AWARD NUMBER: W81XWH-15-1-0257

TITLE: Targeting Trypsin-Inflammation Axis for Pancreatitis Therapy in a Humanized Pancreatitis Model

PRINCIPAL INVESTIGATOR: DR. BAOAN JI

RECIPIENT: Mayo Clinic
Jacksonville, FL 32224

REPORT DATE: DECEMBER 2019

TYPE OF REPORT: FINAL

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

DISTRIBUTION STATEMENT:

[X] Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
DECEMBER 2019	Final	15 Sept 2015 – 14 Sept 2019

4. TITLE AND SUBTITLE
Targeting trypsin-inflammation axis for pancreatitis therapy

in humanized pancreatitis model.

5a. CONTRACT NUMBER
W81XWH-15-1-0257

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Baoan Ji

E-Mail: Ji.baoan@mayo.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mayo Clinic
4500 San Pablo Rd S
Jacksonville, FL 32224

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

Pancreatitis is an inflammatory disease that may be initiated by lifestyle factors such as alcohol abuse, smoking or high fat diet. Acute pancreatitis can become chronic, which is a primary risk factor for developing pancreatic cancer. Since only a fraction of patients that develop pancreatitis report alcohol abuse or smoking, it is very likely that there are also underlying genetic factors for this condition. A well-established genetic defect (R122H) in the trypsinogen gene (PRSS1) results in the development of hereditary pancreatitis. We developed a transgenic mouse model to study the key mutation found in patients with hereditary pancreatitis (PRSS1-R122H). During the final funding year we evaluated whether this novel mouse model predisposed animals to pancreatitis. Our current data show that mice expressing PRSS1-R122H develop a more severe form of pancreatitis than their wild-type littermates, shown by higher serum amylase levels. The addition of sex hormones resulted in no significant difference in pancreatic weight or animal health. Anti-hormone studies generated no differences in pancreatitis compared to the control group. Finally, chemical chaperone studies to reduce pancreatitis in these mice were not able to be carried out to completion due to technical difficulties.

15. SUBJECT TERMS
Pancreatitis, acute pancreatitis, chronic pancreatitis, hereditary pancreatitis, estrogen, testosterone, endoplasmic reticulum stress, alcohol abuse, smoking, trypsin, caerulein.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	20	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

4. **INTRODUCTION:** Pancreatitis is a devastating inflammatory condition that affects the pancreas, resulting in significant morbidity and mortality in both males and females around the world. Pancreatitis may present in acute or chronic variations; however, recurring bouts of pancreatitis is a primary risk factor for the development of pancreatic cancer. There are known risk factors, such as alcohol abuse and cigarette smoking that may encourage the development of pancreatitis, but not all individuals who use those products develop pancreatitis or advance to pancreatic cancer. This suggests there must also be an unidentified, underlying genetic component explaining why some individuals progress to pancreatic cancer, while others exposed to similar conditions and lifestyles remain unaffected. We developed a transgenic mouse model using a bacterial artificial chromosome which contained the full-length human PRSS1 gene containing a key mutation known to occur in hereditary pancreatitis ($PRSS1^{R122H}$), an autosomal-dominant disorder. This mouse model allows us to track changes in enzymatic activity, evaluate ER stress and study inflammation levels, as well as identify how hormonal changes, such as estrogen and testosterone, which may be associated with human hereditary pancreatitis occur in this organism. This research aims to provide insight into the development of human pancreatitis and the advancement to pancreatic cancer.

5. **KEYWORDS:** Pancreatitis, acute pancreatitis, chronic pancreatitis, estrogen, androgen, alcohol abuse, alcoholic pancreatitis, cigarette smoking, endoplasmic reticulum stress, high-fat diet, caerulein-induced pancreatitis, testosterone, trypsinogen, trypsin activity.

6. **OVERALL PROJECT SUMMARY:**

Objectives

Specific Aim 1: Determine the role of $PRSS1^{R122H}$ and the physiological factors in the development of pancreatitis

Several experiments testing the efficacy and dosing of testosterone and estrogen in both wild-type and $PRSS1^{R122H}$ mice produced inconsistent data between the experimental and control groups. After repeating sex hormone injections in both animals to ascertain if the issue of weight loss that was recorded in preliminary experiments in the $PRSS1^{R122H}$ mice was the result of hormone treatment alone we did not see weight loss in the second round of testing. Next, we added caerulein to induce pancreatitis. We observed that the addition of sex hormones in combination with caerulein in vivo resulted in no measurable difference in the severity of acute pancreatitis (tamoxifen only). Experimentation using sex hormone antagonists, tamoxifen and enzalutamide, was not completed due to technical issues; however, we believe they still may provide insight into what role these hormones play in the development of conditions such as hereditary pancreatitis.

Specific Aim 3: Determine the role of ER-stress inflammation cascade in the development of pancreatitis in R122H mice

Data pertaining to observing ER stress in WT and $PRSS1^{R122H}$ mice has proved consistent. Ethanol feeding led to the development of chronic inflammatory changes including acinar atrophy and immune cell infiltrations in 74% of R122H mice. Modest changes were observed in about 45% of WT PRSS1 mice, suggesting the mutation plays a role in the severity of pancreatitis. LPS treatment resulted in increased expression of pancreatic ER stress and oxidative stress response-related genes including Bip, activating transcription factor 4 (ATF4) and CHOP. Evaluating high fat diet, we observed focal chronic inflammatory changes as well as the presence of adipocytes in mice expressing human trypsinogens. Feeding a high-fat diet also induced DNA damage, increased cleaved caspase-3 positive cells, and deposition of collagen with the most significant changes observed in the R122H expressing mice.

SUMMARY OF RESULTS

Characterization of the animal model:

This research is a continuation of work that resulted in the generation of a human-like hereditary pancreatitis (HP) mouse model (Figure 1). The transgenic mice carry the full-length human PRSS1 gene that contains a mutation (R122H) commonly found in patients with HP. That model organism has allowed us to evaluate and monitor changes in the animal's health in response to real world health conditions which commonly occur in humans, such as hormonal increases, resulting from puberty and developmental changes, as well as lifestyle changes including high fat diets, cigarette smoking and the effects of alcohol intake on pancreatic health. One subtask of the study, focusing on sex hormone antagonists such as tamoxifen and enzalutamide, produced no changes in animal health and the resulting pancreatitis showed no changes from the control group. For this reason we did not perform additional experiments with sex hormone antagonists for the prevention or treatment of pancreatitis. The information contained in this report is the result of years of animal studies to track how the addition of sex hormones in wild-type and PRSS1^{R122H} mice impact pancreatic growth and development, as well as how lifestyle factors including high fat diet, drinking and smoking alter pancreatic health. While the humanized mouse model used to evaluate the over-expression of trypsinogen does not produce exactly the same level of protein as recorded in human tissues, it does produce 50% of the human PRSS1 protein which is enough to track and evaluate changes in this model organism.

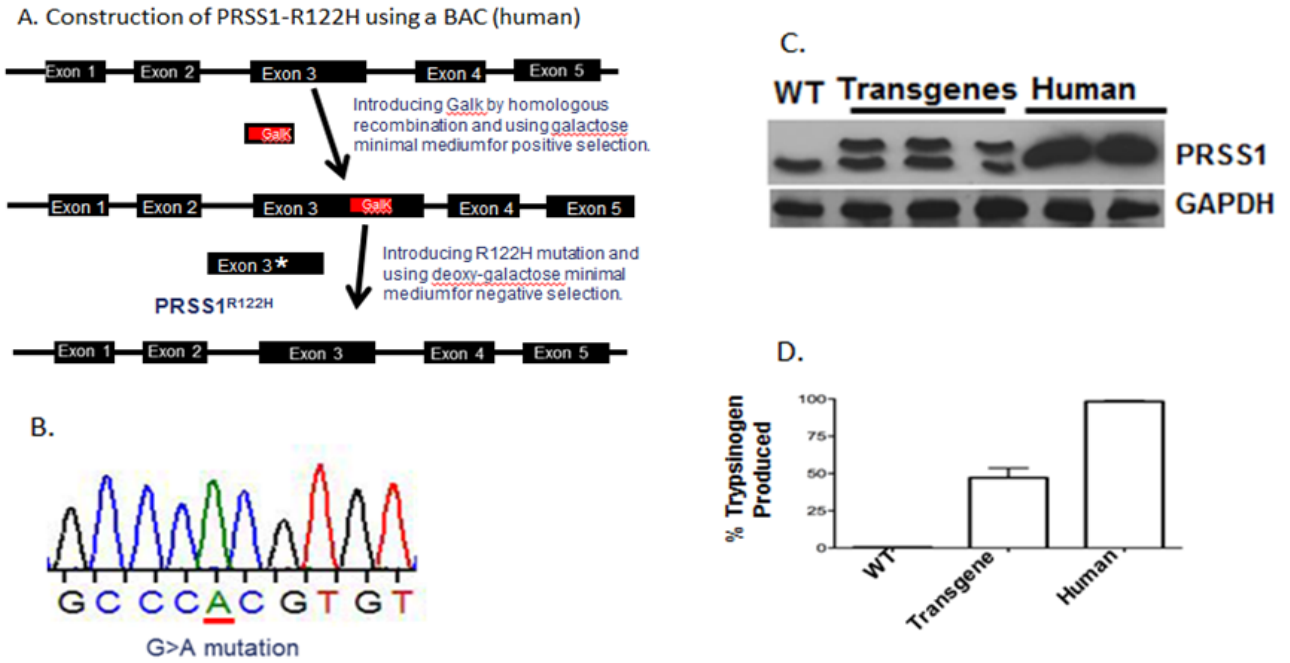

Fig. 1. Transgenic expression of human PRSS1^{R122H} from its native promoter. A single R122H mutation was introduced into a bacterial artificial chromosome (BAC) harboring the full-length human PRSS1 gene by Galk-recombineering (A). The single nucleotide mutation was verified by sequencing methods (B). This transgene allows for high levels of PRSS1^{R122H} expression as detected by WB (GAPDH-loading control) (C) and real-time RT-PCR which shows that the transgenic model produces 50% of PRSS1 (trypsinogen) as was observed in humans (D).

