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TITLE: Transgastric Local Pancreatic Hypothermia: A Novel, Rapid Multimodal Therapy for Acute Pancreatitis

PRINCIPAL INVESTIGATOR: Dr. Vijay P. Singh

CONTRACTING ORGANIZATION: Mayo Clinic Arizona
Scottsdale, AZ 85259.

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Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S)

Dr. Vijay P. Singh

E-Mail: singh.vijay@mayo.edu

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Mayo Clinic Arizona
Scottsdale, AZ 85259.

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14. ABSTRACT

Purpose: Study if transgastric local hypothermia of the pancreas reduces the metabolic demand of the pancreas during acute pancreatitis (AP) and AP severity.

Scope: 1) Using metabolic imaging of the pancreas to predict its severity 2) Using transgastric cooling as a treatment modality for AP. These are proposed in Aim-3 of the grant. Time period of study: Jan to Dec 2015

Major Findings: 1) There is a significant increase in the uptake of Non-Radioactive Near Infra-Red 2-Deoxyglucose (IRDye® 800CW 2-DG) in severe AP over controls which is detectable within an hour after AP induction without cooling. 2) Therapeutic transgastric hypothermia reduces severity in the GTL model of pancreatitis.

Result: 1) Poster presented at the *American Pancreatic Association* annual meeting (November 2015) entitled; "*In Vivo* Imaging of Non-Radioactive Near Infra-Red 2-Deoxyglucose During Mild and Severe Acute Pancreatitis (AP) in Rats". 2) Manuscript reviewed at *PLOS ONE,* minor issues addressed. The title is "*Characterization and predictive value of near infrared 2-deoxyglucose optical imaging in severe acute pancreatitis*". Here we note that *In-vivo* fluorescent imaging of IRDye® 800CW 2-DG can predict the AP severity early during the disease better than conventional markers of severe AP.

Significance: Metabolic imaging of the pancreas can predict AP severity early in the disease.

Ongoing studies: The studies in the intraductal GTL model are almost complete. We shortly will start studying this in the taurocholate model of AP and complete the other studies proposed.

15. SUBJECT TERMS: Transgastric, local hypothermia, pancreatitis

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		88	19b. TELEPHONE NUMBER *(include area code)*
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Table of contents:

1: INTRODUCTION: This Annual Report details the accomplishments and conclusions of the second year of CDMRP funding of the project PR110417 (Transgastric Local Pancreatic Hypothermia: A Novel, Rapid Multimodal Therapy for Acute Pancreatitis), from the period 01/07/2015 till the 01/06/2016. Acute Pancreatitis (AP) is an unpredictable, sudden, painful condition of the abdomen, which is rapidly progressive and has varied outcomes. It has no targeted treatment and affects 274,000 patients in the USA annually. Our veterans are at a higher risk of AP. The basic hypothesis of the project is that the metabolic demand of the pancreas is increased during AP. The project proposes to use imaging studies to determine the metabolic demand of the pancreas during pancreatitis and determine if local cooling of the pancreas through the stomach would be a feasible therapy for AP in rodents, and if so determine the temperatures that should be used, along with determining the efficacy of such an approach. All studies done below have been approved by the Mayo IACUC and the ACURO. Following is a summary of the progress related to project PR110417.

2: KEYWORDS: Acute pancreatitis, hypermetabolic, transgastric, local hypothermia, severity

3: ACCOMPLISHMENTS:

3.1: Goals: Based on the originally proposed studies (Aim 3), the statement of work, and after IACUC protocol A20813, and ACURO approval (see appendix-1); we aimed at determining whether: A) The metabolic demand of the pancreas is increased during AP, B) transgastric hypothermia reduces the severity of AP.

3.2: What was accomplished: The accomplishments were:

A: Confirmation that the pancreas is hypermetabolic in severe pancreatitis: This was part of the ***Major goal of Aim 3, subaim-1;*** i.e. to "Determine the reduction in pancreatic metabolic demand during localized hypothermia using PET". The results of this were presented as

i) Poster, at the American Pancreatic Association meeting (November 2015): "*In Vivo Imaging of Non-Radioactive near Infra-Red 2-Deoxyglucose during Mild and Severe Acute Pancreatitis (AP) in Rats*" (Pancreas: November 2015 – Vol. 44 - Issue 8 - p 1369) (see appendix-2).

ii) Manuscript reviewed at *PLOS ONE*, minor issues addressed (appendix-3): The title is "*Characterization and predictive value of near infrared 2-deoxyglucose optical imaging in severe acute pancreatitis*".

