AWARD NUMBER: W81XWH-17-1-0138

TITLE: Statins prevent pancreatic diseases through mitophagy activation

PRINCIPAL INVESTIGATOR: Stephen Pandol, MD

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
Los Angeles, CA 90048

REPORT DATE: August 2019

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland, 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
August 2019	Final	**1 May 2017 - 30 Apr 2019**

4. TITLE AND SUBTITLE
Statins prevent pancreatic diseases through mitophagy activation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0138

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Stephen Pandol,
MD

Email: stephen.pandol@cshs.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cedars-Sinai Medical Center
Los Angeles, CA 90048

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This proposal addressing FY16 PRMRP topic area Pancreatitis is designed to determine the mechanisms of statin mediated protection from pancreatitis, an observation recently reported using a retrospective cohort analysis of large clinical datasets. Prevention of pancreatitis, especially cases that progress to recurrent acute and chronic pancreatitis is of high relevance because there is no treatment for these disorders and because the progression is associated with an extremely high risk of pancreatic cancer, one of the most lethal human cancers.

15. SUBJECT TERMS-

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	12	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

	Page
1. Introduction	3
2. Keywords	3
3. Accomplishments	3
4. Impact	15
5. Changes/Problems	15
6. Products	16
7. Participants & Other Collaborating Organizations	17
8. Special Reporting Requirements	19
9. Appendices	19

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

The basis for this study was our report of retrospective cohort analysis showed a reduced occurrence of pancreatitis with statin intake (especially simvastatin) in the Southern California Kaiser Permanente Health Care System. Based on work showing that simvastatin attenuates myocardial infarction severity with ischemia-reperfusion injury by increasing mitochondrial autophagy (mitophagy), we hypothesized that the beneficial effects of simvastatin are due to its ability to enhance removal of failing mitochondria from the pancreas to improve the ability of the pancreas to withstand stressors that cause pancreatitis. show to determine if simvastatin promotes autophagy to improve pancreatitis

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

Pancreatitis, mitochondria, mitophagy, simvastatin, statin

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

What were the major goals of the project?

Specific Aim 1: Determine the effects of simvastatin treatment in vitro and in vivo on expression of sXBP1 and pathways of mitophagy in acinar cells of the pancreas using human and murine acinar cells. **Completed**

Specific Aim 2. Determine the effect of the simvastatin treatments on Ca^{2+} signaling and mitochondrial responses to a Ca^{2+} stress. **Completed**

Specific Aim 3. Determine the roles of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and sXBP1 on the pathways of mitophagy, and mitochondrial and pancreatitis responses to Ca^{2+} stress. **Completed**

What was accomplished under these goals?

Progress in the Project

Simvastatin pretreatment reduces inflammation and cell death during acute pancreatitis

A recent cohort analysis showed the reduced occurrence of pancreatitis risk with statin intake (especially simvastatin) in the Southern California Kaiser Permanente Health Care System(1). To explore if simvastatin treatment could reduce the pancreatitis severity and to determine the mechanism of any beneficial effect, we employed an experimental pancreatitis model in mice using cerulein hyperstimulation. We pretreated the mice with simvastatin (20 mg/kg b.w.) for 24 hours or DMSO (vehicle control), followed by 7 hourly intraperitoneal injections of cerulein (50 µg/kg each) to induce acute pancreatitis. The animals were sacrificed 1 hour after the last cerulein injection and tissue was collected for histology and other measurements. We found necrotic cell death, infiltration of inflammatory cells, and edema in the cerulein-treated pancreas, whereas simvastatin pretreatment attenuated this histopathology **(Figure 1A-D)**. Trypsin activation is one of the hallmarks of acute pancreatitis, whereby the cerulein treatment increases the trypsin activity in pancreatic tissue and promotes the pancreatitis response. We observed a significant reduction in trypsin activity with simvastatin pretreatment **(Figure 1E)**. Furthermore, we observed that simvastatin pretreatment reduced the vacuole accumulation **(Figure 1F-G)** commonly seen in pancreatic acinar cells during acute pancreatitis.

Figure 1

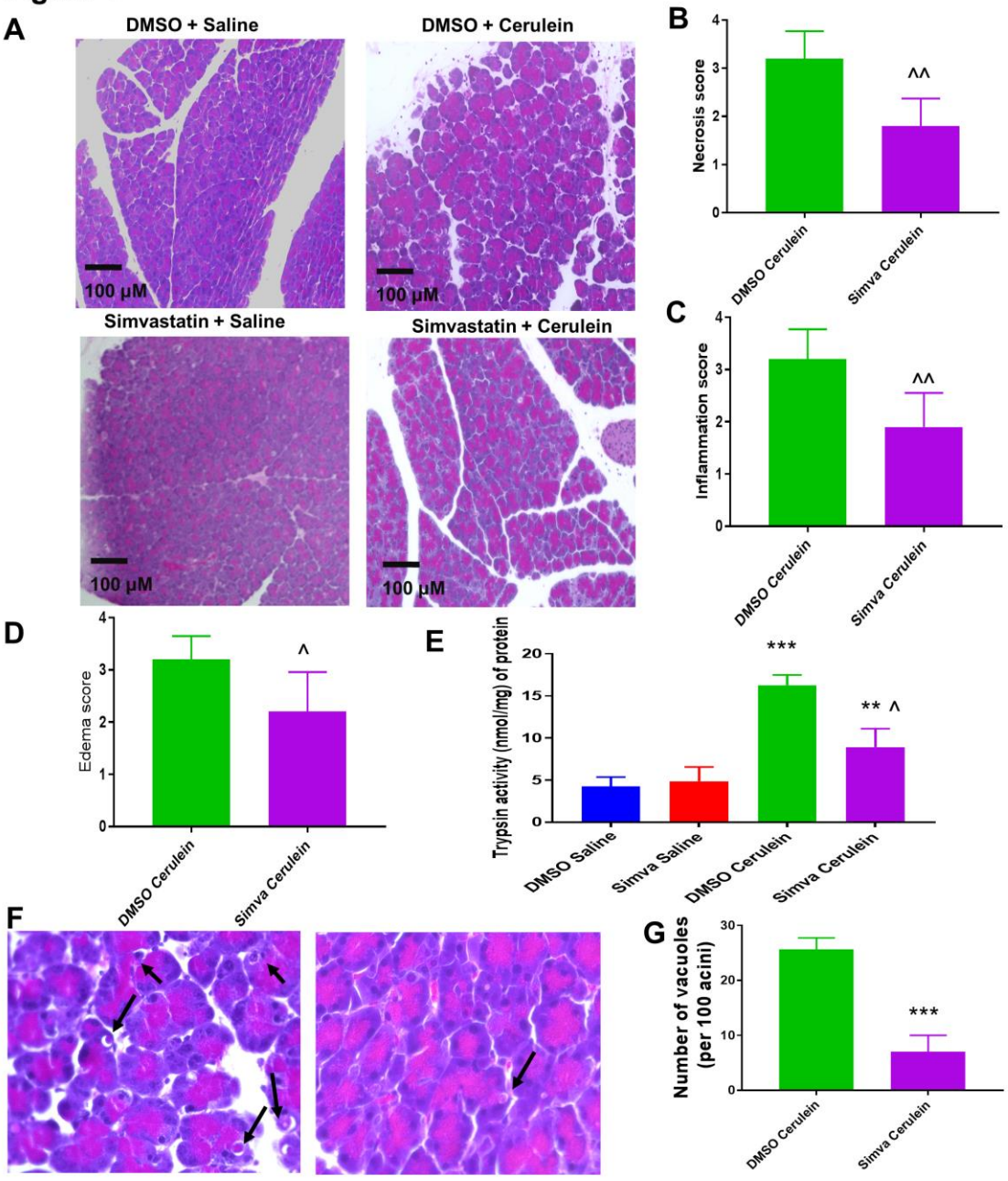

Figure 1. Simvastatin reduces edema, cell death, and trypsin activation during acute pancreatitis. (A) Representative images of H&E staining of pancreatic tissue (n=5). Bar graph indicates Necrosis score **(B),** Inflammation score **(C)** and Edema score **(D)** for each group on a scoring system of 1-4. Data shown are Mean ± SD. **(E)** Trypsin activity was measured by colorimetric assay. Histogram represents the trypsin activity (nmol/mg) for different groups. **(F)** Representative images of H&E staining of pancreatic tissue. **(G)** Bar graph represents the number of vacuoles per 100 acini. Values in graphs are represented as Mean ± SD, **p< 0.01, ***p<0.001 vs DMSO Saline; ^p< 0.05, ^^p<0.01 vs DMSO Cerulein.

