover, patients with lipoprotein lipase deficiency (median serum TGs of 29mM or ≈ 2500 mg/dL) experience one AP episode every 3-5 years(47), and have a lifetime risk of ≈35%(48). Therefore, hyperviscosity or HTG alone do not completely explain the risk of severe AP. These observations and the current study support an alternate mechanism, i.e. intravascular TG lipolysis leads to severity. Previous studies have associated diabetes with the risk of developing HTG-AP(49, 50). While both obesity(51, 52) and HTG (18, 53, 54) are risk-factors for severe AP, the role of diabetes in severe outcomes remains unclear(55, 56). We cannot comment on the role of

alcohol in HTG-AP in the current study since the non-HTG AP group included patients with alcoholic AP (22%) based on clinical history. Moreover, the history-based distinction between “alcohol use disorder with acute pancreatitis” vs. “alcohol-associated pancreatitis” is hard to validate since current guidelines(57) use the history based consensus statement of the Zurich Workshop in 1996(58). Moreover, patients often under-report alcohol intake(59). Whether objective markers like serum fatty acid ethyl esters will improve diagnosis of alcoholic-AP remains to be seen(27).

We note the serum (pancreatic) lipase activity strengthened the correlation of NEFAs with TGFAs (Figure 4), which was most during HTG AP (Red bars in Figure 4D). Moreover, the 8-10-fold higher molar TG (i.e. substrate) concentrations in the sera of HTG AP patients [561-1064 mg/dL; i.e. 8.5mM (6.6 – 12.5mM)] vs NEFA [0.95 (0.6-1.5mM)] (table 1) make the lipolytic generation of NEFA (i.e. product) during early AP a very plausible argument. Thus, we mechanistically studied if intravascular breakdown of TGs to NEFAs can cause the organ failure previously attributed to NEFA(19, 21, 23, 24, 32).

Our experimental studies involved 2 distinct models using mice and rats. TGs peaked at 15000-25000 mg/dL (150-300mM) in mice (Figure 2B), while In rats, GTO infusion increased Serum TGs only to the 150-400mg/dL (2-5mM) range (Supplementary figure 2A). Despite these differences in TG levels, lipolysis of TGs in both models resulted in organ failure, irrespective of whether it was by intravenous lipase or interleukin induced pancreatitis. Moreover, the organ failure was independent of the extent of pancreatic necrosis. The high serum TGs in the HTG alone group (blue dots in Figure 2B) normalized slowly and uneventfully over 6-7 days by normal clearance mechanisms. These experimental studies show organ failure results from rapid NEFA increase due to circulating lipase hydrolyzing the HTG during IL12,18 induced AP (red symbols in Fig 2A-G) or intravenous lipase infusion or (Fig 3), and not by HTG alone or only elevated lipase. These are consistent with previous studies showing that a rapid NEFA increase, which exceeds the binding capacity of their carrier albumin results in higher unbound fatty acids, that eventually mediate the cellular events culminating in organ failure(21, 23, 32). This organ failure is noted as renal injury, lung injury and hypoxia, along with hypotension resulting in reduced survival. Genetic deletion of pancreatic triglyceride lipase (Green dots; Fig 2A-G), or intravenous co-administration of the lipase inhibitor orlistat (Fig 3) prevented this organ failure and improved survival by preventing hydrolysis of circulating TGs to NEFA. These data strongly support the clinical findings

that persistent organ failure, which defines severe HTG-AP is due to lipolysis of the circulating TGs to NEFA. We also note that unsaturated NEFAs like linoleic acid, which are hydrolyzed more in HTG AP (Fig 4D) also cause organ failure in mice (Fig 3H-J; supplementary figure 1), unlike palmitic acid which is hydrolyzed less and is also less aqueous stable(23).

The above findings are also consistent with our data (supplementary figure 7) and previous studies showing that fewer double bonds or saturated fatty acids in a TG molecule reduce its lipolysis by pancreatic lipases(23). For example replacing linoleic acids (L; C18:2) in the triglyceride LLL to LLP and LOP reduced their glide scores for active human pancreatic triglyceride lipase from -7.16 to -4.72 and -1.334 respectively(23). This also increased the distance between the ester linkage in TGs and the lipases' catalytic serine that hydrolyzes the ester linkage from 4.04A° to 9.99A° and 12.42 A° respectively(23). These published data are in concordance with our current correlations of LA as NEFA with corresponding TGFAs being stronger in HTG-AP than for saturated ones (Figure 4D).

The high TG levels in mice given poloxamer-407 is a potential limitation of our studies. However, serum TGs of 100-200 mM have also been noted in humans (60) and in HTG-AP(12, 13). The WT-HTG mice without HTG-AP (blue dots in figure 2A-F) normalized their TGs uneventfully over a week. In rats GTO (stock 100mM) was infused intravenously at ~5% blood volume/hour (i.e. 1ml/hour), thus delivering a blood concentration 5mM/hour. However, this only achieved only a 2-5mM concentration over 8 hours (Supplementary figure 2A). These data support a rapid physiologic TG clearance, and were considered while designing the current experiments, though the relevance of the high serum TGs in mice remains to be determined.

To summarize, the study shows that increased severity noted during hypertriglyceridemia associated pancreatitis may be due to the increased serum NEFAs generated as the result of the lipolysis of elevated circulating triglycerides during pancreatitis. Whether prevention of this lipolysis with agents in ongoing clinical trials (NCT06080789) will improve outcomes compared to modalities using heparin(40), which reduces TGs by releasing lipoprotein lipase (61) and causing lipolysis remains to be seen.