Results: Based on the increased uptake of the near infra-red fluorescent dye, IRDye® 800CW 2-DG, these studies demonstrate that the pancreas has increased metabolic demand during severe (GTL) but not mild AP. Thus this concept/technology/method may be used to make an early distinction between mild and severe AP and predict the course early more reliably than conventional markers.

B: Data showing efficacy of transgastric hypothermia in ameliorating the GTL model of AP: This is detailed under, "Accomplishments compared to statement of work", subheading "Aim 3: Test the therapeutic efficacy of transgastric local hypothermia in experimental pancreatitis."

3.3: What opportunities for training and professional development has the project provided? Nothing to report

3.4: How were the results disseminated to communities of interest? Poster, manuscript.

3.5: Plan during the next reporting period to accomplish the goals?

1. We are analyzing data on reduction in metabolic demand of the pancreas while using transgastric local hypothermia (part of Aim 3, sub aim 3) and whether the presence of a gastric balloon may impact this.

2. We have completed the experiments on the effect of local hypothermia on the GTL model of AP. We are analyzing its effects on local injury (Aim 3, subaim-2) and systemic injury (Aim 3, subaim-3).

3. We will start studying whether the metabolic demand is increased in the taurocholate model of AP and whether transgastric local hypothermia improves outcomes. The effect of local hypothermia will also be studies in the caerulein model, as originally proposed.

These manuscripts should be ready for publication in the next reporting period.

4. We have submitted an IACUC renewal application and will submit an ACURO modification after its approval. This renewal finds a solution to the technical limitations we have encountered, and will help study the efficacy of local hypothermia over longer (3 day) periods, referred to as "Slow models" in the Statement of work (SOW) which are to be accomplished in year 3 of the project.

Accomplishments compared to Statement of Work (SOW):

Below is the timetable for the proposed studies as submitted in the statement of work:

Time / Aim	1-4M	5-8M	9-12M	13-15M	16-20M	21-24M	25-28M	29-32M	33-36M
Aim 1 Sub 1	X								
Aim 1 Sub 2		X	X						
Aim 1 Sub 3	X								
Aim 2 Sub 1	X								
Aim 2 Sub 2		X	X						
Aim 3 Sub 1				X	X	X	X	X	X
Aim 3 Sub 2				X	X	X	X	X	X
Aim 3 Sub 3				X	X	X	X	X	X
				Rapid (caerulein,12 hr. Taurocholate) models			Slow (Arginine, 3-7 day Taurocholate) models		

Timetable for proposed studies:

Each column shows a 4 month (M) interval, progressively increasing from left to right. Each row depicts and aim/subaim of the proposed studies. Details of the aim/subaims are as mentioned in the text. "X" indicates the time when the studies will be performed. The color shades group the tasks. Rapid (blue) and slow (pink) pancreatitis models will be performed in years 2 and 3 respectively.

Aim 1:

This was successfully accomplished in year one, and the annual progress report was submitted in 2014. The main achievements related to this aim reported were: **A)** Presentation of this work entitled "*Transgastric Hypothermia Achieves Temperatures that Synergistically Slow Multiple Signaling Pathways Relevant to Acute Pancreatitis (AP)*" at the 44th annual American Pancreatic Association Annual conference at Miami, Florida (Pancreas: November 2013 - Volume 42 - Issue 8 - p 1368). **B)** presentation of this work at the American College of Cryosurgery annual Conference on January 18th 2014, as an invited lecture entitled: *Local Hypothermia, a Potential Multimodal Reversible Therapy for Acute Pancreatitis*. **C)** Publication of this work in the journal *Pancreatology*: Mishra V, Patel K, Trivedi RN, Noel P, Durgampudi C, Acharya C, Holmes JA, Narla S, Singh VP. *Hypothermia slows*

sequential and parallel steps initiated during caerulein pancreatitis. Pancreatology. 2014 Nov-Dec; 14(6):459-64.

Aim-2 : Optimize the device to safely attain pancreatic TT (Therapeutic temperatures).