Cerulein-induced acute pancreatitis stimulates mitophagy in mice

As we have previously shown that oxidative stress induces mitophagy as a cell survival defense mechanism(2), we next sought to determine if there is a mitophagy induction in our model of acute pancreatitis. Consistent with previous publications(3), we found a significant increase in the levels of total (post-nuclear supernatant) p62 and lipidated LC3 after cerulein injections suggesting autophagy effects of cerulein **(Figure 2A-B)**. Next, we measured the mitophagy marker, parkin, and found that parkin abundance was significantly reduced during acute pancreatitis **(Figure 2A-B)**. Furthermore, we observed increased translocation of parkin to the mitochondria after cerulein exposure **(Figure 2C-D),** which suggests the induction of mitophagy during pancreatitis. This is further confirmed using electron microscopy where mitochondria were present in autophagic vacuoles **(Figure 2E)**. Interestingly, simvastatin treatment (Simva-Cerulein) significantly increased p62 compared to DMSO-Cerulein **(Figure 2A-B)**. Also, there was no significant change in LC3 expression during pancreatitis with simvastatin pretreatment.

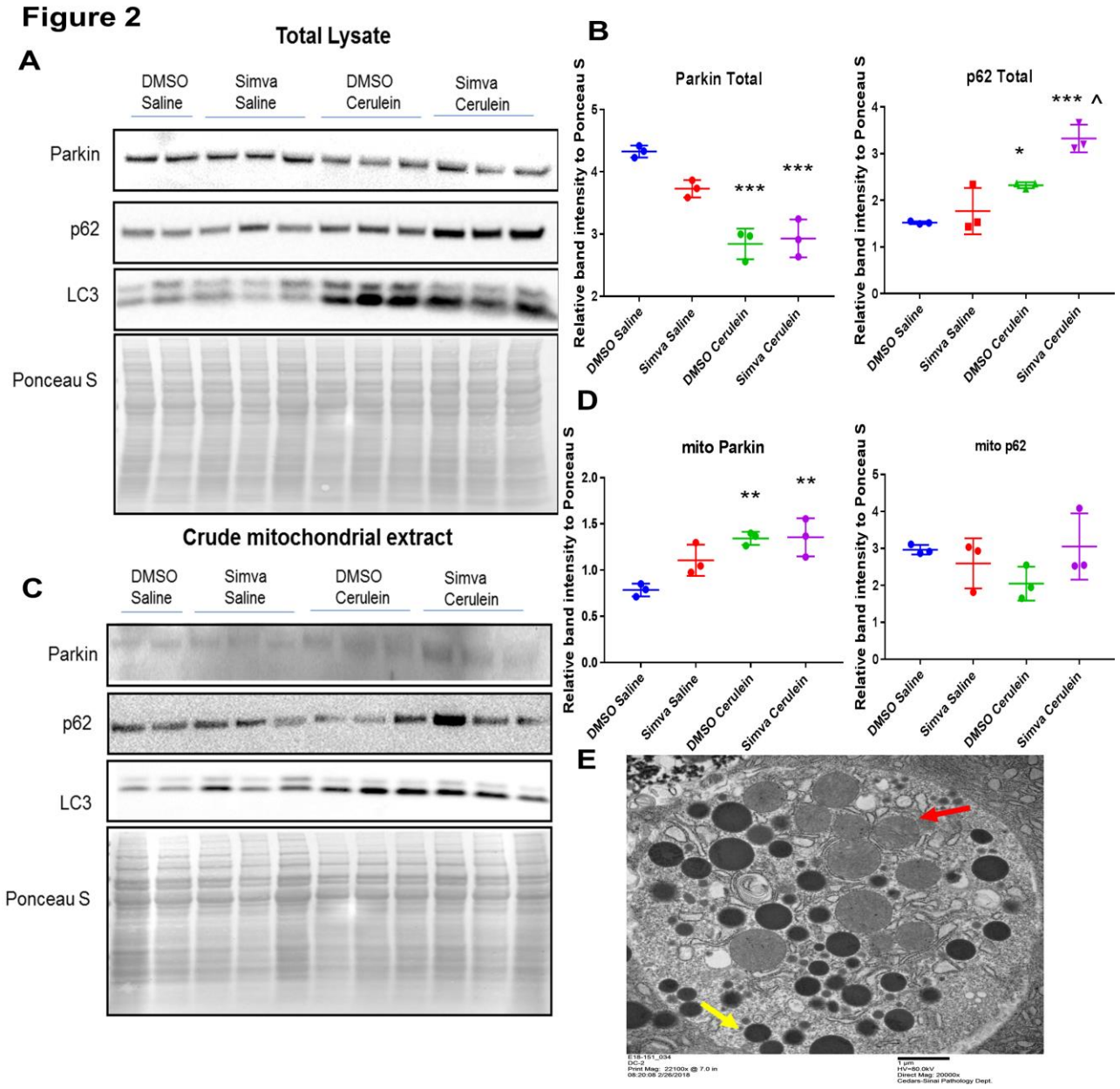

Figure 2. Cerulein-induced pancreatitis triggers mitophagy. Mouse pancreatic tissue was collected and tissue lysates were fractionated for post-nuclear supernatant and crude mitochondrial fraction. **(A)** Western blot of post-nuclear supernatant for parkin, p62 and LC3. Ponceau S staining was used to normalize the protein expression. **(B)** Dot plots representing the expression of total parkin and total p62 normalized to Ponceau S stain. **(C)** Western blot showing expression of parkin, p62 and LC3 in crude mitochondrial fractions. **(D)** Dot plots representing the expression of mitochondrial parkin and p62 normalized to Ponceau S stain. Values in graphs are represented as Mean ± SD (n=3), *p< 0.05, **p< 0.01, ***p<0.001 vs DMSO Saline; ^p< 0.05 vs DMSO Cerulein. **(E)** Representative image of autophagy vacuole containing mitochondria (red arrow) along with other organelles including ER and zymogen Granules (yellow arrow).

Cerulein impairs and simvastatin treatment preserves autophagosome-lysosome fusion

As previously shown, pancreatitis causes an accumulation of vacuoles, leading to cellular dysfunction(4). Using electron microscopy, we found that the total number of vacuoles in the simvastatin pretreatment group was significantly reduced compared to DMSO control during acute pancreatitis **(Figure 3A)**. We counted the number of autophagosomes (recognizable cargo), intermediate vacuoles and autolysosomes (digested cargo) as a percent of the total number of vacuoles **(Figure 3B)**. Simvastatin pretreatment increased the number of intermediate vacuoles and autolysosomes, consistent with enhanced autophagic function with promotion of autophagosomes to functioning autolysosomes after cerulein exposure **(Figure 3C)**. To determine if simvastatin induced autophagy, we used the mRFP-GFP-LC3 reporter in isolated acini. Here, mRFP-GFP-LC3 fluoresces both red and green, represented as yellow in merged images, representing autophagosomes; when the autophagosome fuses with a lysosome to form an autolysosome, the lysosome's acidic pH quenches the GFP; thus, the mRFP-GFP-LC3 fluoresces red(5). Under basal conditions **(Figure 3D-F)**, treatment of mouse pancreatic acini with 1μM simvastatin increased the number of both autophagosomes and autolysosomes, indicating that simvastatin induced autophagic flux. We next investigated the potential mechanisms underlying the preventing effects of simvastatin. Depletion of syntaxin 17 (Stx17), located on the outer membrane of the autophagosome, causes autophagosome accumulation(6) . Interestingly, although we found a decrease in the expression of Stx17 after cerulein injections, simvastatin didn't prevent Stx17 decrease during pancreatitis **(Figure 3G)**, suggesting that cerulein effects on autophagy are in part due to decreased Stx17 but that simvastatin's protective effects are Stx17 independent. We next examined pancreatic levels of LAMP-1, ULK-1 and AMPK, which are essential for mitochondrial targeting to lysosomes(7) and functioning of autophagy(8; 9). We found that cerulein treatment significantly reduced LAMP-1 and ULK-1 protein expression, while AMPK levels didn't change **(Figure 3G-H)**. As AMPK phosphorylates ULK-1(7) to regulate the autophagosome-lysosome fusion process promoting normal autophagic progression, and the activity of both proteins is largely dependent on their phosphorylation states, we next determined the phosphorylation levels of $AMPK^{T172}$ and $ULK\text{-}1^{S555}$ and found that both

AMPK^{T172} and ULK-1^{S555} were dephosphorylated after cerulein treatment **(Figure 3H),** suggesting the possibility that in experimental pancreatitis, the dephosphorylated state of these regulators could lead to cerulein-induced disorders in autophagy, specifically autophagosome-lysosome fusion. Simvastatin pretreatment upregulated LAMP-1, AMPK-1 and ULK-1 as well as the phosphorylated forms of AMPK and ULK-1 **(Figure 3G),** suggesting that simvastatin preserves autophagosome-lysosome fusion to promote autophagic flux.