Methods:

Sex as a biological variable: All data was accrued chronologically over the period of the study, and no alterations were done based on sex of the patient. Difference in outcomes were looked for based on sex and are reported in the results section. All mice that reached ≥20% body fat by weight on the high fat diet were used in the study. These included both females and males and had similar outcomes.

Human serum processing: Patients presenting to Mayo clinic Arizona Emergency room between August 2019 and November 2021 with lipase levels greater than 3 times the upper limit of normal (ULN) were automatically flagged by the system. The initial storge, transportation (4°C), aliquoting and final storage (-80°C) of the residual serum samples collected at admission was done as described previously(21). The transportation, storage time (i.e. hours to processing) were recorded for each sample. All analyses were done at the time of first thaw from -80°C. Colorimetric triglyceride assay was done using Thermo Scientific reagent (Middletown, VA) as mentioned in the manufacturer's protocol. For fatty acid composition of TGs and NEFAs, the serum samples stored at -80°C were shipped on dry ice to Vanderbilt University lipidomic core and were analyzed by gas chromatography as described previously(19). The analysts were blinded to the nature of human serum samples. Total Oleic acid (C18:1) was calculated by adding C18:1ω9 and C18:1ω7.

Patient data collection: Patient details including demographics, diagnosis, etiology of pancreatitis, serum lipase and clinical outcomes including length of stay, intensive care unit (ICU) admission and presence and duration of organ failure were determined based on the chart review and notes by the treating physicians. Diagnosis of AP was based on patient meeting 2/3 diagnostic criteria(30) as recommended. Patients with triglycerides > 500 mg/dL were diagnosed as HTG-AP according to AHA-2018 guidelines(29) and all other patients were classified as non-HTG-AP. Organ failure of patients during hospitalization was determined based on Modified Marshall score of >2(30). The presence of organ failure for > 48 hours was considered to be persistent organ failure and required for a diagnosis of severe AP(30). We compared demographics including age, sex, race, body mass index (BMI), Diabetes, AP etiology, admission serum lipase, TGs, NEFA, ICU admission rates and organ failure rates among HTG and non-HTG-AP patients.

Statistical analysis: After blinded quantification of patients' lipid parameters, the serum TG and NEFA concentrations for palmitic, palmitoleic, stearic, oleic and linoleic acid were entered in separate columns of a master excel with one row for each patient. Other variables including demographics, and AP parameters were retrieved from chart review. Categorical data was compared using the Chi-square test. Normality of continuous sample distributions were determined, and outliers were identified using the ROUT method with a Q=1%. Continuous variables were compared using Mann-Whitney test. A p-value ≤0.05 was considered to be significant. When used, asterisks denoted P values as: "**" for P<0.01, "***" for p< 0.001, "**** for P<0.0001. For studying correlations between proportions (Supplementary figure 6), Pearson correlations (r) were measured between proportions of fatty acids in TG and the corresponding fatty acids in NEFA after excluding outliers. Strengths of correlations in figure 4 were compared using comparison of correlations (cocor) package in R(36, 37). Additionally, differences in Pearson correlations of samples with TG<200 mg/dL vs. > 200mg/dL deduced using cocor were confirmed at Online-Calculator for testing correlations: Psychometrica using the comparison of correlations from independent samples. The analyses were conducted in R 4.3.0 (R Foundation for Statistical Computing, Vienna, Austria) and all graphs were constructed using GraphPad Prism version 9. Continuous data is mentioned in text as median and interquartile range (IQR) and represented graphically as boxes ranging between interquartile range and whiskers extending from minimum to maximum levels. Categorical data was presented as percentages and depicted in the form of bar graphs.

Quality control of serum samples: The impact of storage, transportation (time) on artefactually increasing NEFAs (which are 1/10th the molar concentration of TGs; shown later in study) from TGs was accounted for by 2 methods:

1) Correlation of NEFA concentrations with duration of storage: Normality of distribution was first determined for each parameter and noted to be abnormal for each (Supplementary Figure 3). The sample's NEFA concentrations (total and individual NEFA) were then Spearman correlated with their respective storage duration (in hours). As seen in Supplementary Figure 8, there was no correlation between duration of storage and NEFA concentrations.

2) *Measuring NEFA generation under storage conditions after artificially increasing serum TG:* Baseline serum NEFA were measured. 10mM of the mixed TGs (composition shown below) were then added to patient's sera

(n=10, with serum lipase 1668±902 U/L). NEFA generation was measured daily over 3 days under conditions at which samples were stored and transported (i.e., 4^0C). The TG proportions [in brackets] added were {OOP[6], LOP[6], PLP[2], OPO[4], LLP[1], PO(2)-P[1]}X. The resulting % TGFAs in this mixture represent middle quartiles of our patients, with oleic acid at 43.3%, palmitic acid at 33.3%, linoleic acid at 20% and palmitoleic acid at 3.3%. As shown in Supplementary Figure 9, there was no significant (NS) change in serum NEFA noted on ANOVA compared to baseline whether these were compared in absolute concentrations (Supplementary Figure 9A) or as change from baseline NEFA taken as 100% (Supplementary Figure 9B.)