While this is a noteworthy model to evaluate pancreatitis in mice, it needs to be recognized that these animals did not spontaneously develop the condition from the R122H mutation alone. The PRSS1^{R122H} mice grew normally, were reproductively active, and had healthy pancreatic tissue growth from the histological samples shown (Figure 2), even though they carry the genetic mutation linked to human hereditary pancreatitis. These animals have provided understanding about the changes that are associated with pancreatitis. Relative pancreatic weight compared to the animal's body weight, a common indicator of pancreatic health, was also calculated and showed no statistical difference from the WT animals (not included).

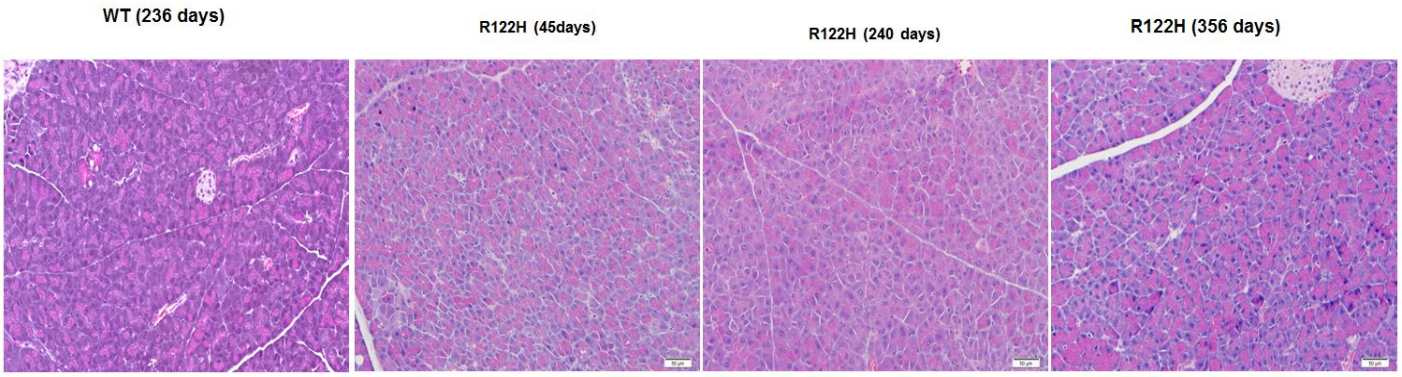

Fig. 2. Pancreatic tissue stained with H+E harvested from wild-type and PRSS1^{R122H} mice showing normal acinar cells, beta cells, and pancreatic ducts. Pancreatic tissue was harvested, weighed, and fixed with formalin before sectioning and staining. Pancreatic tissues show no signs of tumor formation without stimulation, even after 1 year.

The PRSS1^{R122H} mice have allowed us to research how the main genetic mutation associated with human HP may evolve from normal healthy pancreatic tissue and mature into the swollen and inflamed tissue that results from this devastating condition. We examined the pancreata from WT and PRSS1^{R122H} mice, with and without caerulein, which is necessary to stimulate pancreatitis using this animal model. We observed pancreatic samples from animals that were given caerulein by IP injection over a 24 hour period and noticed slight inflammation in the WT animals; however, serious inflammation and edema, which is commonly seen in patients suffering from acute pancreatitis, was observed in the PRSS1^{R122H} mice. These results include increased pancreatic weight, higher serum amylase levels (resulting from acinar cells damage), greater trypsin activity as well as visible acinar cell damage in both WT and PRSS1^{R122H} tissues show that this mouse model can be used to evaluate pancreatic health (Figure 3).

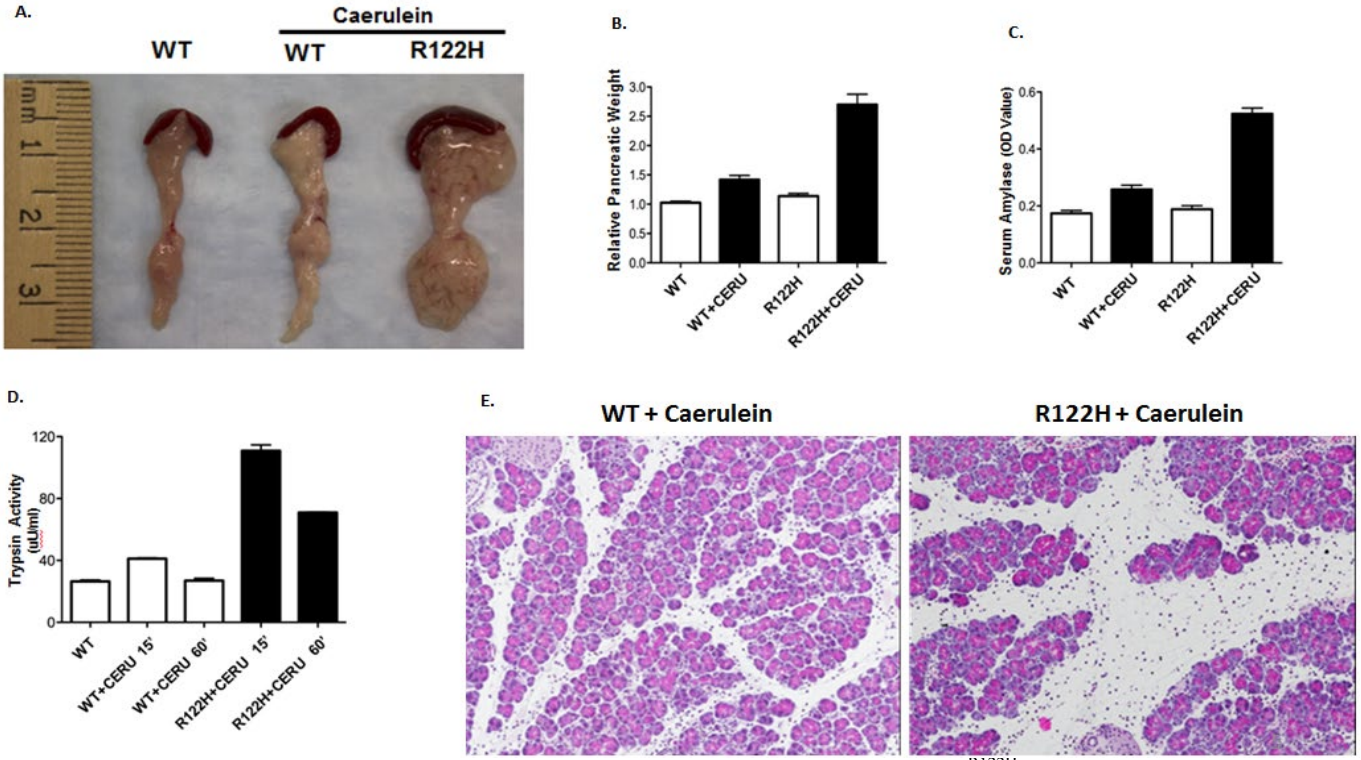

Fig. 3. Acute pancreatitis mouse model using caerulein to induce pancreatitis in WT and PRSS1^{R122H} animals. Mice received caerulein (10µg/kg) by IP injection over a 24 hour time course, then pancreatic tissue samples were collected and show edema associated with AP (A) and the pancreatic tissue was measured to determine relative pancreas weight (B), increased enzymatic secretions were observed by serum amylase levels (C), and increased trypsin levels (D), histological sections were evaluated using H+E staining (E).

Aim 1-1: Determine the effect of sex hormones on trypsinogen (PRSS1) expression and activation; looking at amylase secretion in isolated pancreatic acinar cells.

We have shown that caerulein can result in the development of acute pancreatitis in the mouse model PRSS1^{R122H}, but we next evaluated the health of pancreatic tissue and the animals' ability to become sensitive to caerulein over time. To accomplish this we analyzed WT and PRSS1^{R122H} mice at various concentrations of caerulein ranging from 2.5µg/kg-12.5µg/kg. The WT mice maintained a relatively normal pancreas to body weight ratio and had lower serum amylase levels. However, the PRSS1^{R122H} showed greater inflammation, observable at 10µg/kg, and had increased relative pancreatic weight and serum amylase levels (Figure 4). This information demonstrated that PRSS1^{R122H} mice had greater sensitivity to caerulein-induced acute pancreatitis and were less likely to recover from pancreatic damage than their WT littermates. This model was also used to study chronic pancreatitis with the hope of identifying the subtle cellular changes that signify the shift from CP through PanIN lesion development all the way to pancreatic ductal adenocarcinoma.