We optimized transgastric cooling to obtain the target temperatures during pancreatitis. We chose the IACUC and ACURO approved continuous sedation (Ketamine and Xylazine) method. This ensures animal comfort and can be maintained up to a maximum of 7 hours. This time period is suitable for the "rapid severe" models of pancreatitis, where the endpoints are reached between 2-5 hours in the untreated group. The gastric cooling balloon was procured from Vention Medical (Marlborough, MA). We have successfully used this to induce therapeutic pancreatic hypothermia after induction of GTL pancreatitis (Figure 1). The target temperature of 25-26^{0}C was achieved in about 90 minutes and could be maintained for the duration of cooling needed to study its efficacy in the GTL model of pancreatitis.

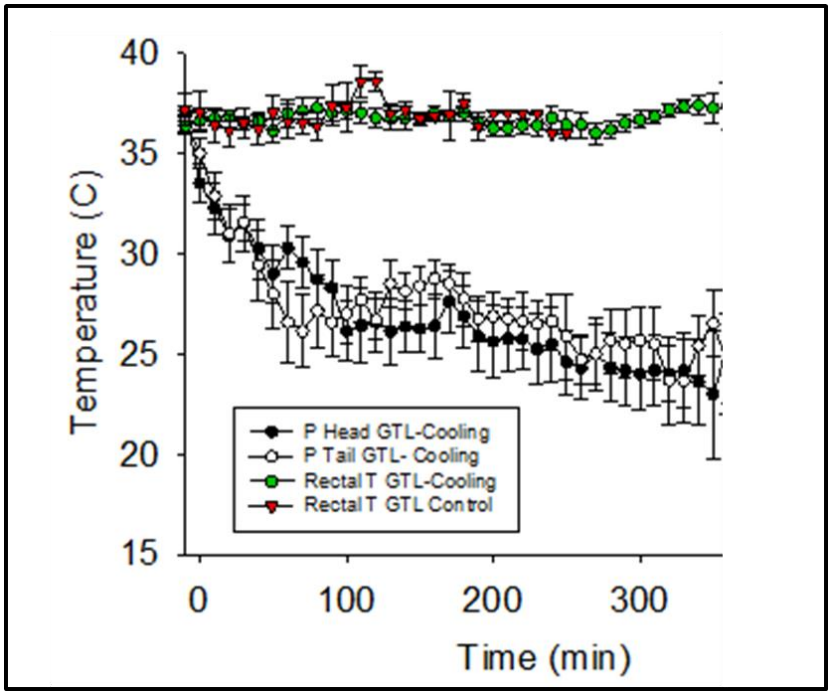

Figure 1: Pancreatic (P) and rectal temperatures (T) in rats with GTL induced pancreatitis: Rectal temperatures of rats without cooling (GTL control, in red triangles) vs. those with GTL pancreatitis and cooling (Green circles) are no different. Note that the target pancreatic temperature of 25-26^{0}C was successfully obtained in about 90 minutes both in the pancreatic head (black circles) and the tail (white circles), and is maintained over the 6 hour duration of the study in the cooled group with no drop in rectal temperatures (Green circles). The rats with GTL pancreatitis without cooling (red circles) survived to a maximum

Cooling with sedation did not affect the pulse oximetry (95±1% vs. 95±1%) plasma lipase (85±28 vs. 76±21 U/L), glucose (120±20 vs. 98±24 mg/dl) in the rats before vs. 6 hours after cooling. A mild reduction in heart rate (476±15 vs. 361±32 beats/min) was likely from sedation and was similar in anesthetized controls (315±28). These studies bring closure to Aim-2 for the "rapid models" proposed in Aim 3, since we have now optimized balloon placement and maintained cooling for the duration needed for these studies.

Aim 3: Test the therapeutic efficacy of transgastric local hypothermia in experimental pancreatitis.

Work on this aim is ongoing. We have accomplished the GTL model. The data are as follows: There was no significant difference between the GTL alone vs. GTL+ balloon without cooling in the parameters studied. The data shown below is pooled for these two groups for simplicity purposes.

Survival: Cooling significantly improved survival vs. rats without cooling. There was 100% mortality by 5 hours in the GTL group (n=15, mean mortality at 3:15 hours). This was reduced to 33% in the GTL+ cooling group (n= 12, p<0.02) for the 6 hours these animals were followed (Figure 2).