Reagents: Glyceryl trilinolein (LLL), triolein (OOO), tripalmitin (PPP) and mixed triglycerides 1,2-dilinoleoyl-3-palmitoyl-rac-glycerol (LLP), 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol (LOP), 1,2-dioleoyl-3-palmitoyl-rac-glycerol (OOP), linoleic acid (L), palmitic acid (P), oleic acid (O), dimethyl sulfoxide (DMSO), and porcine pancreatic lipase (PPL) were purchased from Sigma-Aldrich (St. Louis, MO). 1,2-Dipalmitoleoyl-3-Palmitoyl-rac-glycerol (POA(2)-P) was purchased from Cayman Chemical (Ann Arbor, MI). All triglycerides were sonicated as 20X stocks in phosphate buffered saline pH 7.4 before use. Tripalmitin which is a solid powder, and cannot be sonicated, was first dissolved in DMSO as a 60mM Stock, and diluted 200 times (0.5% DMSO) before use. Poloxamer-407 (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in sterile phosphate buffered saline at 80 mg/ml freshly before use. NEFA in cell media (discussed below) were measured using the LabAssay ™ NEFA kit from Fujifilm Wako chemicals. Lipase assays for cellular studies (discussed below) were done using the manufacturers protocol as previously(23). Lipase assay for human serum samples was performed using cobas® (Mannheim, Germany) according to the manufacturers protocol.

Animal studies:

Experimental pancreatitis: CD1 mice (Charles River Laboratories, Wilmington, MA) or C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were acclimatized for at least 2 days before experimentation. Mice were housed at temperatures ranging from 21-25 °C with a 12-hour light/dark cycle and allowed to drink *ad libitum*. There were 13-14 mice in each group. *Hypertriglyceridemia in mice:* 18-26 week old Male and female C57BL/6J mice were used after feeding an unsaturated high fat diet from age 6 weeks onwards as previously described (23). Hypertriglyceridemia was induced by a single 1gm/kg dose of poloxamer-407 intraperitoneally

on day -1, as described(62). Poloxamer-407 or Pluronic F127 is a nonionic detergent(63) commonly used to induce hypertriglyceridemia in mice(62). This dose was based on standardization to achieve stable hypertriglyceridemia for 48 hours while avoiding repeated dosing. There were no adverse effects noted in such mice followed for up to 1 month. *HTG-AP in mice:* The day after giving poloxamer, AP was induced by administration of the first dose of IL12,18 (i.e., day 0) as described previously(23, 64). For this murine recombinant IL-12 (Peprotech, Cranbury, NJ, 150 ng/30g) and IL-18 (R&D Systems, Minneapolis, MN, 750 ng/30g) were used. A second dose of IL12,18 was repeated a day later as per established protocol(64). Daily monitoring included general activity, carotid pulse distension (MouseOx Oximeter, STARR Life Sciences, Pittsburgh, PA) as a noninvasive measure for blood pressure(31, 65) and blood flow in conscious mice, and rectal temperatures. Mice were euthanized using carbon dioxide if appearing moribund or after 5 days, which ever came first. Blood was processed for biochemical assays (serum) as described below and previously(19, 38). *In vivo NEFA studies in mice and rats:* To minimize strain specific effects, 10-12 weeks old male CD-1 mice were used for this experiment (n=6/group) as part of a study described previously(23). The different groups were control, Palmitic acid (0.3% body weight), Linoleic acid (0.2% body weight). These were followed and euthanized as described above. Blood and histology studies were done as described previously(19, 22, 38).

Intravascular TG lipolysis in Rats: *Infusion in rats:* Pre-cannulated Wistar rats (Charles River Laboratories, Wilmington, MA) were used for the experiments. There were four or more rats in each group. These had an indwelling jugular venous catheter. Maintenance of patency was as per the instructions from the supplier. Triglyceride suspension of sterile 10% glyceryl trioleate (GTO) in sterile lactated ringers were made alone, or with orlistat (50mg/ml GTO). PPL stock (100 mg/ml) was dissolved in sterile lactated Ringers, and syringe filtered. This stock was used as 1/20 in the GTO suspension (as PPL +GTO or PPL+GTO+orlistat). Infusions were done at the rate of 18 microliters/minute for 8 hours or till the rats met euthanasia end points (e.g. moribund, tachypneic or in distress), whichever came first. Monitoring included general activity, heart rate, carotid pulse distension (MouseOx Oximeter, STARR Life Sciences, Pittsburgh, PA) as a noninvasive measure for blood pressure(31, 65) and oxygen saturation (MouseOx Oximeter, STARR Life Sciences, Pittsburgh, PA) as a measure for pulmonary function. Rats were euthanized using carbon dioxide if appearing moribund or

after 8 hours, which ever came first. Blood was processed for biochemical assays (serum) as described below and previously(19, 38). Pre terminal BUN was measured using iSTAT (Illinois, USA) as a marker of renal function. Bronchoalveolar lavage (BAL), and histology studies were done on hematoxylin and eosin-stained formalin fixes paraffin embedded sections as previously(22)

Cell Studies: Pancreatic acini were harvested from CD-1 mice in HEPES buffer as previously described and viability was confirmed to be >95%(19, 23). All triglycerides were used at a final concentration of 300mM (0.3mM) in the cellular studies. Hydrolysis of triglycerides was measured as the increase in NEFA concentrations in the medium over 15 minutes.

Statistical analysis: After blinded quantification of patients' lipid parameters, the serum TG and NEFA concentrations for palmitic, palmitoleic, stearic, oleic and linoleic acid were entered in separate columns of a master excel with one row for each patient. Other variables including demographics, and AP parameters were retrieved from chart review. Categorical data for both mice and human studies was compared using the Chi-square test. Normality of continuous sample distributions were determined, and outliers were identified using the ROUT method with a Q=1%. Continuous variables were compared using Mann-Whitney test. A p-value ≤0.05 was considered to be significant. When used, asterisks denoted P values as: "**" for P<0.01, "***" for p< 0.001, "**** for P<0.0001. For studying correlations between proportions (Supplementary figure 6), Pearson correlations (r) were measured between proportions of fatty acids in TG and the corresponding fatty acids in NEFA after excluding outliers. Strengths of correlations in figure 4 were compared using comparison of correlations (cocor) package in R(36, 37). Additionally, differences in Pearson correlations of samples with TG<200 mg/dL vs. > 200mg/dL deduced using cocor were confirmed at Online-Calculator for testing correlations: Psychometrica using the comparison of correlations from independent samples. The analyses were conducted in R 4.3.0 (R Foundation for Statistical Computing, Vienna, Austria) and all graphs were constructed using GraphPad Prism version 9. Continuous data is mentioned in text as median and interquartile range (IQR) and represented graphically as boxes ranging between interquartile range and whiskers extending from minimum to maximum levels. Categorical data was presented as percentages and depicted in the form of bar graphs.