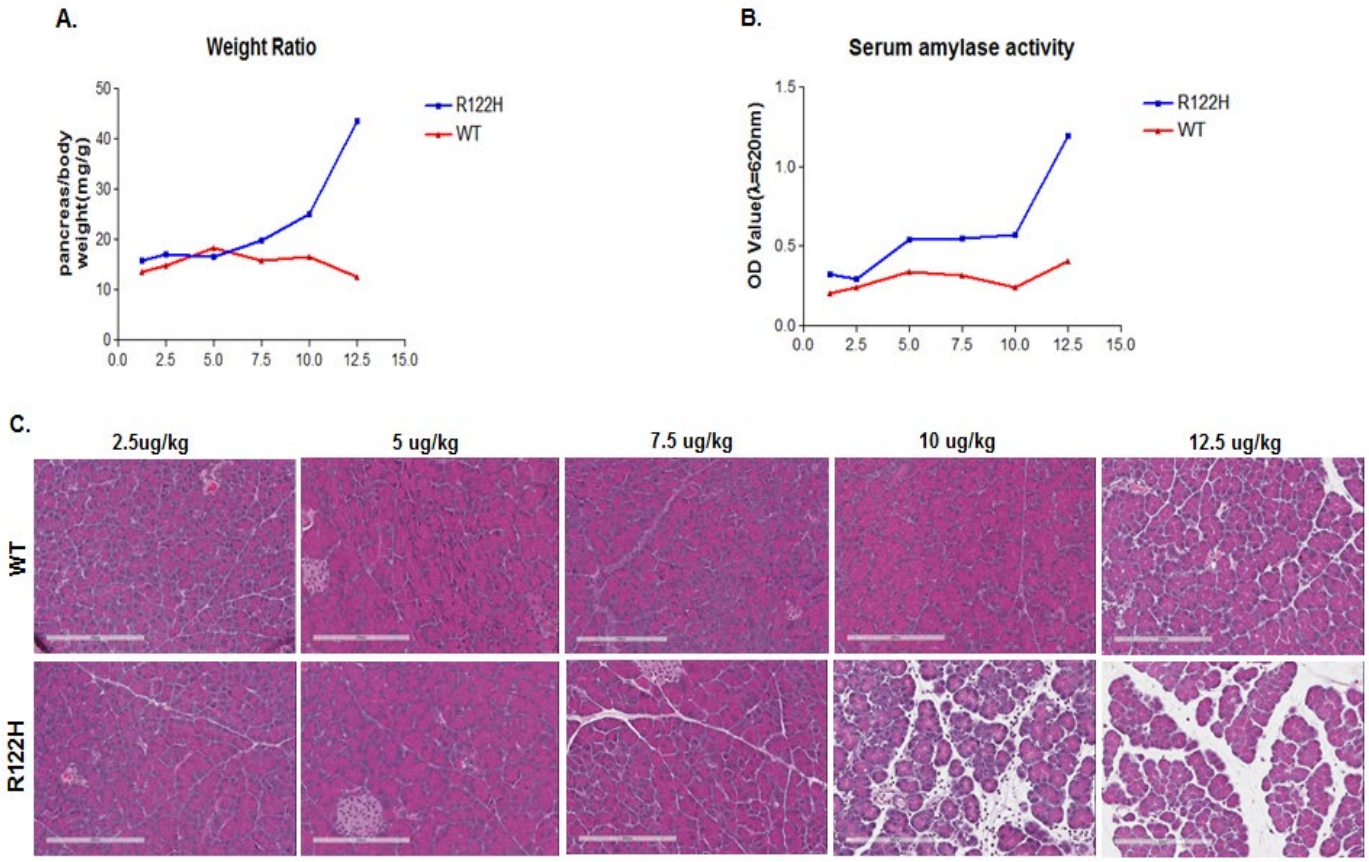

Fig. 4. Sensitization to caerulein induced pancreatitis. Wild type mice show decreases in their pancreas to body weight ratio while PRSS1-R122H mice become more sensitive and develop pancreatitis at lower doses of caerulein than their WT littermates (A). Serum amylase levels were observed to be higher in the PRSS1-R122H mice (B) and pancreatic tissue samples revealed greater acinar cell destruction and swelling in PRSS1-R122H mice (C).

High fat diet stimulation of pancreatitis in R122H mice

In addition to health concerns such as drinking and smoking, the increasing prevalence of obesity and high fat diets has raised health concerns for many individuals. Military veterans are often financially restricted, due to disability and health concerns, and so their personal diets may not be of highest importance. To observe how alterations to diet would impact the development of pancreatitis, a 6-week study was intiated looking at wild-type and PRSS1-R122H mice to evaluate normal and high fat rodent diets. Male and female mice are normally maintained on PicoLab® rodent diet (5053) that is comprised of calories from 25%

protein, 13% fat, and 62% carbohydrates. Mice in this study were switched to Bio-Serv high fat soft pellets (S3282) that is comprised of calories from 20.5% protein, 36% fat, and 36% carbohydrates (the remainder of the pellet comes from ash and moisture, no fiber or other ingredients). Mice were allowed water intake ad libitum. The mice gained considerable weight and there was a visible difference in their coats (oily, shiny hair) due to the increased fat intake. Animals were dissected at the end of the 6-week diet and pancreas weight measured (Figure 5).

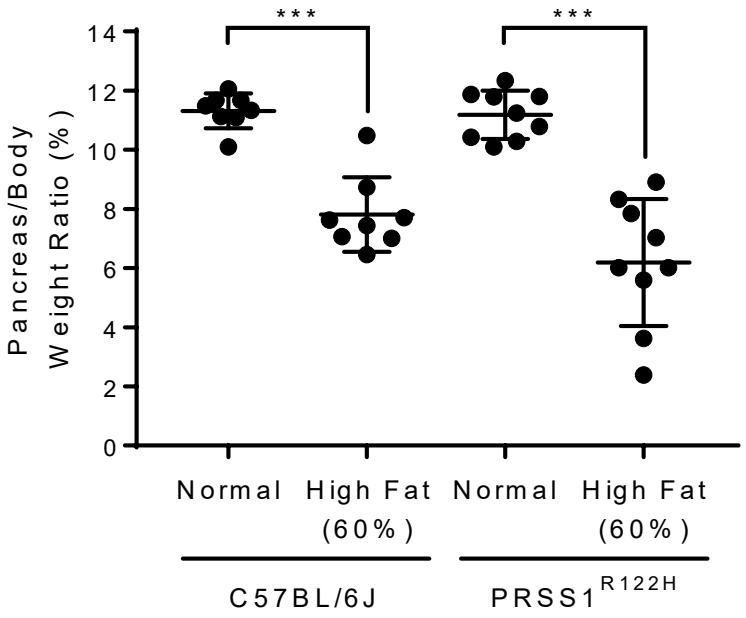

Fig. 5. High fat diet model for pancreatitis using WT and PRSS1^{R122H} animals. Male and female control (n=8) and high fat diet (n=9) C57BL/6J and PRSS1^{R122H} mice were separated into groups and allowed ad libitum access to either normal or high fat diet pellets and water. The pancreatic tissues appeared normal to the naked eye during dissection, but we are awaiting histological examination. Mice on the high fat diet showed a statistically significant decreased in pancreas/body weight ratio (normal is 10-12%) compared to their normal chow counterparts (two tailed student's T-test, $P < 0.05$); however, other than obese, the mice in the study appeared to move, play and groom littermates as usual.

Chronic pancreatitis model

The PRSS1^{R122H} mouse model proved to be successful in recreating human-like pancreatitis conditions following caerulein injections. Not only was pancreatic edema oberseved in the animals, but increased serum amylase activity that is representative of acinar cell damage as well as increased relative pancreatic weight was found to occur in all animals evaluated in this study. Since acute pancreatitis could be studied under these parameters, prolonged administration of caerulein in both WT and PRSS1^{R122H} was necessary to catalogue the changes that would happen under conditions of chronic pancreatitis (Figure 6). This was informative since observation of human tissue samples as they transition from acute to chronic pancreatitis is difficult.

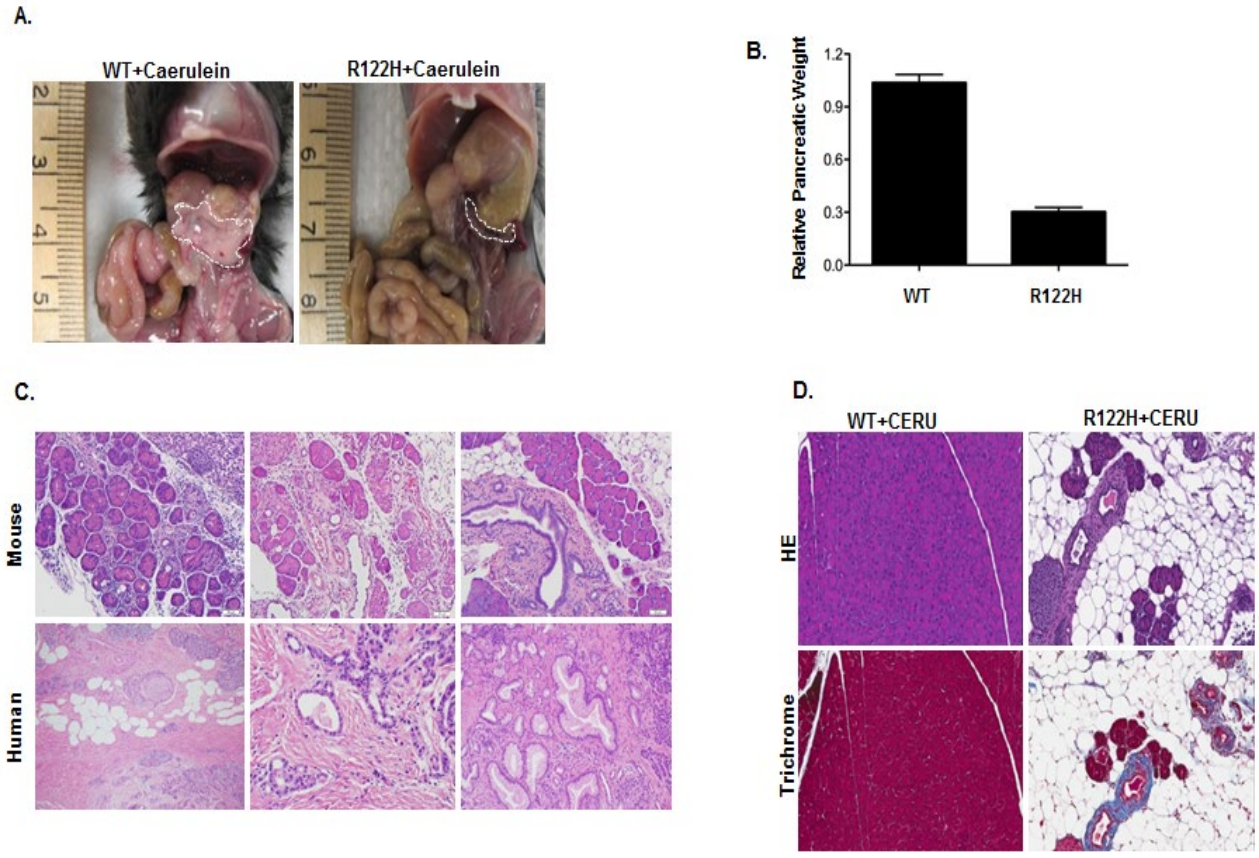

Fig. 6. (A) Prolonged administration of caerulein results in a reduction of the pancreas in mice; this is observed both visually and by calculation of the relative pancreatic weight (B). Wild-type mice following caerulein injection showed slight pancreatic edema, but did not appear to develop acute pancreatitis. However, PRSS1^{R122H} mice showed severe loss of pancreatic tissue that was significant compared to their body weight ($p < 0.05$). The development of chronic pancreatitis in R122H mice shows similar histology to that of humans (C) and both H+E as well as Masson's trichrome staining show parenchymal cell loss, fatty replacement, and fibrosis similar to what is observed in human HP (D).