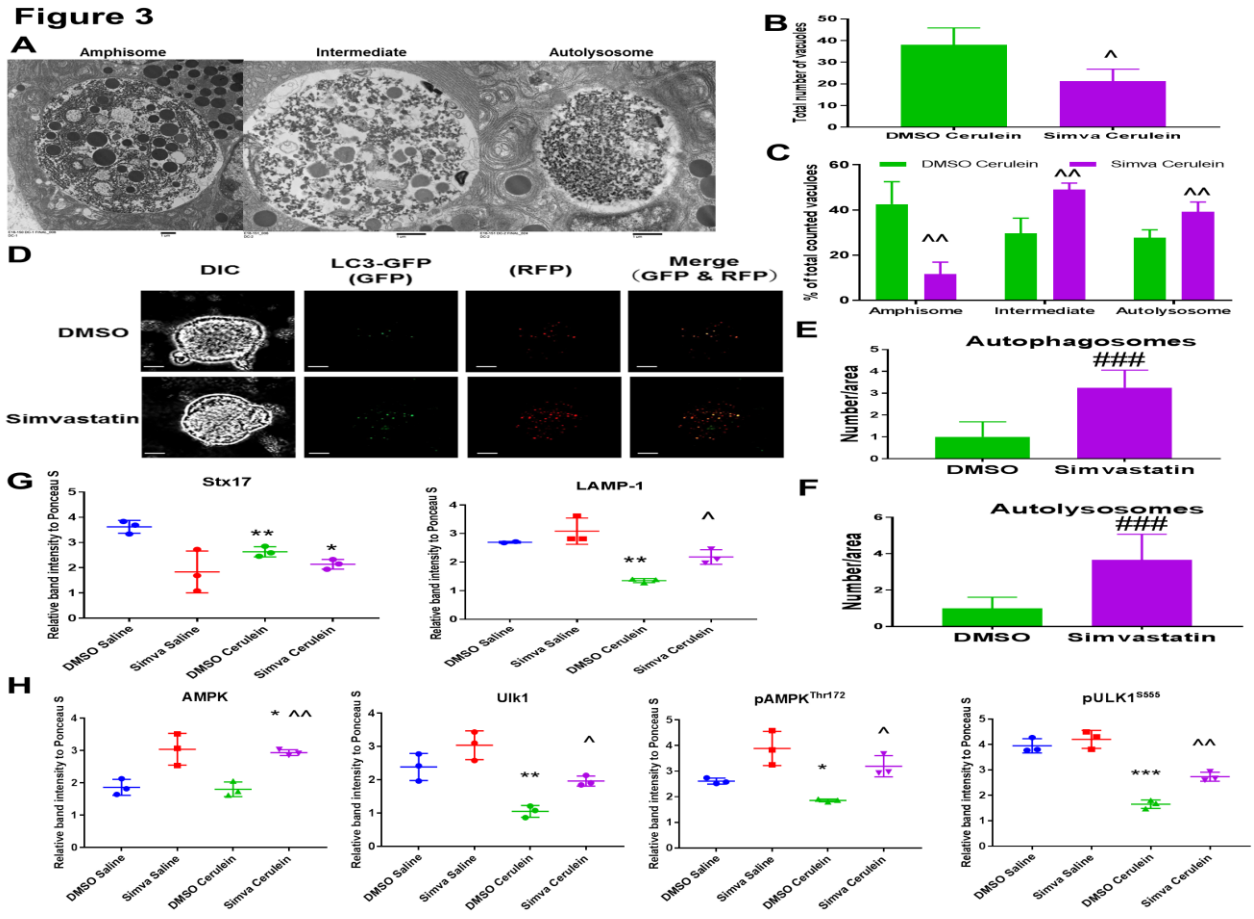

Figure 3: Simvastatin pretreatment improves autophagosome-lysosome fusion by enhanced autophagy signaling and preservation of LAMP-1 protein. (A) Pancreatic tissue was scored for different types of vacuoles by electron microscopy. Representative images show Amphisome, Intermediate vacuoles and autolysosomes in pancreatic sections. **(B)** Bar graph represents the total number of vacuoles during acute pancreatitis with DMSO (DMSO Cerulein) or simvastatin (Simva Cerulein) pretreatment (n=4). Vacuoles were counted from at least 30 different fields for each group. **(C)** Several types of vacuoles (Amphisomes, Intermediate and Autolysosomes) were counted and expressed as percentage of total vacuoles. Values in graphs are represented as Mean ± SD. **(D)** Isolated mouse acini were transduced with Ad-LC3-GFP-RFP along with pretreatment with

1μM simvastatin (dissolved in DMSO) or DMSO (control) for 16 hours, and then fixed with 4% PFA. The slides were then observed under a spinning disc confocal microscope. Images shown are representative data. Bar graph represents the **(E)** total number of yellow (autophagosomes) and **(F)** red dots (autolysosomes) per area. Values in graphs are represented as Mean ± SD of 3 independent experiments, ###p<0.001 vs DMSO. **(G)** Dot plots representing the expression of Stx17 and LAMP-1 **(H)** AMPK, ULK1, pAMPK (T172) and pULK1 (S555) normalized to Ponceau S stain**.** Values in graphs are represented as Mean ± SD (n=3), *p< 0.05, **p< 0.01, ***p<0.001 vs DMSO Saline; ^p< 0.05, ^^p< 0.01 vs DMSO Cerulein.

mtDNA release during acute pancreatitis

Cells such as neutrophils with dysfunctional autophagosome-lysosome fusion are known to release mtDNA and associated protein transcription factor A, mitochondrial (TFAM) into the blood(10). As pancreatitis showed a similar impairment of autophagic flux, we next tested if there was increased mtDNA release with pancreatitis and whether simvastatin could abrogate this effect. Using plasma obtained from healthy individuals and patients with acute pancreatitis, we determined mtDNA content by amplification of *ND4* normalized to a spiked-in control plasmid (*GFP*). Our findings revealed higher levels of mtDNA in plasma from acute pancreatitis patients compared to normal controls **(Figure 4A)**. We next determined if simvastatin could reduce mtDNA release into plasma in the cerulein-induced acute pancreatitis model in mice. We detected a tendential increase (p=0.23) in mtDNA in plasma samples from cerulein-exposed mice, which was attenuated with simvastatin treatment **(Figure 4B).** These findings support the importance of efficient autophagosome-lysosome fusion in preventing the release of inflammatory molecules such as mtDNA, which is a ligand for TLR9(11) and the NLRP3 inflammasome(12).

Figure 4

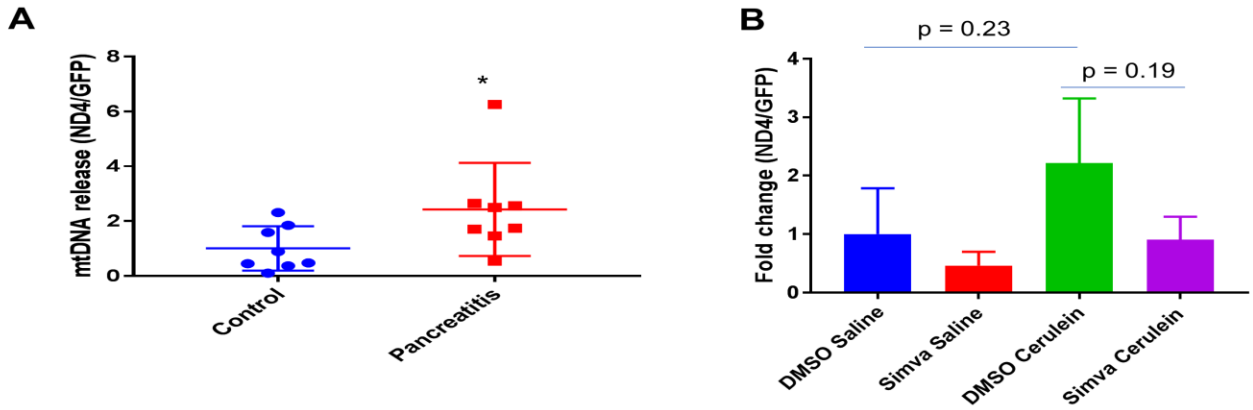

Figure 4. Effects of pancreatitis and simvastatin treatment on mtDNA release. (A) Quantification of mtDNA release in blood assessed by qRT-PCR of mitochondrial gene

ND4 (fold change) present in plasma of normal control (n=8) and acute pancreatitis patients (n=8). GFP plasmid was spiked in before DNA extraction to normalize the samples. **(B)** Bar graph represents the quantification of *ND4* gene normalized to GFP, from plasma of mice treated with DMSO or simvastatin for 24 hours followed by saline or cerulein (7 hourly) injections (n=3). Values in graphs are represented as Mean ± SD, *p< 0.05 vs Control.