Study approval: All human studies were approved by the Institutional Review Board of Mayo Clinic. All experiments were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. Animals were handled in accord with the Guide for the Care and Use of Laboratory animals by the Institute for Laboratory Animal Research.

Data availability: Supporting data and values can be made available from the corresponding author upon reasonable request.

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Figures with Legends:

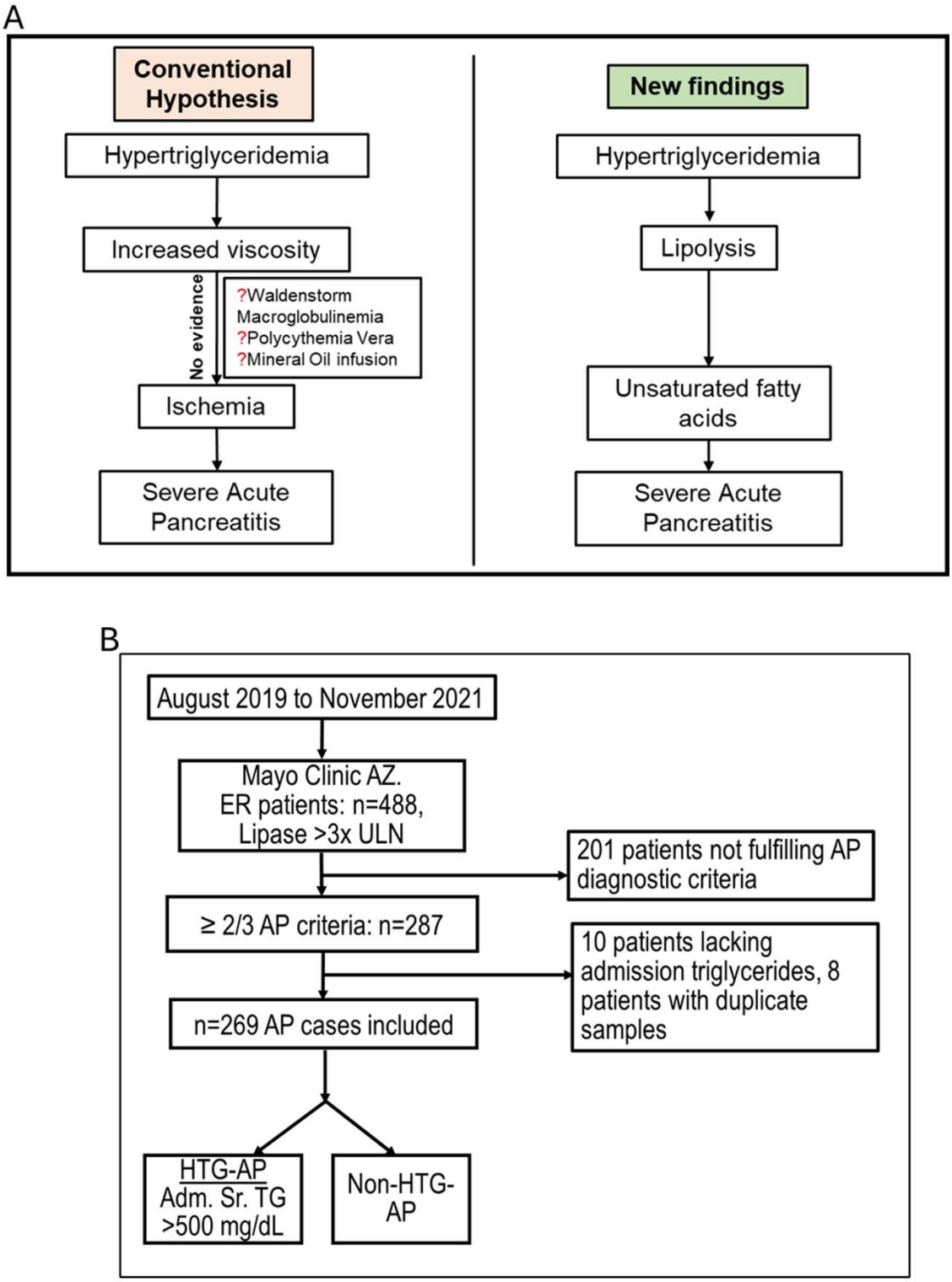


Figure 1: A) Schematic of conventional hypothesis of severity of hypertriglyceridemic pancreatitis compared to new findings. **B)** Flow chart of patient selection for the study population of patients with acute pancreatitis (AP), and classification as HTG-AP vs. Non-HTG AP**.** ULN; upper limit of normal. ER; Emergency room.