Progression of pancreatitis to PDAC and possible recovery following acute pancreatitis

Upon further examination using the caerulein method to induce pancreatitis, the beginning stages of cellular transition toward a cancerous phenotype (Pan-IN lesion formation) was observed in the PRSS1^{R122H} animals. Following caerulein treatment in both wild-type and PRSS1^{R122H} mice, the pancreatic acinar cells became altered and began to appear more duct-like, signaling they have undergone acinar to ductal metaplasia (ADM) which permanently reconfigures this tissue and may serve as a marker for beginning stages of pancreatic cancer (Figure 7). We received help from a pathologist to evaluate pancreatic histology samples and focus on identifying pancreatic intraepithelial neoplasm (PanIN) lesion formation in the PRSS1^{R122H} mouse model. This animal model mimics the cellular changes observed in human HP.

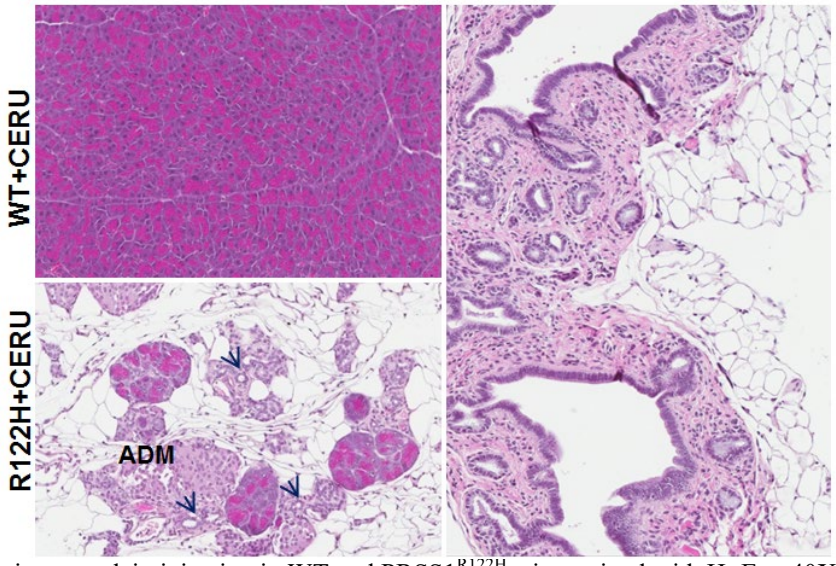

Fig. 7. Pancreas tissue following caerulein injection in WT and PRSS1^{R122H} mice stained with H+E at 40X. The pancreas from the WT mice appear normal, while the tissue isolated from PRSS1^{R122H} mice shows pancreatitis development as well as acinar to ductal metaplasia (ADM) that is associated with the transition from normal to cancerous tissue. The right panel shows clearly the alteration to a duct-like phenotype at 100X.

Finally, we used our pancreatitis mouse model to evaluate whether or not if mice that had several bouts of pancreatitis could recover once the caerulein stimulus was alleviated. This experimentation was conducted because people usually experience acute pancreatitis several times before it progresses to chronic pancreatitis, then deal with chronic pancreatitis for some time before the advancement to pancreatic cancer. Mice were either given no treatment or six IP injections of caerulein during day 1; 24 hours later a few mice were sacrificed and evaluated for pancreatitis, 72 hours later another round of mice were evaluated to see how pancreatic tissue would recover following caerulein-induced pancreatitis (Figure 8). Both groups of animals experience inflammation relating to acute pancreatitis; however, the WT cohort was able to recover from the acute pancreatitis and the tissue looks normal 72 hours after the initial caerulein injections. But the PRSS1^{R122H} mice that are genetically predisposed to pancreatitis were unable to recover once the caerulein stimulus was eased. Instead those tissues showed signs of developing fibrosis to replace the damaged acinar cells that could not be repaired. This information shows that even small genetic changes can be the difference in recovery or disease progression.

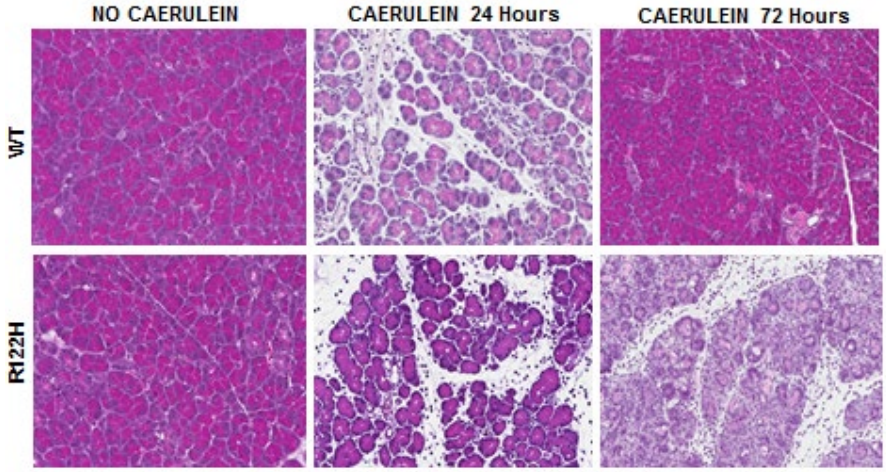

Fig. 8. Repair and recovery of pancreatic tissues following caerulein-induced pancreatitis in both WT and PRSS1^{R122H} mice. Mice were left untreated or given six IP injections of caerulein during day 1. Tissues were examined for pancreatitis 24 or 72 hours later to determine if cellular repair and recovery would be able to override the caerulein-induced pancreatitis in these animals. The WT mice recovered, but the PRSS1^{R122H} mice showed a damaged and fibrotic pancreas upon examination which did not show signs of repair.

7. PROGRESS AND ACCOMPLISHMENTS

The transgenic model used in these experiments will help to decipher the genetic mechanism underlying the development of hereditary pancreatitis. This model allows for transgenic expression of the human PRSS1 gene with the R122H mutation that is commonly associated with this disease (Figure 1). While the transgenic mouse models only produce about 50% of the $PRSS1^{R122H}$ of the endogenous human protein level, this novel model is expressing $PRSS1^{R122H}$ at a physiologically relevant level, so it can be studied in mice. We have also developed a wild-type PRSS1, wild-type PRSS1-dead (possessing the human gene, but lacking functional expression), and PRSS1-R122H-Dead (the human gene with PRSS1 mutation, but lacking functional expression) transgenic mutant mouse models to help better characterize if the R122H mutation alone is the main culprit to hereditary pancreatitis or if it the over-expression of trypsin that initiates ER stress and inflammation that is the main cause. Due to the fact that people with HP are usually diagnosed at a mean age of 10 years, there may be a physiological link associated with hormones during sexual development that may provide more information about the disease.

Aim 1-2: Determine the effect of sex hormones on the development of pancreatitis.

It was for this reason this research focused on treating both WT and $PRSS1^{R122H}$ mice with both male and female sex hormones and study what outcome that would have on their overall health and pancreatic development. Of several experiments that were conducted the first was to administer 5mg/kg of the male sex hormone testosterone, 5mg/kg of either female sex hormone 17α-ethynylestradiol or 17β-estradiol and evaluate the pancreatic health of the animals (Figure 9). Male mice showed slight decreases in body weight and decreases in the pancreas to body weight ratio following sex hormone treatment. While this decrease would appear to result from the addition of estrogen to male mice, female mice in this study that were given 17α-ethynylestradiol also showed a slightly decreased pancreas to body weight ratio. We observed no statistically significant change in relative pancreatic weight compared to normal (10mg/g).

We also initiated an alternative physiological concern associated with pancreatitis, focusing on high fat diet consumption (Figure 5). This study only recently concluded, but the appearance of the mice is markedly different from their control littermates mostly by increased body weight and oily coat. This study compared mice on normal or high fat diet pellets over the period of 6 weeks with wild-type and PRSS1-R122H mice of various ages to determine if lifestyle changes, such as poor deitary nutrition, can also be a factor contributing to pancreatic issues in individuals predisposed to developing pancreatitis.

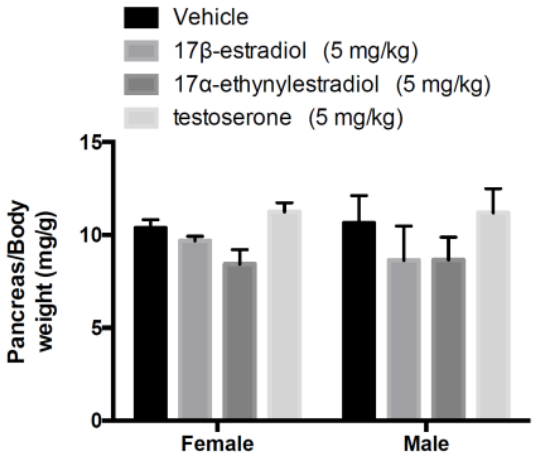

Fig. 9. Evaluation of hormone involvement in the development of pancreatitis. Wild-type (WT) C57BL/6J mice were treated with vehicle alone (corn oil), 17β-estradiol, 17α-ethynylestradiol, or testosterone once daily over 5 days. Mice were euthanized on day 6 and evaluated for pancreatic health by comparing the pancreas to body weight ratio (normal level is 10 mg/g).