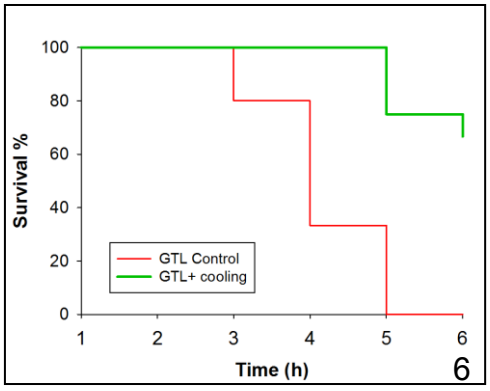

Figure 2: Kaplan-Meyer curve showing the effect of cooling on survival in rats after GTL induced AP. The red line shows rats with GTL induced AP and the green line rats with therapeutic transgastric cooling initiated after induction of GTL AP

Local injury: While there was no difference in the pancreatic edema (measured as % water content) between the pancreas of rats with GTL pancreatitis alone or with cooling (85±1.7% vs 84±2.4%); there seems to be a reduction in pancreatic necrosis both grossly and microscopically (Figure 3). The morphometric data of this is currently being analyzed before final quantification of results.

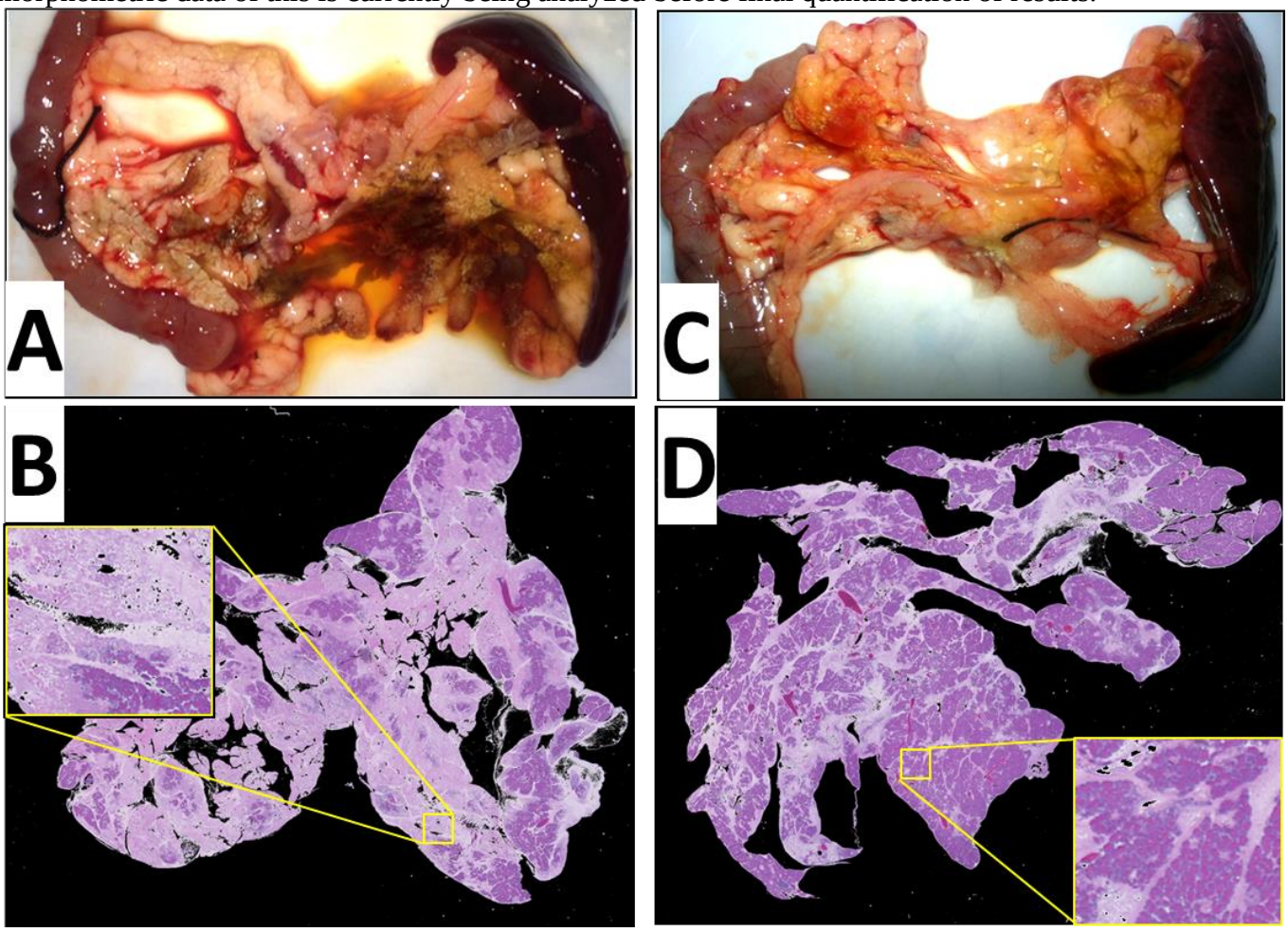

Figure 3: Gross (A, C) and microscopic appearance (B, D) of the pancreas in rats with GTL pancreatitis without cooling (A, B) and with transgastric cooling (C, D). Note the higher amount of grossly visible hemorrhages in the pancreas without cooling. Necrosis seen as an amorphous pink appearance with loss of cell outlines is much more in the pancreas without cooling (B) than in the cooled pancreas.

Systemic Injury:

Preliminary data suggest that transgastric cooling of the pancreas also reduces systemic injury. As seen in figure 4, there is a statistically significant reduction in the BUN of the cooled rats measured at 3:20 hours, which is the mean mortality time of the GTL group. The lung injury and Kidney histology studies in these animals are pending.

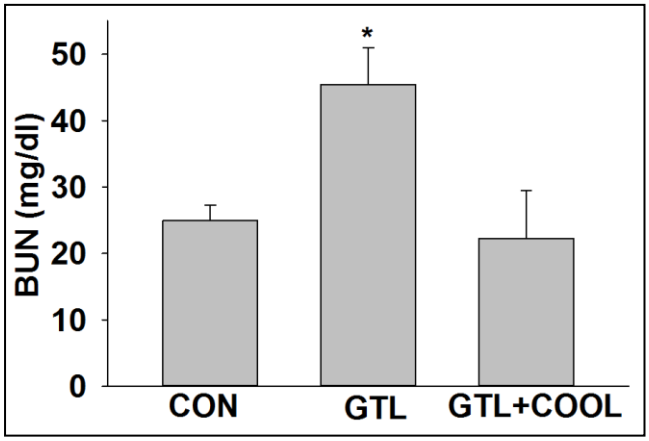

Figure 4: Serum BUNs of rats at the time of mortality in the GTL group, and blood samples collected at the same time (mean; 3:20 hours) in the GTL+ cooling (GTL+COOL) group. * indicates $p<0.02$ on ANOVA.

Studies are ongoing to make the total number 8-12/group, and analyze the remaining parameters.

4. IMPACT:

4.1 Impact on the development of the principle discipline of the project:

A: Use of 2-DG mimetics can predict the severity of pancreatitis: Based on the data in appendix-3, which was a part of Aim-3 of the grant, we can say the 2-DG mimetics such as IRdye 800CW 2-DG can be used to predict AP severity early in the disease. This is a significant advance because AP has a sudden onset with a rapid, variable and unpredictable course ranging from resolution with minimal care over a few days (mild AP; which occurs in 70-80% cases), to a severe course (severe acute pancreatitis; SAP) progressing to extensive pancreatic necrosis, requiring intensive care, a prolonged hospitalization with high costs and sometimes resulting in death.

We currently lack reliable tools to predict the course of AP early in the disease. This study shows that severe biliary AP is associated with an early and sustained retention of the near-infrared 2-DG probe (IRDye® 800CW 2-DG; NIR 2-DG; Li-Cor) in the pancreas of rats with GTL induced SAP. This retention is associated with worse pancreatic necrosis along with mortality. Our discovery of 2-DG mimetics to predict AP severity early on in the disease course is a significant advance.

The study is unique for the following reasons: **1)** It uses a rapidly evolving inflammatory disease model which is unlike the much longer duration cancer [1,2,3] or chronic inflammation [4,5] models commonly employing 2-DG probes. **2)** it shows the feasibility of detecting near infra-red (NIR) signals in large (250-350 gm) rats in contrast to mice which are commonly used for such imaging studies [1,2,6]. **3)** It shows that the increased retention of NIR 2-DG between 1 and 3 hours of AP induction has a stronger relation with severe outcomes than commonly used blood parameters used to determine AP severity in animal models. Translationally, the early identification of SAP patients, will allow management strategies to be focused on patients who need them most.