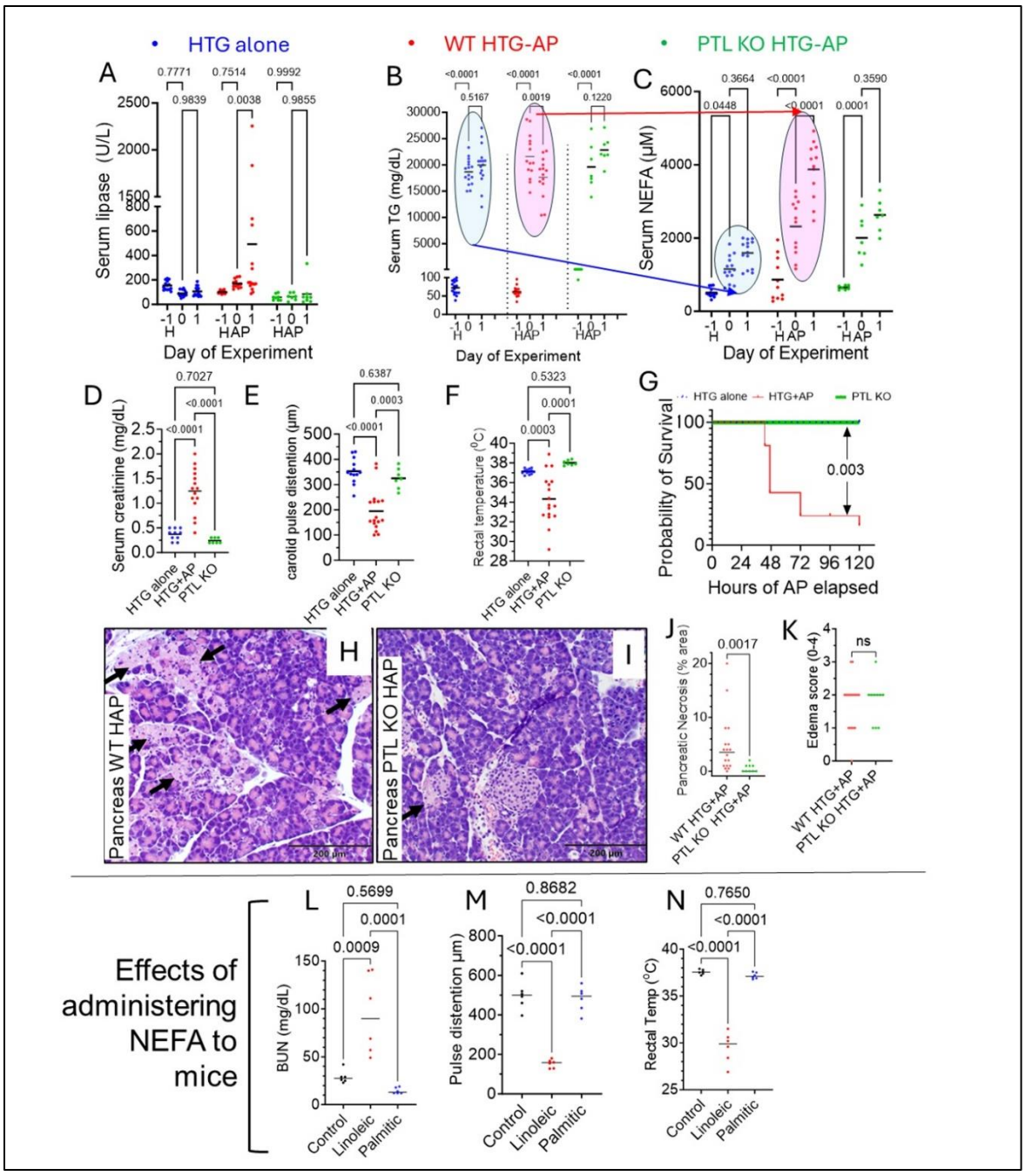


Figure 2: Parameters of mice with HTG alone or HTG-AP (A-H), those given NEFA (I-K), and *invitro* studies using pancreatic acini (L-0). A-H: Mice were given poloxamer-407 (P) on Day -1 to induce HTG (H) alone (blue). Hypertriglyceridemic AP (HAP) was induced on day 0 (i.e. one day after poloxamer-407) in C57bl/6 wild type (WT) mice (red) or pancreatic triglyceride lipase KO mice (PTL KO; green dots). Dot plots with means are shown for serum lipase (A), serum triglycerides (B), serum NEFA (C) for days mentioned on the x-axis. Serum creatinine at necropsy (D), along with carotid pulse distention (E) and rectal temperature (F) recorded before euthanasia are also shown. **G** compares the survival curves for the two groups with p-values based on the Log-rank (Mantel-Cox) test. **H, I:** Images of Hematoxylin and eosin staining pancreatic sections from WT mice (**H**) and PTL KO mice (**I**) with HAP. **J, K:** Dot plot graphs showing pancreatic necrosis **(J**) and pancreatic edema (**K**) in WT and PTL KO mice with HAP. **L-N** show dot plots with means comparing the effects of administering linoleic acid (red dots) or palmitic acid (blue dots) on serum BUN at necropsy (**L**), along with carotid pulse distention (**M**) and rectal temperature (**N**) recorded before euthanasia. Two- way ANOVA was done for panels A-F. A Log-rank (Mantel-Cox) text was done for panel G. A Mann-Whitney test was done for panel J. A Student's t-test was done for panel K and an ordinary one-way ANOVA was done for panels L-N.