Histological examination of pancreatic tissue isolated from low dose sex hormone treated wild-type mice resulted in no observable difference in the tissue (Figure 10). We moved forward with this research in the PRSS1^{R122H} mice to evaluate if hormone changes in those animals, harboring the mutation for hereditary pancreatitis, will result in increased tumor formation or tumor development. Pancreatic tissue was analyzed from both male and female animals in the sex hormone treatment group to determine if low doses of testosterone or estrogen alone would be enough to initiate pancreatitis. We observed no histological differences that could be considered pancreatitis, either acute or chronic, or edema in these animals. All tissue samples appear similar to those animals treated with vehicle (corn oil) alone.

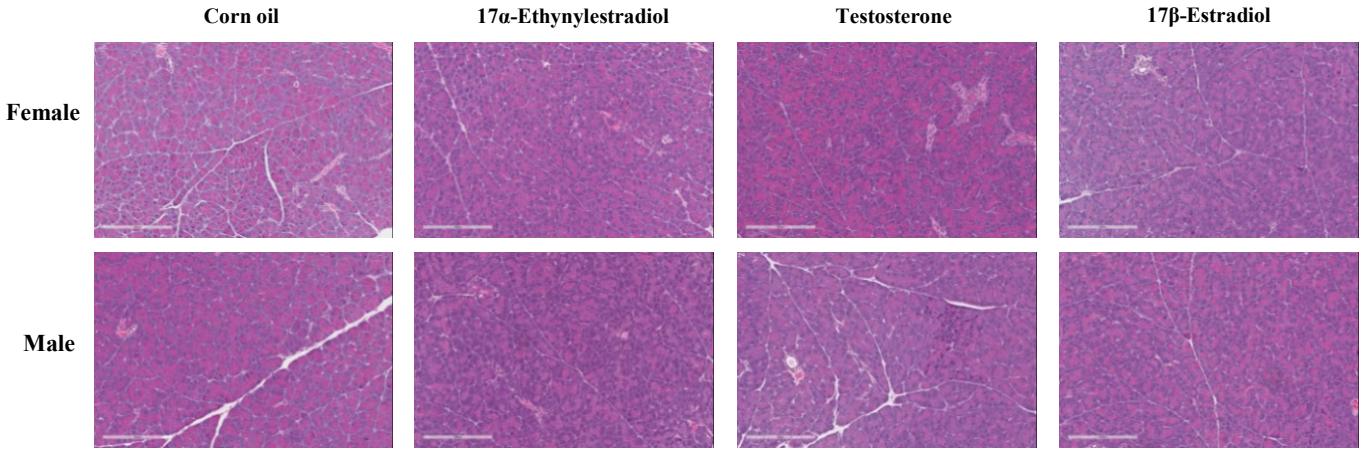

Fig. 10. Histological examination of hormone treated pancreas sections from WT (C57BL/6J) mice using H+E stain at 40x magnification. Wild-type (WT) C57BL/6J mice were treated with vehicle alone (corn oil), 17β-estradiol, 17α-ethynylestradiol, or testosterone once daily over 5 days. Mice were euthanized on day 6. Tissue sections appear normal and these mice did not develop pancreatitis or show a reduction in general body weight following low dose treatments (5 mg/kg) of sex hormones.

Due to the fact that hormone treatment alone did not appear to induce pancreatitis or result in pancreatic edema, we adopted a two-step approach which increased the sex hormone treatment to 25 mg/kg and incorporated the caerulein-induced model to evaluate changes to the pancreas along with drug therapy evaluation. While caerulein induction is not a natural method by which pancreatitis would develop in humans, it does allow for laboratory research to be conducted on animal models so that we may extrapolate and gain understanding regarding the development of pancreatitis in humans. Sex matched male and female WT or PRSS1^{R122H} mice were given 25 mg/kg testosterone, 17α-ethynylestradiol, or 17β-estradiol dissolved in corn oil by oral gavage over the course of 5 days. Two mice from each group were euthanized and body weight, pancreas weight, pancreas health, and histology samples obtained (Figure 11). As figure 11 shows, there was no histological difference between the treated and untreated male and female mice following the high dose sex hormone treatment.

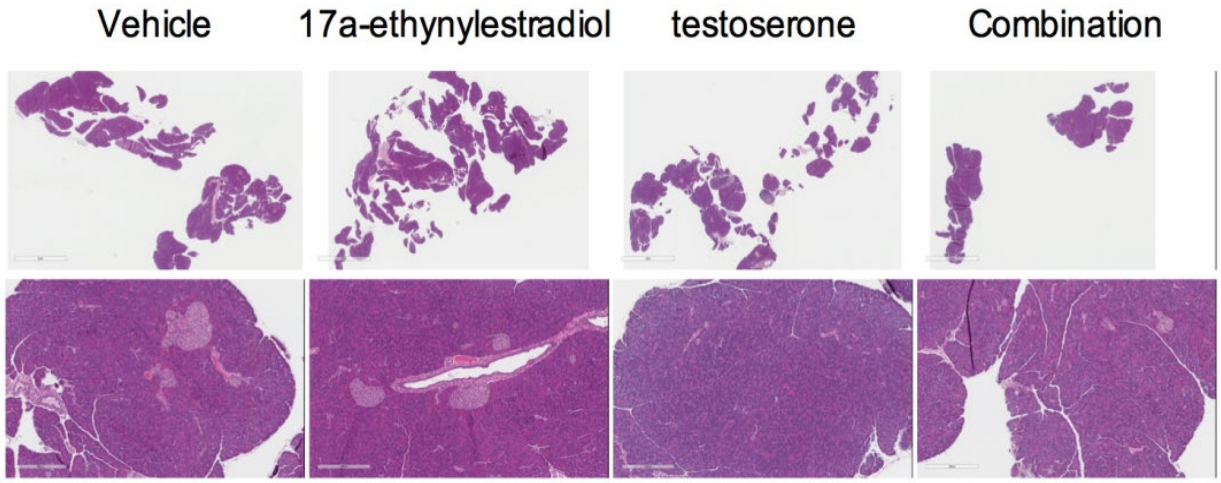

Fig. 11. Histological examination of high dose sex hormone treated PRSS1^{R122H} mice. Male and female mice were given 25 mg/kg testosterone, 17α-ethynylestradiol, or 17β-estradiol dissolved in corn oil by oral gavage over the course of 5 days. Both low 1X (top) and high 20X (bottom) magnification of H+E staining shows that hormone treatment alone was not enough to cause these animals to develop edema, fibrosis, or fat replacement.

Since sex hormones alone resulted in no pancreatitis in this study, the remaining mice in the sex hormone study were given several rounds of caerulein treatment by IP injection, over a 24 hour period, then mice were sacrificed and tissue samples collected (Figure 12). However, the pancreatic damage that was observable can be attributed to the addition of caerulein and cannot be considered to be acute pancreatitis resulting from high doses of sex hormones due to the fact that none of the mice in the same study developed pancreatitis 1 day prior (Figure 11).

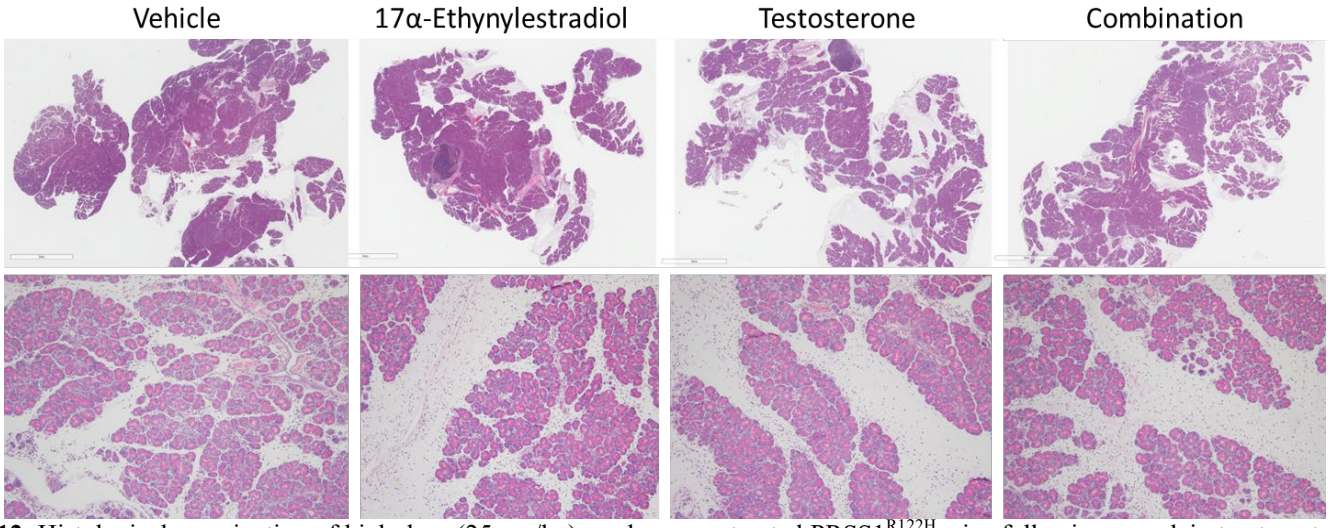

Fig. 12. Histological examination of high dose (25 mg/kg) sex hormone treated PRSS1^{R122H} mice following caerulein treatment. Both male and female mice were given sex hormones by oral gavage in corn oil over 6 days, but the last 24 hours the mice were also treated with caerulein by IP injection. The pancreatic tissue shows acinar cell destruction, at both low 1X (top) and high 20X (bottom) magnification of H+E staining, resulting from the caerulein injections. There was no additional destruction of pancreatic tissue nor was there any observable benefit in the pancreatic tissue in mice that were treated with sex hormones and given caerulein.

Aim 3-2: Test the role of ER stress signaling in PRSS1-R122H induced pancreatitis.