Mitochondrial biogenesis is an early event in acute pancreatitis

Previous studies have shown that cellular stress signaling pathways can increase mitochondrial biogenesis to meet increased energy demand(13). To determine if pancreatitis induces mitochondrial biogenesis, we determined the abundance of representative Oxidative phosphorylation (OXPHOS) subunits (CI-NDUFB8, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, CV-ATP5A) and COXIV by western blot. We found that cerulein administration significantly increased the abundance of mitochondrial proteins **(Figure 5A-B)** in both DMSO- and simvastatin-treated mice, although there was a trend of smaller increase of these proteins with simvastatin treatment. To understand the mechanism of mitochondrial biogenesis during pancreatitis, we performed a time-course study where mice were injected with 1, 3, or 7 cerulein injections (50µg/kg per injection at hourly intervals) and sacrificed 1 hour after last injection. We detected an increase in mitochondrial proteins as early as 1 hour after the first cerulein injection **(Figure 5C-D)**, raising the possibility of a translationally-controlled process, possibly regulated through microRNAs.

Figure 5

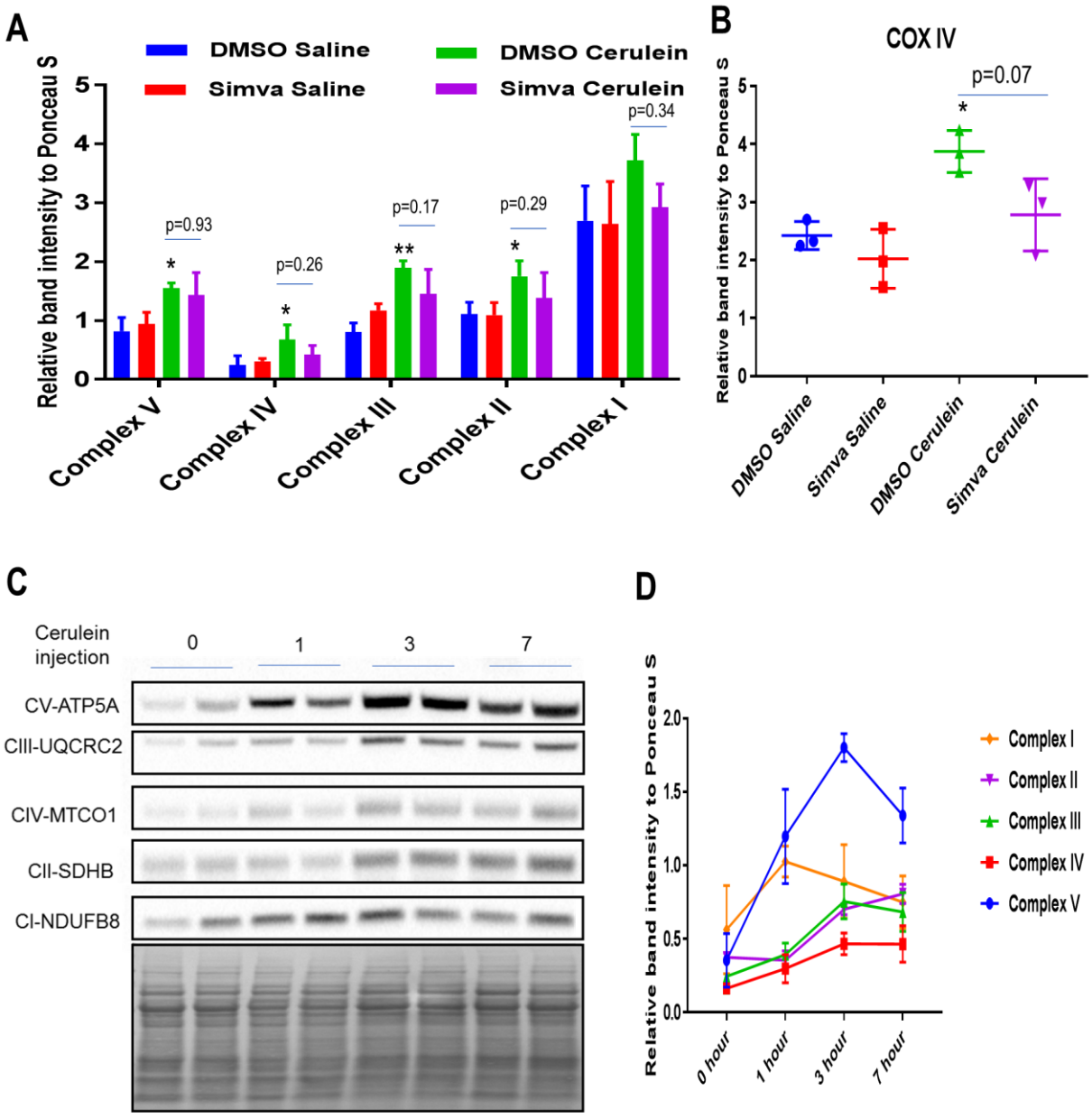

Figure 5. Upregulation of mitochondrial proteins occurs rapidly after cerulein exposure. (A) Graphs representing the Ponceau S stained normalized protein expression of ATP5A (Complex V), MTCO1 (Complex IV), UQCRC2 (Complex III), SDHB (Complex II), NDUFB8 (Complex I) and **(B)** COX IV. **(C)** Different number of (1, 3 or 7) Cerulein injections (50 µg/kg, i.p. per injection, each at 1-hour interval) were administered and mice were sacrificed 1 hour after indicated cerulein injections to collect pancreatic tissue. Control mice received no injection (0 hour). Western blot showing expression of ATP5A (Complex V), MTCO1 (Complex IV), UQCRC2 (Complex III), SDHB (Complex II), NDUFB8 (Complex I) subunits of mitochondrial electron transport machinery. **(D)** Line graph represents the Ponceau S staining normalized expression of these proteins at different

times after cerulein injections. Values in graphs are represented as Mean ± SD (n=3), *p< 0.05, **p< 0.01, ***p<0.001 vs DMSO Saline.

Simvastatin increases stress resistant mitochondrial population

Previously, we reported that simvastatin induces mitophagy to provide cardioprotection during ischemia-reperfusion injury(14). We next questioned if simvastatin induced mitochondrial quality control to prevent acinar cell injury. We assessed mitophagy in mouse pancreatic tissue obtained at various times after simvastatin administration, using mitophagy-related markers, parkin and mfn2, as well as autophagy-related markers, p62 and LC3. We observed a decrease in p62 at 3 hours which returned to baseline (or slight increase) at 6 hours, which was paralleled (with smaller changes) by Mfn2. Parkin and LC3 levels were lowest at 6 hours and returned to baseline by 12 hours **(Figure 6A)**. Mitochondrial protein abundance reached a nadir at 12 hours, followed by partial recovery at 24 hours **(Figure 6B)**. To determine if simvastatin depended on autophagic flux to induce clearance of dysfunctional mitochondria, we used chloroquine to prevent lysosomal acidification and fusion of autophagosomes with lysosomes. We then assessed the levels of p62 and OXPHOS proteins. Consistent with our earlier results **(Figure 6B)**, simvastatin triggered a decrease in OXPHOS proteins and p62, which was abolished by chloroquine administration **(Figure 6C-D)**. We hypothesized that simvastatin treatment would leave behind a population of mitochondria that were more stress-resistant. We performed mitochondrial swelling assays on mitochondria isolated from pancreas 24 hours after treating the mice with DMSO or simvastatin. Mitochondria were resuspended in mitochondria swelling buffer and the decrease in absorbance was monitored after the addition of calcium (100 μM). Mitochondria from simvastatin-treated mice showed less swelling (decrease in absorbance) over the 20-minute observation **(Figure 6E)**, suggesting that simvastatin-induced mitochondria quality control resulted in a mitochondrial population that was more resistant to calcium overload.