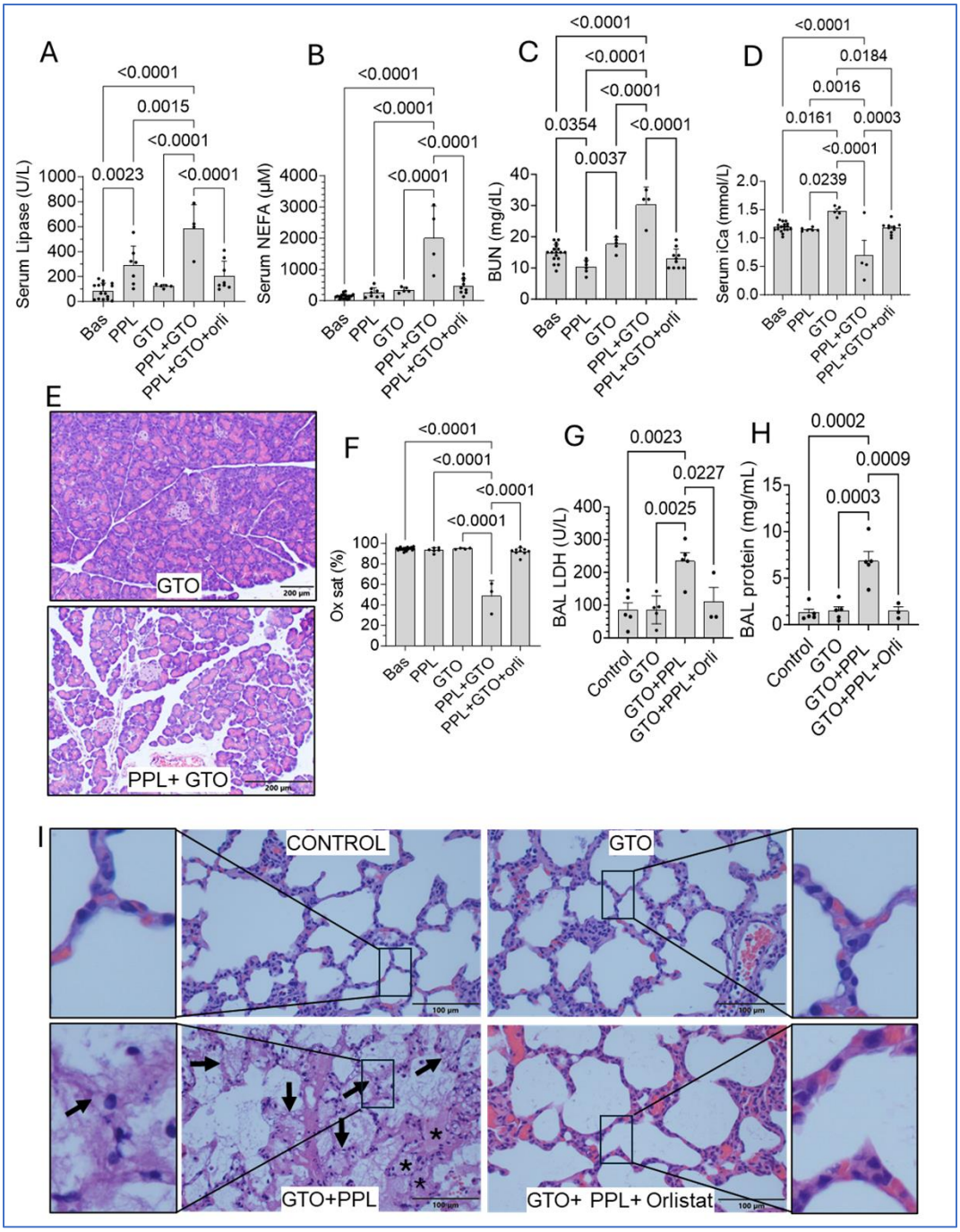
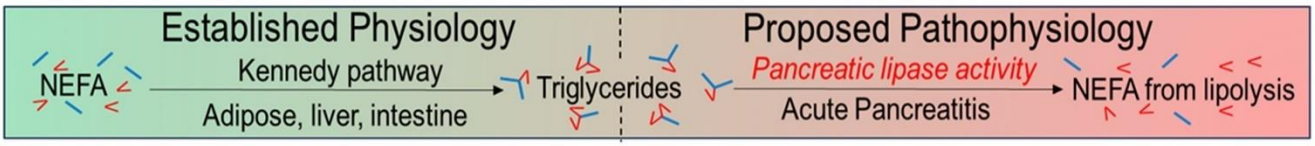


Figure 3: Effect on intravenously infusing triglyceride, compared to infusing triglyceride with pancreatic lipase with or without the lipase-inhibitor orlistat in rats. Bar graphs (with standard deviation and individual values) comparing the effects of infusing pancreatic lipase (PPL), triglyceride (GTO), pancreatic lipase and triglyceride (PPL + GTO) and pancreatic lipase, triglyceride and lipase inhibitor (PPL + GTO + Orlistat) at baseline (Bas) and after infusion. Parameters are serum Lipase (**A**), NEFA (**B**), BUN (**C**), Ionized calcium (iCa; **D**). **E** shows representative images of Hematoxylin and eosin staining pancreatic sections from rats infused GTO alone (upper panel) or GTO+PPL (lower panel). **F-H:** Bar graphs of pre-terminal oxygen saturation (**F**), lactate dehydrogenase (LDH) activity in bronchoalveolar lavage (BAL) fluid from the lungs (**G**), and protein concentrations in the BAL (**H**). **I** shows representative lung histologic images after Hematoxylin and eosin staining from each group mentioned at the top of the image. The rectangle inset is zoomed outside the image. In the PPL+GTO group, arrows point to alveolar wall damage, and asterisk show fluid filled alveoli. P-values show significance by ANOVA. PPL: Porcine Pancreatic Lipase; GTO: Glyceryl trioleate; triglyceride of oleic acid. All data in graphs are compared by ordinary one-way ANOVA with multiple comparisons.