B: Transgastric cooling therapy reduces the severity and improves outcomes of an SAP model:
As shown in figures 1-4, transgastric pancreatic cooling initiated after induction of pancreatitis took about 90 minutes to achieve the target therapeutic temperature of < 26^0C which is relevant to the GTL model. This temperature was chosen based on studies done as part of Aim-1 in which we noted that there was an 80% reduction in GTL induced LDH leakage at 26^0C compared to that at 37^0C (Figure 5). Details are in 2013 progress report.

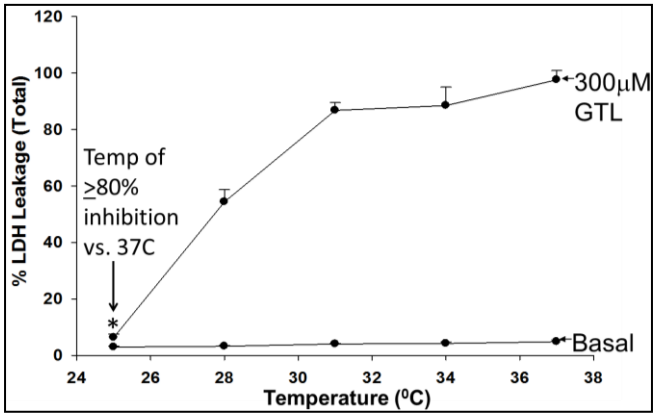

Figure 5: Graph showing the effect of different temperatures on GTL (300μM) induced LDH leakage over 4 hours from rat pancreatic acini.

These studies prove that therapeutic use of transgastric hypothermia after the onset of acute pancreatitis is a feasible strategy to ameliorate the severity of pancreatitis. This hypothermia improves outcomes in the GTL model. The improved outcomes include a lower mortality, reduced systemic severity, and while the data related to pancreatic necrosis are being analyzed, the preliminary findings suggest a reduction in pancreatic necrosis. The studies so far are over a 6 hour period (rapid models) and need to be done in other severe models and over a longer period of time.

4.2: Impact on other disciplines: <u>This is hypothetical</u>, and could be executed in the future:

Imaging studies of 2-DG mimetics may be used to predict severity of other acute events also. These include burns, trauma and severity of acute inflammatory conditions such as appendicitis.

4.3: Impact on technology transfer: nothing to report.

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

5. CHANGES/PROBLEMS:

5.1 Changes in approach and reasons for change: Nothing to report

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

DELAY RESULTING FROM WRITING PAPER FOR *PLOS ONE*: As part of the execution of Aim 3, we noted a significantly increased uptake of 2-DG mimetic IRdye 800CW 2-DG to occur within the first 2 hours of in the GTL model of pancreatitis. The increased uptake was a better predictor of AP severity than conventional markers of biliary AP such as serum ALT or amylase. We therefore elected to report this very exciting finding, which is the manuscript currently under review at PLOS ONE (appendix 3). Working on this manuscript delayed the execution of the "rapid" taurocholate and caerulein models. We however anticipate their execution by the end of March 2016, during which time we will also get IACUC and ACURO approval for modifications in executing the slow models as described below.

DELAY DUE TO NECESSITY OF CONTINUOUS ANESTHSIA WHILE USING COOLING BALLOON:
While gastric balloons are routinely used in humans for weight loss, and we have been successful in ameliorating severe AP with the gastric cooling balloon; this success did not come without its pains. The balloon had to be finally used with the rats under continuous anesthesia (this has IACUC/ACURO approval). The continuous anesthesia strategy was necessary, since after balloon placement, the rat would chew out the catheter while we were giving him time to recover. Additionally, even with buprenorphine, the rat would get agitated when the balloon would be inflated. We thus had to use the last IACUC/ACURO approved option, which is studying the effect of the cooling balloon while the rat was under continuous sedation. We have approval to use 7 hours of continuous sedation. This is sufficient in executing the "rapid" models, i.e. caerulein and taurocholate, and its success is apparent in the benefits we note in the GTL model described above. However, to make this suitable for the "slow models" we are making IACUC and ACURO modifications described below under the next point. These are after consulting with Ms. Sheron Westbrook, RVT, LAT, CPIA (Animal Use Review Specialist, Ctr: Charles River, USAMRMC Animal Care and Use Review Office, 810 Schreider Street Fort Detrick, MD 21702-5012). While the use of continuous anesthesia slows us down and is much more labor intensive, needing 2 people continuously and allowing a maximum of 2 cooled animals at a time; this has not hindered us in achieving our study goals.