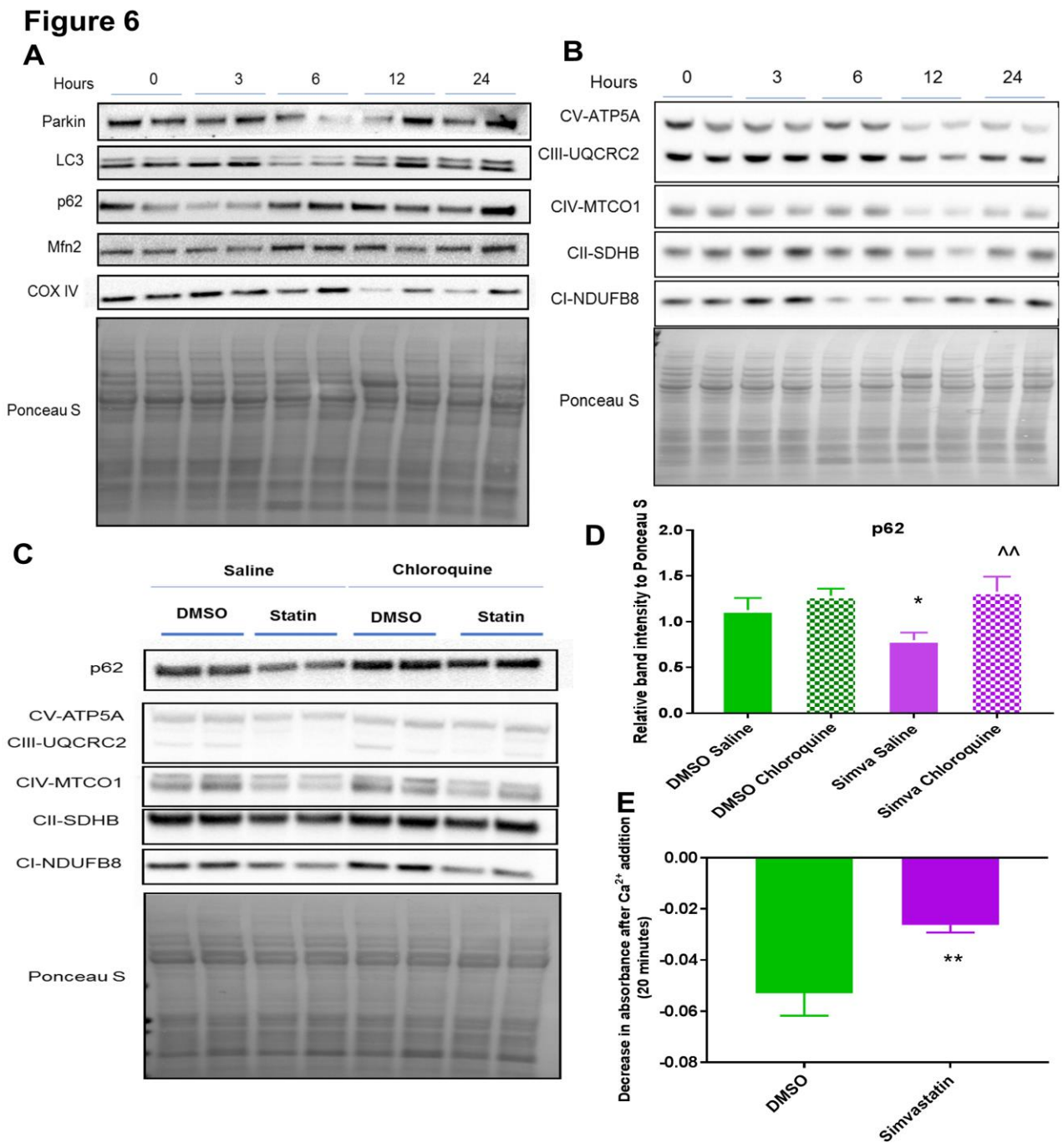

Figure 6. Simvastatin induces mitophagy for mitochondrial quality control. Mice were treated with simvastatin and sacrificed 3, 6, 12, 24 hours later. **(A)** Western blot for pancreatic tissue lysate showing the expression of Parkin and Mfn2, p62 and LC3. COX IV is used to indicate mitochondrial content in the lysate. Ponceau S staining was used as loading control. **(B)** Western blot for different mitochondrial subunits of OXPHOS complexes (ATP5A, UQCRC2, MTCO1, SDHB and NDUFB8) and used to infer mitochondrial content present in the lysate at the indicated time points. **(C)** Mice were pretreated with DMSO or simvastatin (20mg/kg, i.p.) for 6 hours followed by saline or

chloroquine (40mg/kg, i.p.) for 4 hours and pancreatic tissue was processed to obtain post-nuclear supernatants. Representative western blot showing expression of p62, ATP5A, UQCRC2, MTCO1, SDHB and NDUFB8. **(D)** Bar graph represents the expression of p62, normalized to Ponceau S stain. Values in graphs are represented as Mean ± SD (n=3), *p< 0.05 vs DMSO Saline; ^^p< 0.01 vs Simva Saline. **(E)** Mitochondrial swelling was used as an indicator of mitochondrial quality in pancreatic tissue from mice treated with DMSO or simvastatin (20 mg/kg) for 24 hours. Bar graph represents the decrease in absorbance (540 nm) after incubating the isolated mitochondria with 100 µM Ca2+ for 20 minutes. Values in graphs are represented as Mean ± SD (n=3), *p< 0.05, **p< 0.01 vs DMSO control.

Simvastatin protective effects are abolished with parkin genetic deletion

As previously shown by our group(14) in ischemia-reperfusion model of cardiac injury, simvastatin protection is parkin-dependent. We elucidated the role of parkin in pancreatic injury and whether parkin is required for simvastatin-induced protection. We used the same model as above and compared the effects of simvastatin in preventing pancreatic injury in parkin knockout (PKO) mice to wild type mice (wt-mice). At baseline, pancreatic tissue had normal histological appearance (Data not shown). However, simvastatin's protective effects against cerulein-induced pancreatitis were abolished with parkin deletion **(Figure 7A-D)**. Examination of p62 and AMPK revealed that both proteins were upregulated in PKO mice even in control saline groups, while simvastatin pretreatment further increased p62 and AMPK during pancreatitis **(Figure 7E-G)**. Also, decrease in COXIV expression with simvastatin pretreatment was abolished in PKO mice **(Figure 7E-F)**. We also noted increased accumulation of LC3 in PKO mice pretreated with simvastatin.