A



B

All patients (n=263)	C16:0	C16:1	C18:0	C18:1	C18:2	Total
Correlation of lipase to TGFA (*p* below correlation)	-0.040 (0.527)	-0.061 (0.332)	-0.057 (0.363)	0.004 (0.817)	0.034 (0.580)	-0.017 (0.791)

C

All patients (n=263)	C16:0	C16:1	C18:0	C18:1	C18:2	Total
Correlation of individual NEFA to TGFA (all *p*<0.001)	0.360 (<0.0001)	0.451 (<0.0001)	0.306 (<0.0001)	0.311 (<0.0001)	0.334 (<0.0001)	0.319 (<0.0001)
Significance of correlations (COCOR *p* value)	0.019	0.000	0.134	0.104	0.109	0.103
Correlation of individual NEFA to Lipase (p value below correlation)	***0.192 (0.002)***	***0.150 (0.015)***	***0.232 (0.000)***	***0.208 (0.000)***	***0.235 (0.000)***	***0.215 (0.000)***
Significance of correlations (COCOR *p* value)	0.013	0.001	0.057	0.063	0.031	0.04
Correlation of individual NEFA to Lipase x TGFA (all *p*<0.001)	0.370 (<0.0001)	0.409 (<0.0001)	0.358 (<0.0001)	0.332 (<0.0001)	0.383 (<0.0001)	0.356 (<0.0001)

D

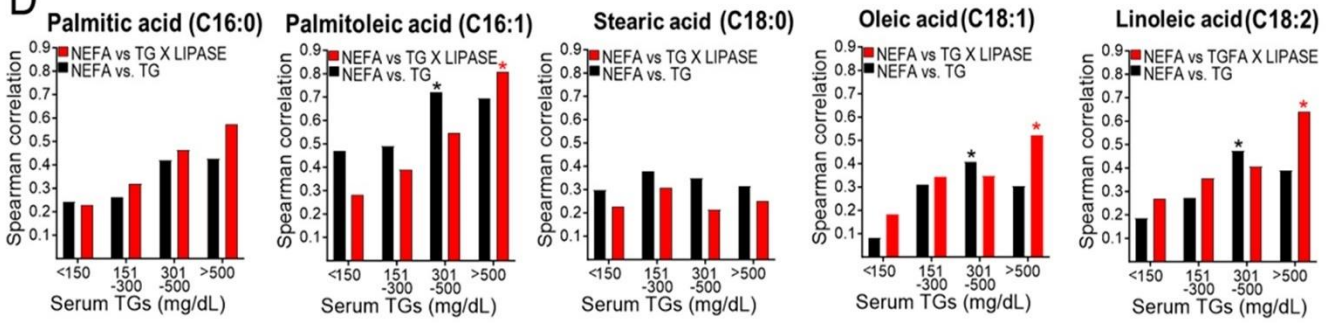


Figure 4: Effect of lipase activity on the relationship between individual NEFA and triglyceride fatty acids (TGFA) in pancreatitis patients A: Schematic comparing the Kennedy pathway (left side, green background) by which NEFAs are physiologically incorporated into the TGs for storage (adipose, liver) or transport (intestine) vs. the pathological release of NEFAs from intravascular triglyceride lipolysis by pancreatic lipases during HTG-AP (red background on the right side). **B:** correlation of serum lipase activity with individual TGFAs for all patients. Each column shows a unique fatty acid. The upper value shows the correlation coefficient, and the lower number the *p* value. **C:** All patient data; formatted as in B. Middle row (dark grey background) correlates individual serum NEFAs with serum lipase. Top row (white background) correlates individual NEFA and their TGFA concentrations. Bottom row (white background) correlates individual NEFAs and the product of serum lipase x TGFA concentrations. The light grey rows show p. values comparing the strength of correlations (COCOR as described in methods) between the middle row and corresponding top or bottom rows. Those with a p value < 0.05 are shown in red. **D:** Bar graphs of correlations (R-values) arranged by serum triglyceride concentrations (x-axis) for individual fatty acids. Each graph is for a fatty acid (mentioned above) and shows correlations of its NEFAs with corresponding TGFAs (back bars) or NEFAs with the product of the corresponding TGFA concentration x serum lipase (red bars). The asterisks show the bar with significantly stronger correlations vs. normal triglycerides i.e. <150 mg/dl. All correlations are Spearman correlations and p-values are two-tailed. The comparison of correlations COCOR between two spearman coefficients are done as mentioned in methods and p values are shown. Asterixes in panel D indicate a COCOR or <0.05 vs. the normal (<150mg/dl) triglyceride group.