Expression of human R122H trypsinogen in mouse acinar cells sensitized the pancreas to LPS

Next, we assessed if chronic pancreatitis would be observed in the transgenic mice after administration of LPS (4mg/kg twice per week for 2 weeks), which mimic bacterial infection. The pancreas weights were significantly

reduced only in R122H mice treated with LPS. Histological examination showed that the development of chronic inflammatory changes was more prominent in R122H mice with comparatively few small lesions observed in PRSS1 mice and nearly no pathological changes in BAC control mice (Fig. 13A). Immunohistochemical analysis revealed increased infiltration of leukocytes (CD45 positive inflammatory cells) and macrophages (F4/80 positive cells) in R122H mice treated with LPS compared to control or PRSS1 mice (Fig. 13A-B). LPS treatment significantly increased DNA damage (nuclear expression of γH2AX) in the areas of chronic inflammation in R122H mice treated with LPS to a greater extent than that in BAC control or WT PRSS1 mice (Fig. 13A, C). LPS treatment resulted in upregulated expression of the pro-inflammatory cytokine interleukin-1 (IL-1β). Fibrosis markers of α-SMA and Sirus Red staining of collagen were significantly higher in R122H mice than in BAC control or PRSS1 mice. In LPS treated mice, trypsin activity was higher in PRSS1 and R122H mice than BAC controls. There was a trend that trypsin activity was higher in R122H mice than PRSS1 mice, but the difference did not reach statistical significance. LPS treatment also led to increased expression of pancreatic ER stress and oxidative stress response-related genes including Bip (Fig. 13D), activating transcription factor 4 (ATF4) and CHOP. In areas of tissue damage, increased acinar apoptosis (cleaved caspase 3 positive cells) was prominent.

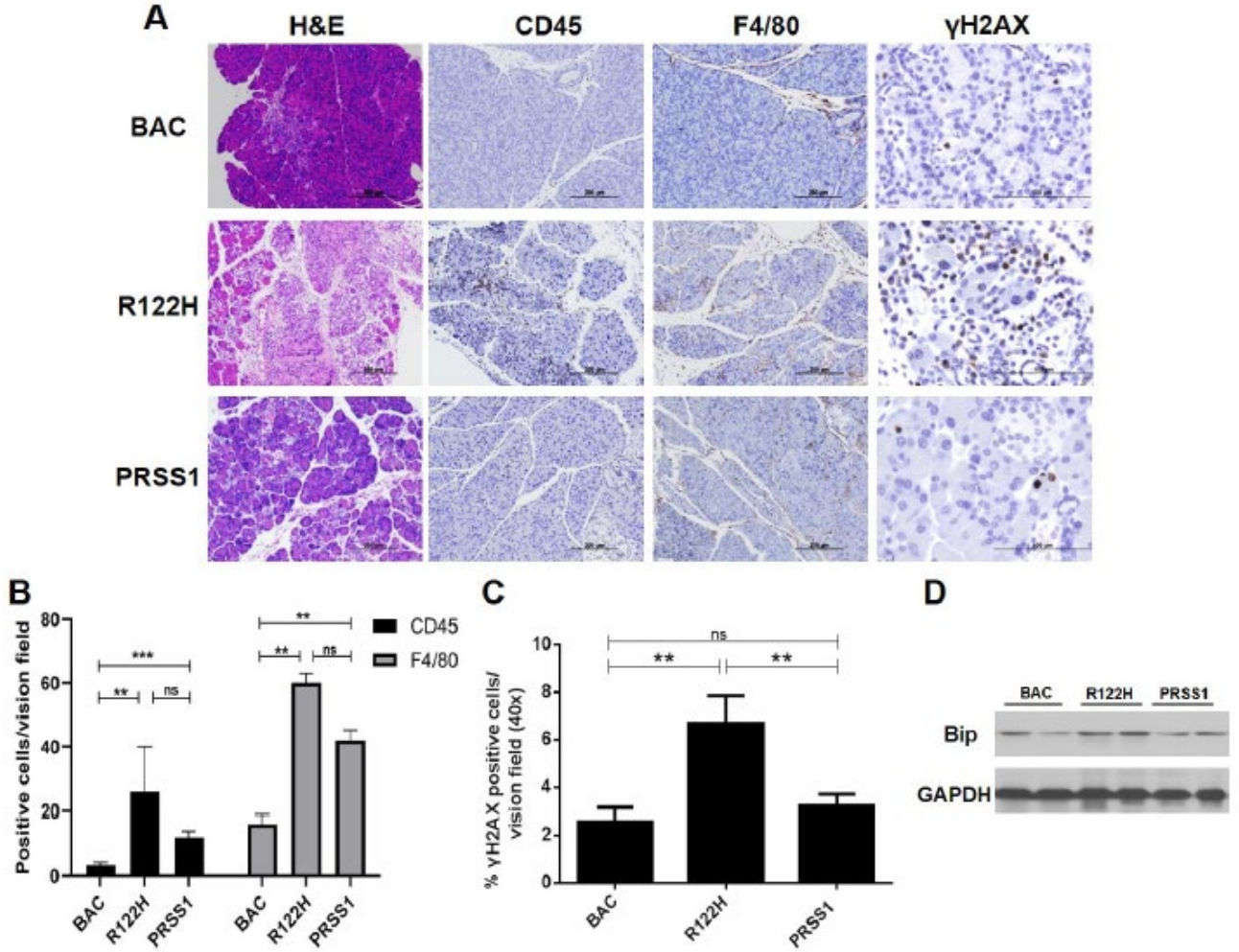

Fig. 13. Human mutant PRSS1^{R122H} transgenic expression caused extensive chronic inflammatory changes after LPS stimulation. (A) The representative histological images of pancreas of R122H, PRSS1, and BAC mice examined by H&E staining. The treatment with LPS led to development of prominent chronic inflammatory changes in R122H mice. In contrast, smaller chronic lesions were observed in PRSS1 mice and only small focal changes in BAC controls. The representative immunohistochemical images of pancreas indicating an increased infiltration of leukocytes (CD45 positive inflammatory cells), macrophages (F4/80 positive cells), and phosphorylated form of histone 2A (γH2AX) (reflecting DNA damage) that were prominent in R122H mice treated with LPS. Quantification under light microscope (40x) for control (BAC), R122H and PRSS1 mice treated with LPS of CD45 positive cells, F4/80 positive cells (B), and γH2AX positive cells (C) (**, $p<0.01$; ***, $p<0.001$; ns, $p>0.05$). (D) Western blot of Bip, which is induced by ER stress, indicated that LPS treatment increased ER stress in R122H compared to PRSS1 mice.

Aim 3-1: Characterize ER stress signaling pathways induced by alcohol/NNK in R122H mice.

Expression of human R122H trypsinogen in pancreatic acinar cells sensitized the pancreas to ethanol and high-fat diet

To determine the interaction between expression of human trypsinogens and clinically relevant environmental insults, we assessed if administration of ethanol or a high-fat diet initiated CP in the trypsinogen transgenic mice. First, we determined if increased human trypsinogen expression could potentiate the effect of ethanol, a significant risk factor for CP. We fed BAC and trypsinogen transgenic animals with a liquid Lieber-DeCarli diet for 4 weeks (Fig. 14A). Ethanol feeding did not result in apparent histological changes in the pancreas of BAC control mice (Fig. 14B). In contrast, ethanol feeding led to the development of chronic inflammatory changes including acinar atrophy and immune cell infiltrations in 74% of R122H mice. Modest changes were observed in about 45% of WT PRSS1 mice (Fig. 14B), suggesting the mutation plays a role in the severity of pancreatitis. Ethanol feeding induced DNA damage (γ-H2AX) (Fig. 14B-C), elevated trypsin activity (Fig. 14D), and collagen deposition. Obese patients are more prone to develop pancreatitis and hyperlipidemia is one of the toxic risk factors of CP. Therefore, we tested if transgenic expression of PRSS1 or R122H would exacerbate pancreatic damage with a high-fat diet (60% of fat) (Fig. 15A). After 6 weeks feeding of high fat diet, we observed focal chronic inflammatory changes as well as the presence of adipocytes in mice expressing human trypsinogens (Fig. 15B). Feeding a high-fat diet also induced DNA damage (Fig. 15B-C), increased cleaved caspase-3 positive cells (Fig. 15D), and deposition of collagen with the most significant changes observed in the R122H expressing mice.

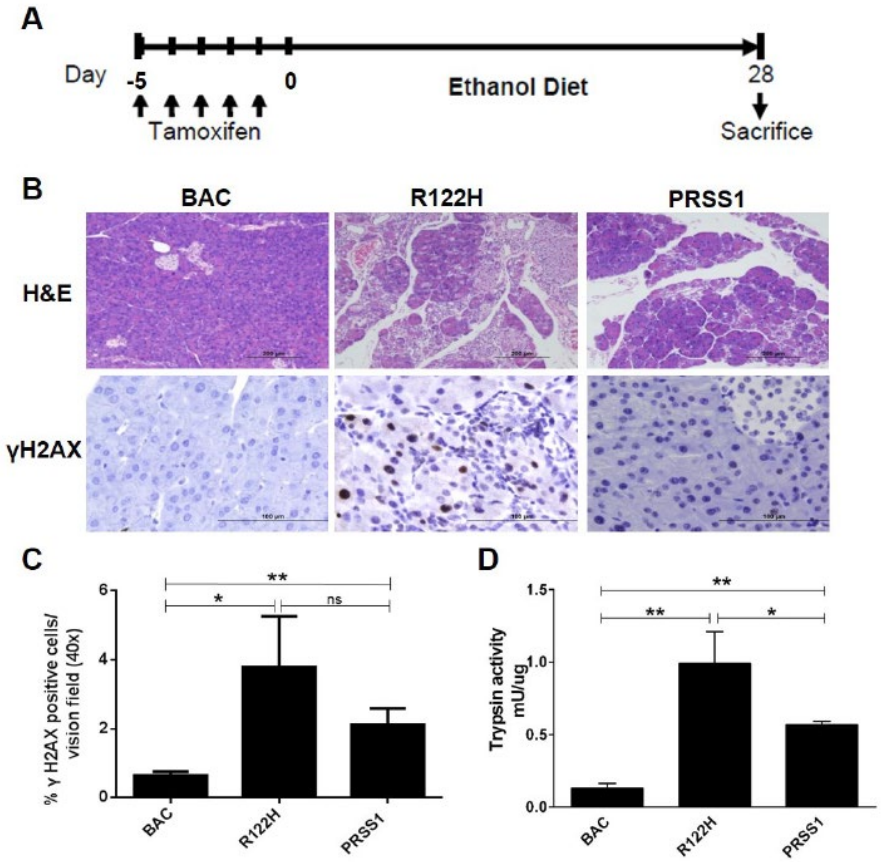

Fig. 14. Feeding with ethanol or high fat diet induced chronic inflammatory changes in pancreas of transgenic trypsinogens expressing mice. (A) The scheme of treatment. The trypsinogen expressing and control mice were fed with a Liber –De-Carli Diet for 6 weeks. (B) The representative H&E images of pancreas of control (BAC) and trypsinogens (R122H and PRSS1) expressing mice fed with ethanol diet for 4 weeks. Corresponding representative immunohistochemical images showing focally localized DNA damage (nuclear staining for γH2AX) (magnification: 40 x, error bar: 100μm). (C) Quantification of γH2AX positive cells under light microscope (40x) for control (BAC), R122H and PRSS1 mice fed with ethanol diet. (D) Trypsin activity in control (BAC), R122H and PRSS1 mice (*, $p<0.05$; **, $p<0.01$; ns, $p>0.05$).