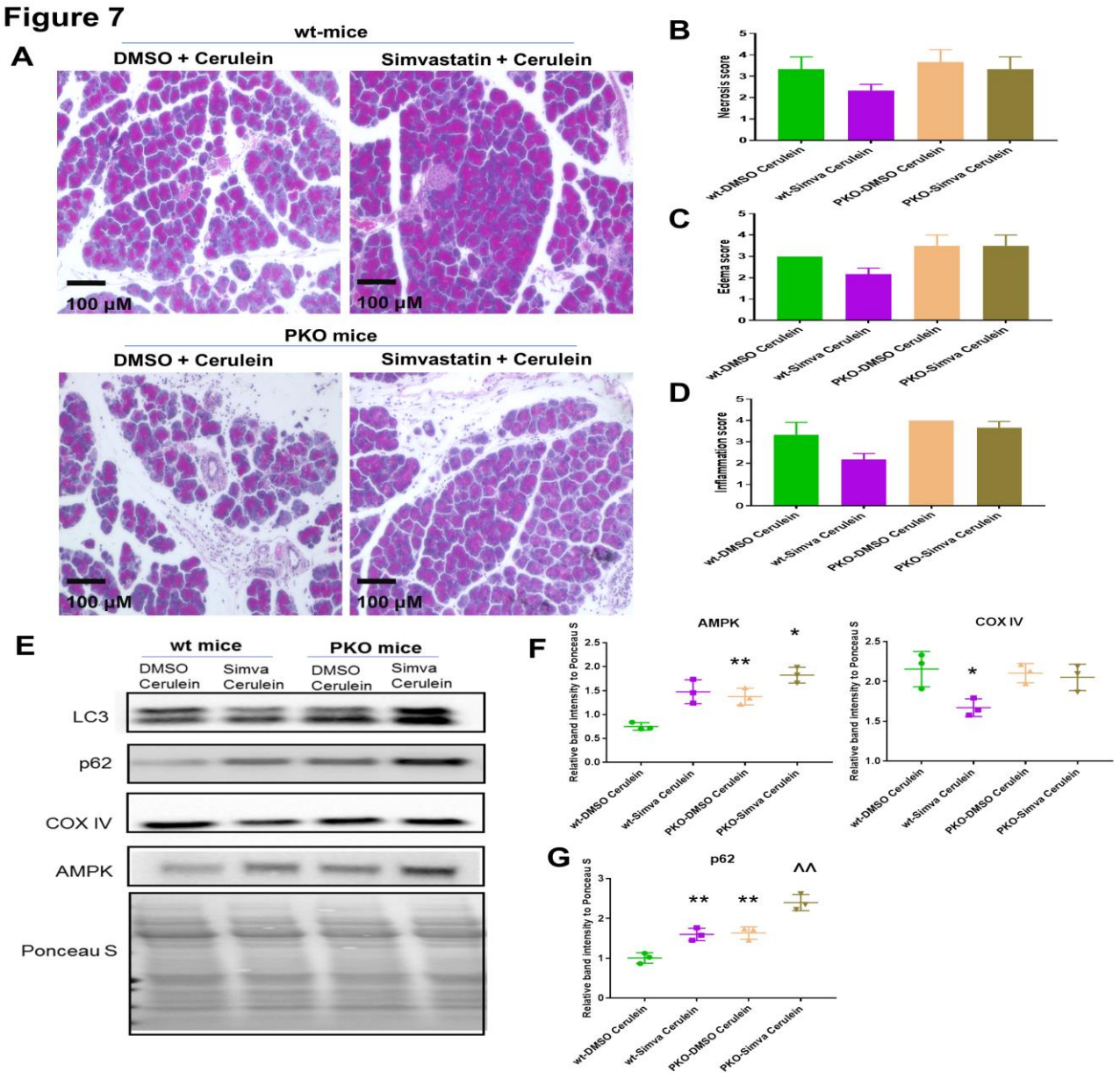

Figure 7. Simvastatin-mediated protection is dependent on Parkin. Acute pancreatitis was induced in wild type (wt-mice) and Parkin knockout mice (PKO mice) with 7 hourly injections of cerulein. DMSO or simvastatin (20 mg/kg) was given 24 hours before inducing acute pancreatitis. **(A)** Representative images of H&E stained pancreatic tissue from different groups. Bar graph indicates Necrosis score **(B),** Inflammation score **(C)** and Edema score **(D)** for each group on a scoring system of 1-4. Data shown are Mean ± SD. **(E)** Western blot showing the expression of LC3, p62, COX IV and AMPK. Dot plots representing the expression of **(F)** AMPK and COX IV, and **(G)** p62, all normalized to Ponceau S stain**.** Values in graphs are represented as Mean ± SD (n=3), *p< 0.05, **p< 0.01 vs wt-DMSO Cerulein; ^^p< 0.01 vs PKO-DMSO Cerulein.

Network analysis for biological processes

As impaired flux is known to be involved in progression of pancreatitis to pancreatic cancer(15) and simvastatin helps resolve the process by activating AMPK and ULK-1, we next tried to determine the biological processes which can be affected by AMPK and ULK-1 dysregulation. We generated a protein-protein interaction network from whole human proteome showing 156 and 55 interacting proteins of AMPK and ULK-1 respectively **(Figure 8)**. Based on the network, biological processes involved were extracted using ClueGo-CluePedia. ULK1 and PRKAA2 and their shared interacting partners are enriched for pathway terms as well as biological processes. Network shows ULK-1 and AMPK (PRKAA1 and PRKAA2) linked with pathway terms showing their participation in biological processes especially mTOR signaling, p53 signaling, and energy homeostasis. Many of the enriched biological processes especially mTOR signaling, p53 signaling, and energy homeostasis are the key hallmarks of pancreatic cancer progression, underscoring the importance of activating AMPK and ULK-1 by simvastatin to mitigate pancreatitis.

Figure 8

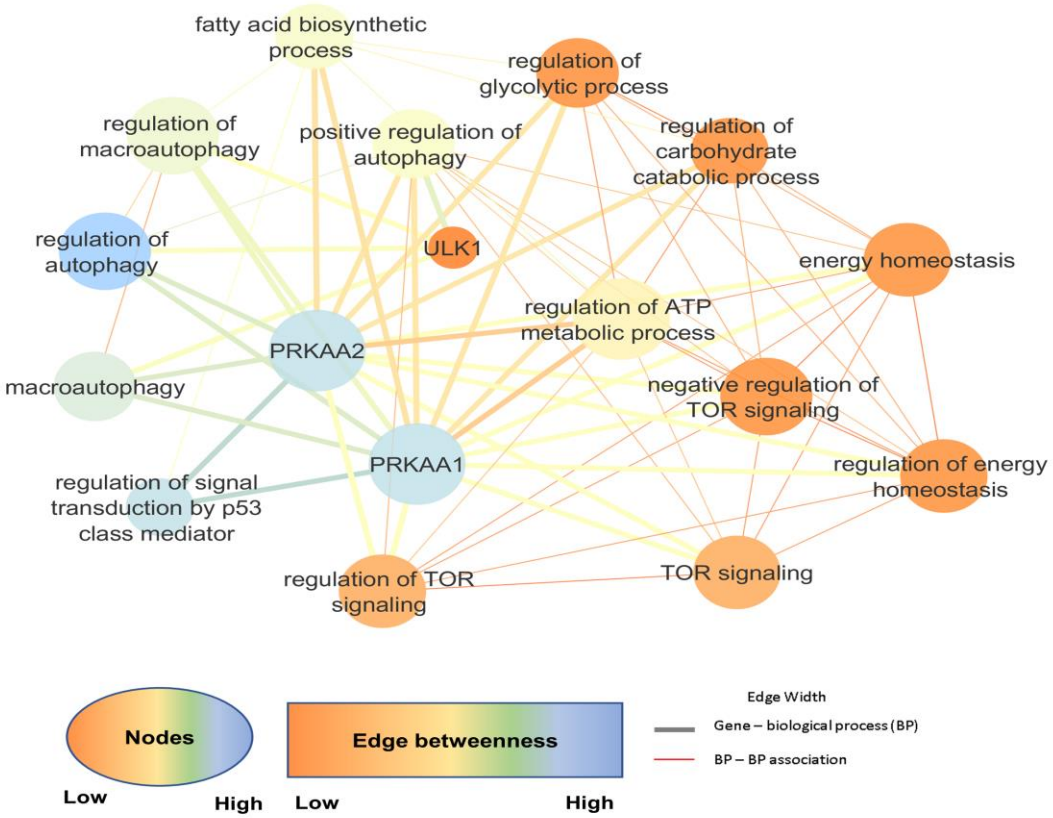

Figure 8. BINGO/ClueGo Network analysis for protein-protein interaction of AMPK (*PRKAA2*) and ULK1 to identify different biological processes generated using Cytoscape. Edge betweenness centrality is the indicator of essentiality of that edge in the network. Degree is the number of connections related to the node in the network. Thick lines indicate the Gene to biological process (BP) association and thin lines indicate BP to BP association. Color of the node represents the importance of the node in the network.

Molecular docking studies

To investigate the interaction of the simvastatin with various proteins involved in autophagy, molecular docking studies were carried out using LigandExpress (CYCLICA). The binding analysis was first performed with the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (PDB ID: 1HW9) and simvastatin. Binding analysis showed that simvastatin effectively binds to HMG-COA reductase **(Figure 9A)** with 98.97% accuracy and predicted effect is negative confirming that the LigandExpress program to be efficient in the present docking studies. Molecular docking studies of simvastatin with 5'-AMP-activated protein kinase catalytic subunit alpha-1 (AMPK; PDB ID: 4RER) predicted that simvastatin can also bind to AMPK **(Figure 9B)** with accuracy 95.73%. However, the predicted effect is unknown, but our initial studies shows that simvastatin can activate AMPK.