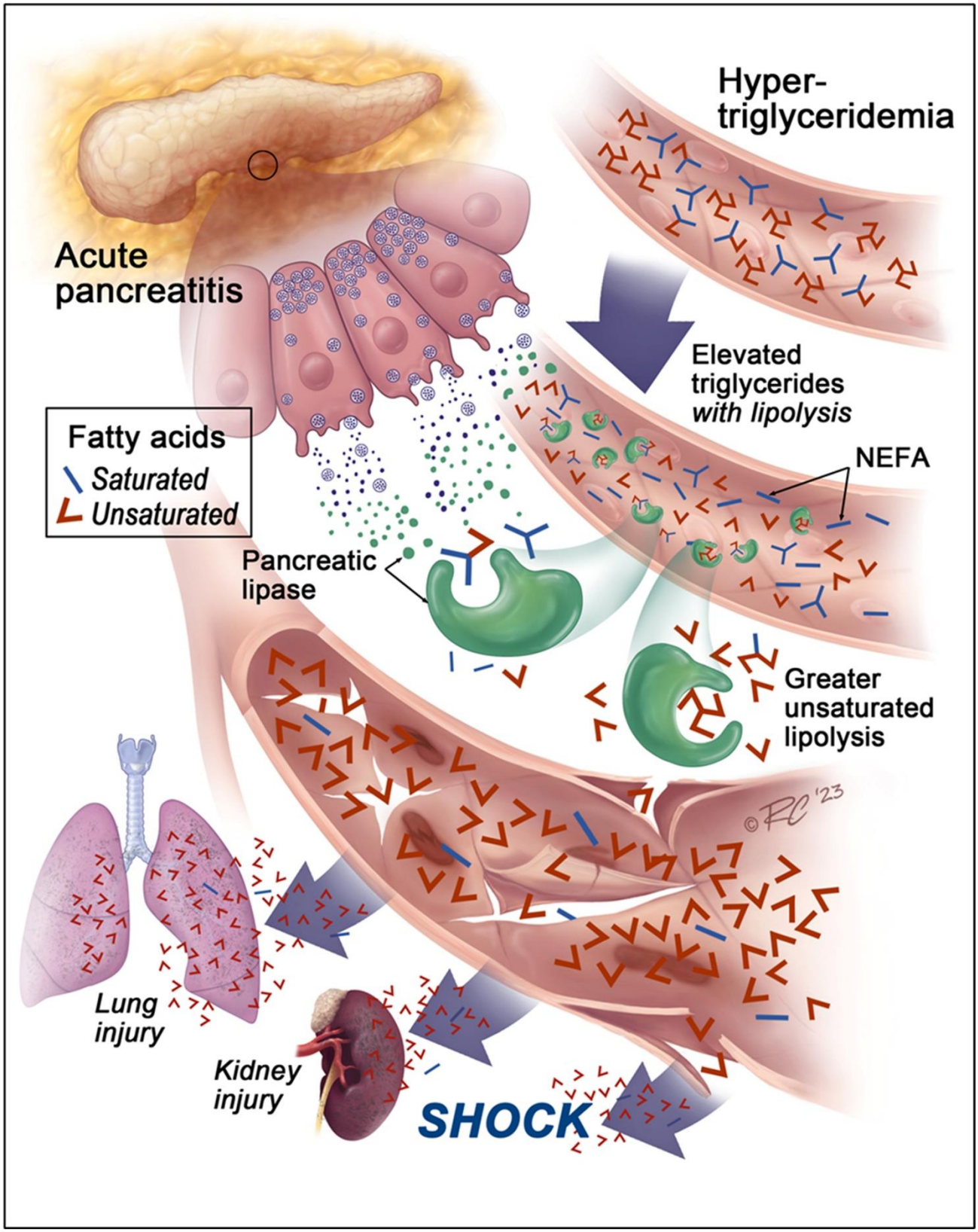


Figure 5: Schematic showing mechanisms underlying severity of HTG-AP: Image shows elevated triglycerides (three limb structures in blue, red shown within upper blood vessels) being cleaved by pancreatic lipases (green) into fatty acids during HTG-AP (middle vessel). The images, inset depict saturated fatty acids as blue lines, and unsaturated fatty acids as red. The unsaturated fatty acids cause vascular leak resulting in shock, along with renal failure and lung injury- resulting in severe AP (lower vessel).

Table 1

	Non-HTG-AP	HTG-AP	P-value
Total Patients (n)	242	27	
Age (years)	57 (39-68)	56 (44-63)	0.63
Females, n(%)	103 (43%)	13 (48%)	0.58
BMI	28 (25-32)	30 (27-33)	0.34
Race, n (%) African American Asian Hispanic Native American White	11 (5%) 3 (1%) 8 (3%) 6 (2%) 214 (88%)	2 (7%) 0 (0%) 2 (7%) 1 (4%) 22 (82%)	>0.2853
Hours to processing	17 (8-26)	20 (11 – 30)	0.2762
Days from pain onset	1 (1-2)	1 (0.5 – 2.0)	0.4255
Serum Lipids			
Triglycerides (mg/dL)	161 (111-238)	730 (561-1064)	<0.0001
Lipase (U/L)	860 (410-1696)	703 (457 – 1080)	0.38
***Serum NEFA** (μM)*	616 (371-876)	948 (602-1502)	<0.0001
Palmitic acid; C16:0 (μM)	181 (115-244)	286 (189-382)	<0.0001
Palmitoleic acid; C16:1 (μM)	23 (10-39)	36 (22-67)	0.0015
Stearic acid; C18:0 (μM)	52 (38-75)	68 (46-94)	0.0222
Oleic acid; C18:1 (μM)	229 (129-336)	340 (206-609)	0.0002
Linoleic acid; C18:2 (μM)	99 (55-139)	148 (113-237)	<0.0001
Clinical outcomes			
Organ Failure, n(%)	23 (10)	8 (30)	0.0019
Severe AP, n(%)	16 (7)	5 (19)	0.0287
Length of Stay (days)	3 (2-5)	5 (3-12)	0.0002
ICU admission rate, n(%)	19 (8)	7 (26)	0.0026

Table 1: Table comparing HTG-AP vs. Non-HTG-AP patient populations: Patient number**,** Demographics, body mass index (BMI), pain duration, sample storage, lipase levels (U/L), serum lipid parameters at the time of admission and clinical outcomes of non-HTG-AP and HTG-AP patients are shown. Data is shown as median (interquartile range) for continuous data and as percentages in categorical data. P. values were calculated as discussed in methods, with continuous variables (e.g. serum lipids and NEFA) compared by a Mann-Whitney test, and categorical variables (e.g. Clinical outcomes) compared by a Chi square test.