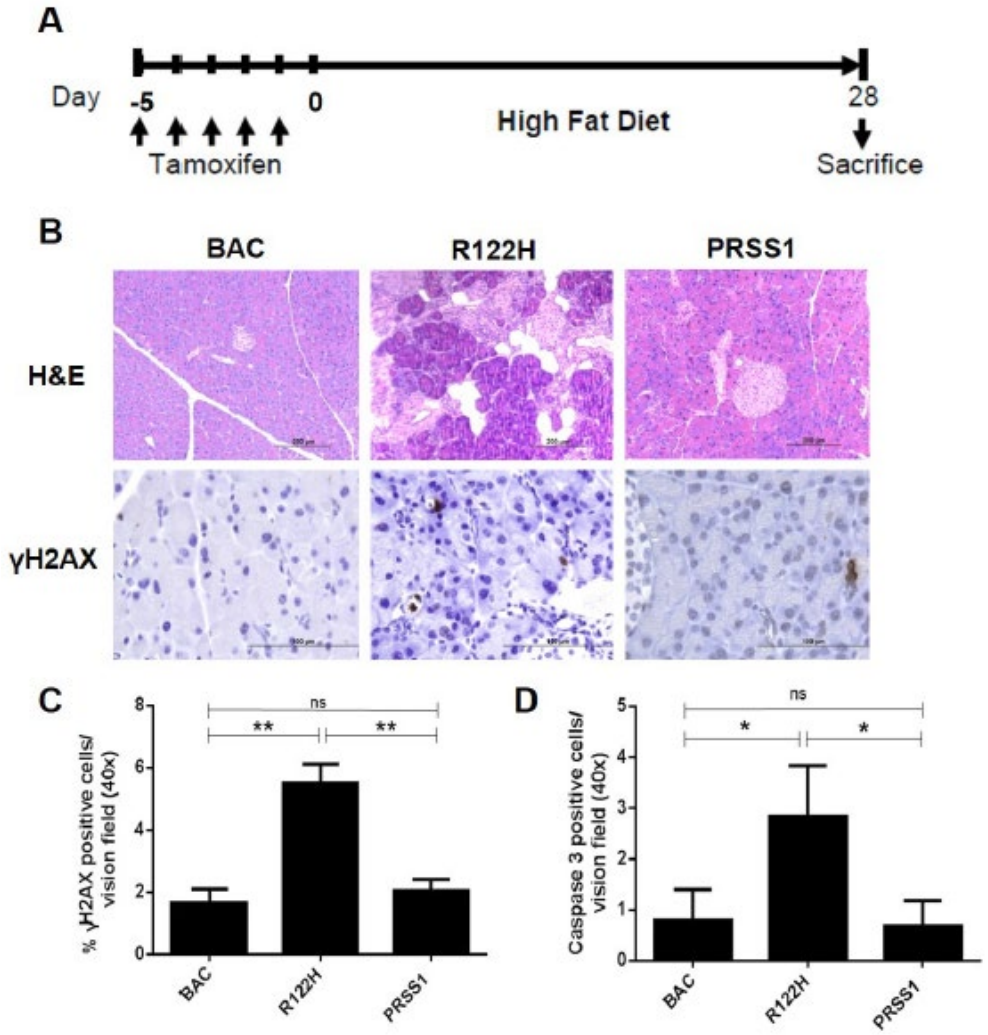

Fig. 15. (A) The scheme of treatment. The trypsinogen expressing and control mice were fed with a high fat diet (60% fat) for 6 weeks. (B) The representative H&E images of pancreas of control (BAC) and trypsinogens (R122H and PRSS1) expressing mice fed with high fat diet for 4 weeks. Corresponding representative immunohistochemical images showing focally localized DNA damage (nuclear staining for γH2AX). (C) Quantification of yH2AX positive cells (D) and cleaved caspase 3 under light microscope (40x) for control (BAC), R122H and PRSS1 mice fed with high fat diet (*, $p<0.05$; **, $p<0.01$; ns, $p>0.05$).

8. DISCUSSION

During the final reporting period we repeated several sex hormone experiments. This research hoped to gain a better understanding of whether the hormonal changes that occur around the first episode of acute pancreatitis in patients with HP could be linked to the disease. However, the data shows that while there may be small changes in relative pancreatic weight (not statistically significant) with the addition of sex hormones in control and $PRSS1^{R122H}$ mice, the addition of sex hormones alone in wild-type mice was not sufficient to trigger the development of pancreatitis. Furthermore, sex hormone treated mice responded similarly to caerulein-induced pancreatitis and showed no further pancreatitis or beneficial effects from the addition of sex hormones in this study. Anti-hormone treatment with Tamoxifen resulted in no pancreatic benefit with or without the stimulation from caerulein, so anti-androgen studies with Enzalutamide were not conducted. Further work is needed to identify the precise role these hormones play in the development of hereditary pancreatitis in humans.

Caerulein treatment in $PRSS1^{R122H}$ mice has been shown to alter the pancreatic tissue, changing it from healthy acinar cells to a more ductal phenotype, which may be a transitional state before the development of pancreatic cancer. Work is continuing to better understand the cellular and genetic changes that occur in each step in pancreatic tissue and their role in pancreatic cancer. It is still unclear how the R122H mutation in PRSS1

contributes to the development of hereditary pancreatitis. This study focused on evaluating trypsin levels in the various mouse genotypes to determine how active trypsin affects the pancreas. Cell death and inflammation are key pathological responses of acute pancreatitis. The severity of experimental acute pancreatitis positively correlates with the extent and the type of cell death. Although multiple forms of cell death occur in physiological and pathological conditions, apoptosis and necrosis play a major role and are most widely studied in clinical and experimental acute pancreatitis. Important signaling pathways in pancreatitis include: NFAT, NF- B, and Ras pathways.

Elevated trypsin is associated with severe acute pancreatitis, which was demonstrated by dramatically increased levels of edema, serum amylase, inflammatory cell infiltration, and acinar cell damage. Furthermore, high intra-acinar trypsinogen activation resulted in a significant increase in the severity of chronic pancreatitis. Chronic pancreatitis is characterized by progressive pancreatic damage that eventually results in significant impairment of both exocrine and endocrine functions of the gland. The pathophysiological mechanisms of chronic pancreatitis are not well understood. A popular notion is that intrapancreatic trypsinogen activation could be responsible for the pathogenesis of chronic pancreatitis. The strongest support of this idea is the identification of trypsinogen mutations in hereditary pancreatitis, an uncommon form of chronic pancreatitis. Compared with pancreatic tissues from wild-type mice, those trypsinogen knockout mice had similar levels of atrophy, histological features of chronic pancreatitis, and chronic inflammation.

Recently, it has been recognized that there is extensive cross-talk between coagulation and inflammation pathways. Activation of one system may amplify activation of the other. Inflammation causes endothelial damage, increased fibrinogen concentration, and tissue factor expression on the cell surface of leukocytes, upregulates both platelet count and platelet reactivity, and downregulates anticoagulation factors. Conversely, the tissue factor/VIIa complex stimulates PAR-2 signaling, which induces release of inflammatory cytokines. With the exception of coagulation, factor Xa and thrombin (factor IIa) are also involved in inflammation, predominantly mediated via the activation of proteinase activated receptors (PARs). PAR-1, -3, and -4 are thrombin receptors. PAR-1 and PAR-2 can be activated by the TF–factor VIIa complex and factor Xa. PAR activation plays important roles in pancreatitis and other inflammatory diseases. Therefore, reciprocal interaction between coagulation and inflammation forms a positive feedback loop which may result in tissue damage or even lethal multi-organ failure — a condition frequently observed in severe AP. Some of the next areas of focus for pancreatitis will be on identifying how signaling pathways communicate and understanding the roles that coagulation and inflammation play in the transition from AP to CP and from CP to pancreatic cancer.

Regarding aim 3-3: Improving ER stress with chemical chaperones will ameliorate pancreatitis. We planned to evaluate how chemical chaperones that are believed to help reduce ER stress, sodium phenylbutyrate (4-PBA) and tauroursodeoxycholic acid (TUDCA), would impact the development and severity of pancreatitis following caerulein stimulation. We tried the addition of 4-PBA, but we observed no improvement upon pancreatitis in animal studies (data not shown) for that reason we did not do additional studies evaluating the role of TUDCA and its ability to prevent ER stress in these animal models. Difficulties with acinar cell preparation, calcium signalling using Fura-red and machine breakdown/maintenance of the Spectramax i3X plate reader resulted in delays and the inability to produce the desired data for this aim. The control acinar cells were still responsive to calcium fluctuations in the absence of CCK or chemical chaperones to reduce ER stress (TUDCA and 4-PBA). The reported wavelengths for Fura-red (Thermofisher) produced inconsistent data even after modifying the protocol for injection to prevent the movement of the non-adherent acinar cells. Finally, the plate reader necessary for this protocol was down for several weeks for bulb replacement and additional maintenance.