Figure 9

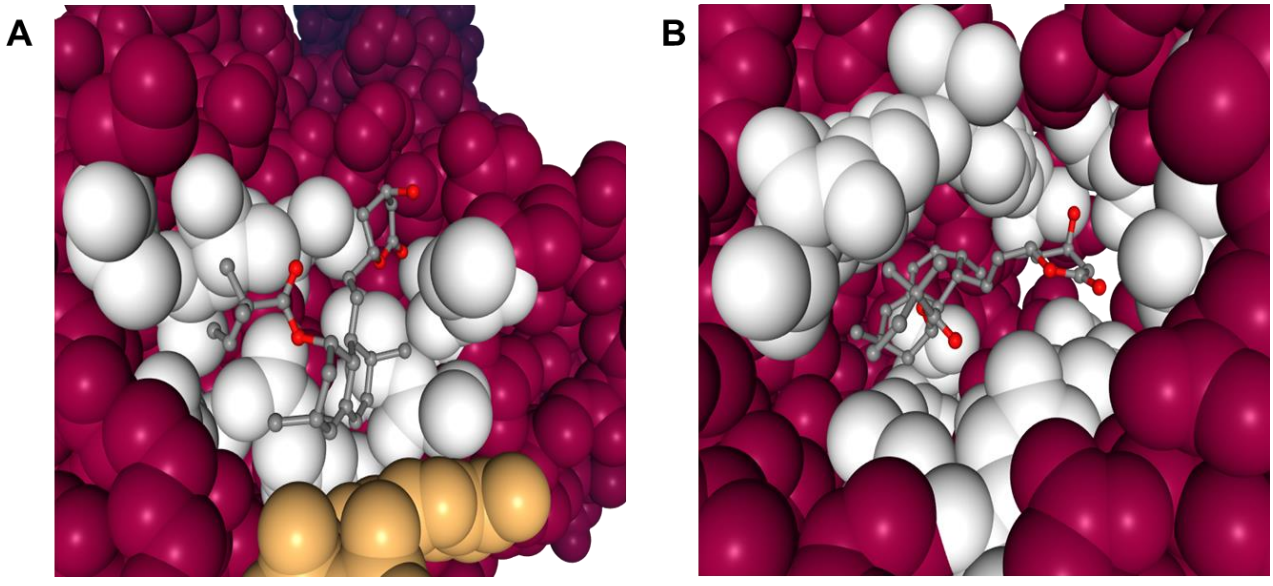

Figure 9: (A) Docked complex of simvastatin and HMG-CoA reductase. HMG-CoA reductase is shown as a space fill and simvastatin as a stick representation. **(B)** Docked complex of simvastatin and AMPK. AMPK is shown as a space fill and simvastatin as a stick representation.

Milestone

We have presented this work on the effects of simvastatin on mitophagy and pancreatitis at the American Pancreatic Association meeting in November 2018 and 20th annual symposium of Southern California Research Center for ALPD & Cirrhosis in December 2018. We have also published the work as a research article in Journal Biochim Biophys Acta Molecular Basis of Disease.

Simvastatin prevents inhibition of sXBP1 during pancreatitis

We have shown that the transcription factor and member of the unfolded protein response member of cellular signals, spliced X-box protein-1 (sXBP1) maintains the exocrine pancreas in its differentiated and physiologic state; that this transcription factor is upregulated with alcohol intake in animal models; and that inhibition of this transcription factor makes the pancreas vulnerable to pancreatitis with an alcohol abuse stressor.(16; 17). Importantly, during this project we showed that the protection afforded by sXBP1 with intake is abolished during an additional smoking insult because smoking molecules inhibit sXBP1 expression making the pancreas vulnerable to alcohol toxicity and pancreatitis.(18)

Considering that simvastatin is protective of pancreatitis, we posited that simvastatin would protect the pancreas against the decrease of sXBP1 occurring during pancreatitis and that this prevention of a decrease would be associated with the lessened severity of pancreatitis found above. The results shown in **figure 10** below indeed supported this posit showing that simvastatin treatment protected against a decrease in sXBP1 occurring with an acute pancreatitis; and that this effect was associated with decreased trypsin activity and acinar vacuolization (expected from the benefits on autophagy).

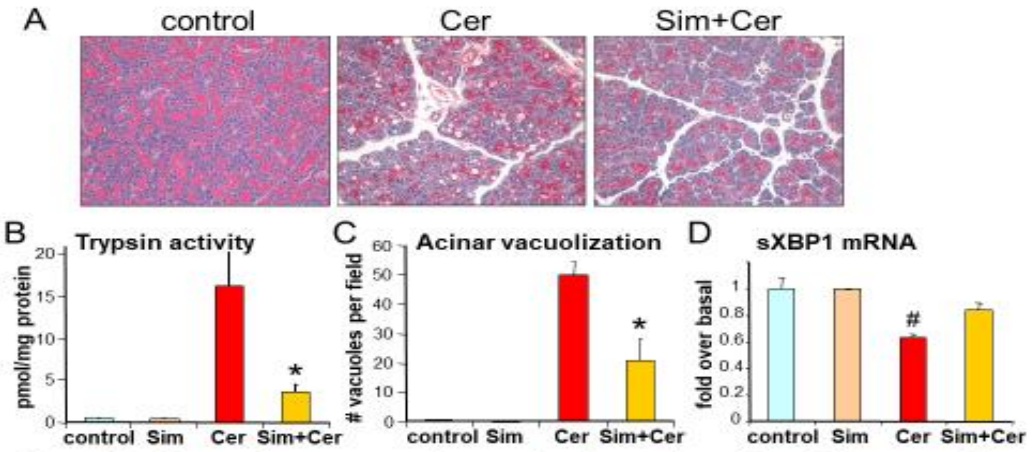

Figure 10. Simvastatin attenuates the severity of experimental cerulein pancreatitis and prevents cerulein-induced XBP1s reduction. Male Sprague-Dawley rats (3-6 per group) were pretreated for 3 days with simvastatin (Sim, 40 mg/kg i.p. once per day) and then treated with cerulein (Cer, 50 µg/kg, 4 hourly i.p. injections) to induce pancreatitis. (A) Panels show representative pancreas histology; H&E staining. (B) Pancreatic trypsin activity was measured in pancreas homogenates. (C) Acinar cell vacuolization was quantified in H&E stained pancreatic tissue sections; 10-20 fields per tissue section were analyzed. (D) XBP1s expression in pancreatic tissues. *P<0.05 vs. Cer; # P<0.05 vs control.

Effects of inhibition of sXBP1 on cellular and mitochondrial metabolism

To understand the effects of decreases in sXBP1 on mitochondrial and cellular metabolism, we developed a regulatable model of XBP1 deletion and measured the effects of inhibition of XBP1. As shown in **figure 11**, inhibition of XBP1 expression led to a decrease in mitochondrial oxygen consumption associated with decrease oxidative phosphorylation activity of mitochondrial complex I. These changes were also associated with an increase in glycolytic rates (likely to compensate for decreased energy production from mitochondrial metabolism); and a failure to maintain cellular ATP despite the increased glycolytic rate. We additionally used the genetically engineered animals to determine the effects of inhibition of XBP1 on multiple pathways using RNA seq. As shown in **figure 12**, inhibition of XBP1 had a significant effect on a large number of processes in the pancreatic acinar cell but prominent were effects on mitochondrial metabolism.

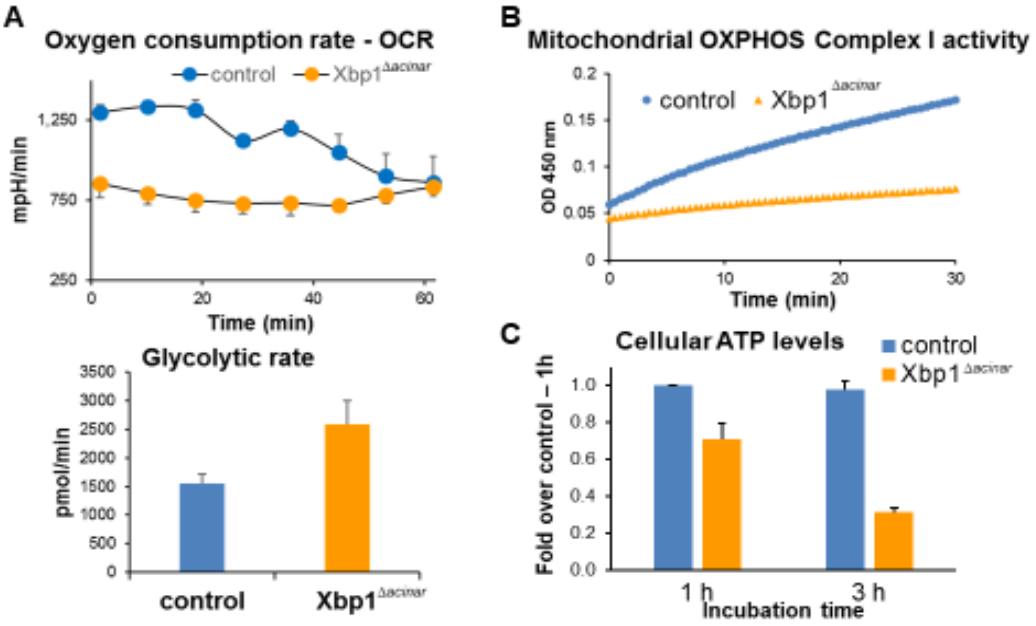

Figure 11. Compared to control cells, Xbp1 deficient acinar cells are energetically inefficient and display reduced mitochondrial oxidative phosphorylation (OXPHOS) activities, increased glycolytic rate and lower ATP levels. Acinar cells were isolated from control mice (normal Xbp1 expression) and mice with specific genetic deletion of Xbp1 in pancreatic acinar cells (*Xbp1*$^{\Delta acinar}$ mice). Freshly isolated cells were assayed as indicated. (A) Mitochondrial OXPHOS and cellular glycolytic rate were measured by Seahorse analysis. (B) OXPHOS complex I activity was measured using an antibody-based colorimetric activity assay. (C) Total cellular ATP levels were measured after 1 and 3 h incubation.