9. KEY RESEARCH ACCOMPLISHMENTS:

- IACUC approval (A00003730-18) The Mayo Clinic IACUC protocol (PI-Dr. Baoan Ji) was approved in August of 2018, so we could continue work on this project.

- USAMRMC Animal Care and Use Review Office (ACURO) approval was received for the new IACUC in September of 2018.

- The major goals described in the SOW remain the same.

- We have included the lifestyle condition of a high fat diet to see how that contributes to the development or progression of pancreatitis in our hereditary pancreatitis mouse model.

- A transgenic mouse line that expresses the most common mutation in the trypsinogen gene that is associated with hereditary pancreatitis (HP), $PRSS1^{R122H}$, was engineered and allows us to study and evaluate changes associated with human pancreatitis without exploiting individual patients with this disease.

- Breeding and expansion of the R122H mouse colony took longer than planned. While there were delays due to construction projects on buildings that house animal facilities that slowed animal mating and breeding as well as tropical storms and hurricanes that altered the barometric pressure resulting in numerous failed pregnancies.

10. CONCLUSION:

This research focus is of high relevance to the military as it pertains directly to clinical health issues related to current and former servicemen and women. A recent study from the Veterans Health Care System showed that 10.7% of patients with a diagnosis of pancreatitis were found to have advanced to pancreatic adenocarcinoma within a 2 year period after the initial pancreatitis diagnosis. Furthermore, alcohol abuse is one of the most common causes of pancreatitis. Cigarette smoking further increases the risk for the development of pancreatitis. The prevalence of alcoholism and tobacco use is high in the veteran population. A 2018 report by The Truth Initiative showed that 15-30% of service members are active smokers. Among military personnel, service members between the ages 18 and 25 had a 27.3% prevalence of heavy drinking, compared to 15.3% among civilians in the same age group. In 2005, a total of 43.2% of active-duty military personnel reported binge drinking within a recent 30-day period. Additionally, a recent survey by The United Health Foundation found that veterans smoked and drank more than the general population. Finally, dietary concerns further increase the risk of developing pancreatitis. Using a hereditary pancreatitis mouse model allows us to stimulate the development of pancreatitis in a controlled setting and measure subtle chemical and genetic changes that may be the impetus for the advancement from pancreatitis to pancreatic cancer. Development of pancreatitis from sex hormones alone did not occur during the reporting period, but this work can add to the understanding of pancreatic tissue and how subtle changes may be the driving factor towards whether a person develops acute pancreatitis that goes on to pancreatic cancer. This research has the ability to directly contribute to the understanding of how one's environmental and biological components interact, either resulting in a normal or diseased state. The work performed in this study includes Dr. Pandol, a world-renown pancreatologist with many years of experience in the Veterans Health Care System. He is collaborating with us as a physician and investigator of the mechanisms for risk factors (i.e. alcohol, smoking) in the promotion of pancreatitis.

11. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Mutant Human PRSS1-R122H Expression in Mice Greatly Exacerbates Pancreatitis. Yuebo Zhang, Xianbao Zhan, Guowei Zhang, Lu Zhuang, Yinghua Li, Jia Guo, Lizhi Zhang, Stephen J. Pandol, Craig D. Logsdon, Yan Bi, Baoan Ji.

Gastroenterology. April, 2017. Volume 152, Issue 5, Supplement 1, page S893. DOI: http://dx.doi.org/10.1016/S0016-5085(17)33051-2.

Pancreatic acinar cells with human PRSS1(R122H) expression display higher susceptibility to stress induced by cholecystokinin or a combination of ethanol and cigarette smoke extracts. Hu, C; Su, HY; Waldron, R; Xia, Q; Ji, B; Lugea, A; Pandol, SJ. GASTROENTEROLOGY. Volume: 152, Issue: 5, Pages: S899-S900; April 2017.

Ethanol and Smoking Promote Inflammation and Cell Death in Pancreas of Humanized PRSS1-R122H Transgenic Mice. Author(s): Hu, C; Su, HY; Waldron, RT ; Lugea, A; Xia, Q; Ji, B; Pandol, SJ. **Source:** PANCREAS **Volume:** 46 **Issue:** 10 **Pages:** 1407-1407 **Published:** October 2017.

Effects of Pentoxifylline and Indomethacin on a Genetic Mouse Model of Hereditary Pancreatitis. Zhuang, L; Guo, J; Yao, Y; Bi, Y; Ji, B. PANCREAS. Volume: 46, Issue: 10, Pages: 1447-1448**:** NOV-DEC 2017.

Aurora Kinase A Improves Acinar Cell Survival and Regeneration in Experimental Pancreatitis of Mice. Zhuang, L; Zhan, X; Yao, Y; Zhang, Y; Guo, J; Gui, F; Chen, J ; Haddock, A; Bi, Y; Ji, B. PANCREAS. Volume: 46, Issue: 10, Pages: 1447-1447. NOV-DEC 2017.

Trypsin Activity Governs Increased Susceptibility to Pancreatitis in Mice Expressing Human PRSS1^{R122H}. Gui, F; Zhang, Y; Wan, J; Zhan, X; Yao, Y; Li, Y; Haddock, A; Shi, J; Guo, J; Chen, J; Zhu, X; Edenfield, B; Zhuang, L; Hu, C; Wang, Y; Mukhopadhyay, D; Radisky, E; Zhang, L; Lugea, A; Pandol, S; Bi, Y; Ji, B. The Journal of clinical investigation. 2019 September 24.

Transgenic Expression of PRSS1^{R122H} Sensitizes Mice to Pancreatitis. Huang H, Swidnicka-Siergiejko AK, Daniluk J, Gaiser S, Yao Y, Peng L, Zhang Y, Liu Y, Dong M, Zhan X, Wang H, Bi Y, Li Z, Ji B, Logsdon C. Gastroenterology. 2019 August 13.

Elevated intracellular trypsin exacerbates acute pancreatitis and chronic pancreatitis in mice. Zhan X, Wan J, Zhang G, Song L, Gui F, Zhang Y, Li Y, Guo J, Dawra RK, Saluja AK, Haddock AN, Zhang L, Bi Y, Ji B. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2019 April 3.

Presentations- 2018 American Pancreatic Association, Miami, FL

High-efficient pancreatic specific gene manipulation in FLPo transgenic mice
Xiaohui Zhu[1], Fu Gui[1], Jiaxiang Chen[1], Jianhua Wan[1], Yan Bi[2], Baoan Ji[1]

Effective Experimental Therapeutics in a Novel Humanized Animal Model of Hereditary Pancreatitis
Fu Gui[1], Yuebo Zhang[1], Xianbao Zhan[1], Jianhua Wan[1], Jiaxiang Chen[1], Xiaohui Zhu[1], Ashley Haddock[1], Yinghua Li[1], Lizhi Zhang[2], Stephen Pandol[3], Craig Logsdon[4], Yan Bi[5] and Baoan Ji[1]

The tumorigenic roles of mutant KRas isoforms in pancreatic cancer
J.Chen[1], A. Haddock[1], F. Gui[1], J. Wan[1], X. Zhu[1], Y.Bi[2], B.Ji[1]

Activation of muscarinic receptor 3 on pancreatic acinar cells causes both acute and chronic pancreatitis
Jianhua Wan[1], Fu Gui[1], Jiaxiang Chen[1], Xiaohui Zhu[1], Ashley N Haddock[1], Pei Wang[2], Yan Bi[3], Baoan Ji[1].

12. INVENTIONS, PATENTS AND LICENSES:

Nothing to report

13. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Dr. Baoan Ji is the PI and main contributor to the recent findings. Dr. Fu Gui has left the lab. An amendment to the IACUC protocol was submitted 11/7/2018 to remove Dr. Gui and to add a new lab member Ji Shi who has contributed to this study. A new IACUC protocol was submitted and approved to allow continuation of this project. The ACURO approval was finalized October 1, 2018.

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?
Previously, this project worked in collaboration with Dr. Stephen Pandol from Cedars Sinai Medical Center, Los Angeles, CA. Aim 2 of the project was his area of research and he has completed all projects focusing on "Determining the role of PRSS1-R122H and alcohol/smoking in the development of pancreatitis". He is still an active partner and tele-conferences between our labs occur bi-monthly.

14. REPORTABLE OUTCOMES:
Nothing to report

15. OTHER ACHIEVEMENTS:
Nothing to report

16. REFERENCES:
No change from previous

17. APPENDICES:
Trypsin Activity Governs Increased Susceptibility to Pancreatitis in Mice Expressing Human PRSS1^{R122H}. Gui, F; Zhang, Y; Wan, J; Zhan, X; Yao, Y; Li, Y; Haddock, A; Shi, J; Guo, J; Chen, J; Zhu, X; Edenfield, B; Zhuang, L; Hu, C; Wang, Y; Mukhopadhyay, D; Radisky, E; Zhang, L; Lugea, A; Pandol, S; Bi, Y; Ji, B. The Journal of clinical investigation. 2019 September 24.

Transgenic Expression of PRSS1^{R122H} Sensitizes Mice to Pancreatitis. Huang H, Swidnicka-Siergiejko AK, Daniluk J, Gaiser S, Yao Y, Peng L, Zhang Y, Liu Y, Dong M, Zhan X, Wang H, Bi Y, Li Z, Ji B, Logsdon C. Gastroenterology. 2019 August 13.

Elevated intracellular trypsin exacerbates acute pancreatitis and chronic pancreatitis in mice. Zhan X, Wan J, Zhang G, Song L, Gui F, Zhang Y, Li Y, Guo J, Dawra RK, Saluja AK, Haddock AN, Zhang L, Bi Y, Ji B. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2019 April 3.

NOTE: No awards

QUAD CHARTS:
Nothing to report

MARKING OF PROPRIETARY INFORMATION:
None