A

Biological process; Down	*P value*
• Protein processing in ER	5.3E-14
• Oxidation-reduction process	3.2E-10
• Response to ER stress	6.1E-07
• ER unfolded protein response	2.3E-06
• ER-associated proteolysis	1.9E-05
• ER to Golgi vesicle transport	9.3E-05
• Vesicle-mediated transport	7.4E-04
• Exocrine pancreas development	9.3E-04
• Fatty acid metabolism	7.0E-03

B

Biological process; Down	*P value*
• Metabolic processes	7.4E-36
• Oxidative phosphorylation	7.3E-04
• Respiratory electron transport chain	6.6E-06
• Electron transport, NADH to ubiquinone	6.0E-05
• Tricarboxylic acid cycle	3.9E-05
• ROS metabolic process	5.0E-06
• Fatty acid beta-oxidation	2.0E-05
• ATP biosynthesis	4.6E-04
Biological process; Up	
• Regulation Ca uptake	2.0E-04
• Apoptotic signaling	1.7E-04
• Regulation of necroptosis	3.4E-06

Fig. 12: RNAseq analysis was performed in pancreas tissues from control mice and mice with specific genetic deletion of Xbp1 in pancreatic acinar cells (Xbp1$^{\Delta acinar\ mice}$). Tables show (A) endoplasmic reticulum (ER) and (B) mitochondrial processes altered in Xbp1$^{\Delta acinar}$ compared to control mice.

Conclusions

The combination of results combined thus far indicate that simvastatin has effects to promote mitochondrial health by promoting mitophagy to maintain a healthy population of mitochondria; and by effects on the transcription factor sXBP1 to maintain mitochondrial function. These effects are certainly of clinical importance.

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

The project provided training for postdoctoral fellow (now Research Scientist I), Honit Piplani, PhD, in a novel area in pancreatic research with Drs. Pandol and Gottlieb who are experts in pancreatitis and mitophagy, respectively. The project will allow him to be a leader in this new field or research.

What do you plan to use the results for future developments?

We have recently received approval for a Focused Program Award entitled “Mechanisms and Treatment Development for Pancreatitis Resulting from Alcohol Abuse and Smoking (PR182623)” and the results of the present study were used to develop the preliminary results needed for one of the projects of the Focused Program.

4. **IMPACT:**

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

This project will have an impact on the clinical treatment of pancreatitis where there are no currently available treatments. The pending Focused Program Award provides a step toward clinical application

What was the impact on other disciplines?

This project will have an impact on the clinical treatment of other inflammatory disorders where there are no currently available treatments.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

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

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Piplani H, Iannucci SM, Germano J, Hou J, Sin J, Sang Y, Gulla A, Wu BU, Waldron RT, Lugea A, Andres AM, Gottlieb RA, Pandol SJ (2018). Simvastatin Induces Mitophagy and Restores Cerulein Impaired Phagosome-lysosome Fusion to Reduce the Severity of Acute Pancreatitis. Pancreas 47 (10): 1417-1418.

Journal publications.

Piplani H, Marek-Iannucci S, Sin J, Hou J, Takahashi T, Sharma A, de Freitas Germano J, Waldron RT, Saadaeijahromi H, Song Y, Gulla A, Wu B, Lugea A, Andres AM, Gaisano HY, Gottlieb RA, Pandol SJ. Simvastatin induces autophagic flux to restore cerulein-impaired phagosome-lysosome fusion in acute pancreatitis. Biochim Biophys Acta Mol Basis Dis. 2019 Aug 6;1865(11):165530.

Lugea A, Gerloff A, Su HY, Xu Z, Go A, Hu C, French SW, Wilson JS, Apte MV, Waldron RT, Pandol SJ. Combination of Alcohol and Cigarette Smoke Induces Endoplasmic Reticulum Stress and Cell Death in Pancreatic Acinar Cells. *Gastroenterology* 2017;153:1674-86

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

Nothing to report

Other publications, conference papers and presentations.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

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

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Stephen Pandol, MD
Site PI
Months 2
Develops goals and direction for the project. Oversees direction and analyzes results.

Roberta Gottlieb, MD
Site PI
Months 2
Provides expertise in cell biology, mitophagy and autophagic function. Oversees direction and analyzes results.

Honit Piplani PhD
Research Scientist I
Months 12
Performs experiments, organizes and analyzes results interacting with site PIs and Dr. Lugea.

Aurelia Lugea PhD
Research Scientist
Months 2
Provides expertise on animal models, treatments, measurements of pathways.

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

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

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

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

9. APPENDICES:

References

1. Wu BU, Pandol SJ, Liu IL. 2015. Simvastatin is associated with reduced risk of acute pancreatitis: findings from a regional integrated healthcare system. *Gut* 64:133-8
2. Hamacher-Brady A, Brady NR, Gottlieb RA. 2006. Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. *J Biol Chem* 281:29776-87
3. Gukovskaya AS, Gukovsky I. 2012. Autophagy and pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 303:G993-G1003
4. Mareninova OA, Hermann K, French SW, O'Konski MS, Pandol SJ, et al. 2009. Impaired autophagic flux mediates acinar cell vacuole formation and trypsinogen activation in rodent models of acute pancreatitis. *J Clin Invest* 119:3340-55
5. Kimura S, Noda T, Yoshimori T. 2007. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 3:452-60
6. Itakura E, Mizushima N. 2013. Syntaxin 17: the autophagosomal SNARE. *Autophagy* 9:917-9
7. Laker RC, Drake JC, Wilson RJ, Lira VA, Lewellen BM, et al. 2017. Ampk phosphorylation of Ulk1 is required for targeting of mitochondria to lysosomes in exercise-induced mitophagy. *Nat Commun* 8:548
8. Zhao M, Klionsky DJ. 2011. AMPK-dependent phosphorylation of ULK1 induces autophagy. *Cell Metab* 13:119-20
9. Eskelinen EL. 2006. Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. *Mol Aspects Med* 27:495-502

10. Caielli S, Athale S, Domic B, Murat E, Chandra M, et al. 2016. Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus. *J Exp Med* 213:697-713
11. Oka T, Hikoso S, Yamaguchi O, Taneike M, Takeda T, et al. 2012. Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. *Nature* 485:251-5
12. Shimada K, Crother TR, Karlin J, Dagvadorj J, Chiba N, et al. 2012. Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 36:401-14
13. Jain SS, Paglialunga S, Vigna C, Ludzki A, Herbst EA, et al. 2014. High-fat diet-induced mitochondrial biogenesis is regulated by mitochondrial-derived reactive oxygen species activation of CaMKII. *Diabetes* 63:1907-13
14. Andres AM, Hernandez G, Lee P, Huang C, Ratliff EP, et al. 2014. Mitophagy is required for acute cardioprotection by simvastatin. *Antioxid Redox Signal* 21:1960-73
15. 2018. Pancreatitis-Induced p62 Accumulation Promotes Pancreatic Cancer. *Cancer Discov* 8:OF16
16. Lugea A, Tischler D, Nguyen J, Gong J, Gukovsky I, et al. 2011. Adaptive unfolded protein response attenuates alcohol-induced pancreatic damage. *Gastroenterology* 140:987-97
17. Lugea A, Waldron RT, French SW, Pandol SJ. 2011. Drinking and driving pancreatitis: links between endoplasmic reticulum stress and autophagy. *Autophagy* 7:783-5
18. Lugea A, Gerloff A, Su HY, Xu Z, Go A, et al. 2017. The Combination of Alcohol and Cigarette Smoke Induces Endoplasmic Reticulum Stress and Cell Death in Pancreatic Acinar Cells. *Gastroenterology* 153:1674-86