ANTICIAPTED CHANGE IN IACUC/ACURO FOR EXECUTION OF THE SLOW MODELS:
We anticipate that the slow models (3 day) planned in the last year of the study will need a modification to the IACUC protocol, and approval by the ACURO. This is based on the following facts:

1. Rats with a cooling balloon need to be kept sedated. This can be done for a maximum of 7 hours.

2. The severe end points in the rats without cooling (e.g. mortality) are achieved within this period (Figure 2), mostly by 3-5 hours.

3. Cooling up to 6 hours post pancreatitis induction reduces mortality (Figure 2), and AP induced injury (Figure 3, 4). Rats without cooling experience adverse outcomes between 2-5 hours of AP induction.

4. The GTL model of pancreatitis involves biliopancreatic duct ligation after GTL injection [7].

   Therefore to study the maximum benefit of cooling over long term survival (i.e. in the slow

models), the ligature on biliopancreatic duct will need to be removed, at the time when the group of rats without cooling are having adverse outcomes (e.g. 2-5 hours), and we will need to remove the cooling balloon at @ 6 hours. The sedation can then be discontinued. These unsedated rats with the previously placed cooling balloon can then be followed over the 3 day course. They will be given all the post-operative care (e.g pain control, hydration, monitoring) as previously planned.

Since these animals will be under continuous sedation from the time of balloon placement, AP induction, till the 6 hours after AP induction when the balloon is removed, the whole process will be counted as a single surgery.

This protocol modification will allow studying hypothermia's effects on the originally proposed 3 day taurocholate pancreatitis in lean and obese rats, and also the long term benefits in the GTL model (which we have IACUC and ACURO approval to replace the originally proposed L-arginine model, see appendix-1)

5. We will include a separate sub-group of animals in the local hypothermia +AP group that is electively sacrificed at the time when we note mortality in the severe AP groups without hypothermia. This sub-group will help determine the efficacy of local hypothermia in reducing paramaters (e.g. necrosis in the pancreas) which cannot be judged by serum markers, are reduced by cooling at the time of sacrificing the group without cooling, but may still progress over the duration of the study.

6. We plan to increase the number of animals per group to 12 animals/group, since there are several groups (up to 7 groups) and with the 30-40% variation from animal to animal per parameter, we need a number of 12 rather than the originally proposed 8 to see a statistically significant difference ($p<0.05$)with an 80% probability on ANOVA.

5.3 Changes that had a significant impact on expenditures: Nothing to report.

5.4 Significant changes in the use or care of human subjects, vertebrate animals, biohazards, and/or select agents: Nothing to report in the previous year. However, the anticipated changes for the next year are mentioned under point 5.2.

6: PRODUCTS

6.1 Journal publications: Manuscript under review at *PLOS ONE*. Please see appendix-3**.**
Title: *Characterization and predictive value of near infrared 2-deoxyglucose optical imaging in severe acute pancreatitis.*
Authors: Cristiane de Oliveira, Krutika Patel, Vivek Mishra, Ram N. Trivedi, Pawan Noel, Abhilasha Singh, Jordan R. Yaron, Vijay P. Singh
Acknowledgement of Federal Support: Yes.

6.2 Books: Noting to report.

6.3 Other publications or conference presentations: Poster, at the American Pancreatic Association meeting (November 2015): "In Vivo Imaging of Non-Radioactive Near Infra-Red 2-Deoxyglucose During Mild and Severe Acute Pancreatitis (AP) in Rats" (*Pancreas*: November 2015 – Vol. 44 - Issue 8 - p 1369) (see appendix-2).

6.4 Website or other internet sites: Nothing to report.

6.5 Technologies or techniques: Nothing to report.

6.6 Inventions, patents and/or licenses. Previously reported in 2013 progress report. US Patent No. 8,529,612 titled "Gastroduodenal Balloon Tubes and Methods for Use in Localized Hypothermia"

6.7 Other products: Nothing to report.

7: PARTICIPANTS AND OTHER COLLBORATING ORGANIZATIONS

7.1 what individuals have worked on this project?

Following are the individuals with more than 1 person month effort on the project:
PI: Dr. Vijay P. Singh, 3.5 person months. Role: Oversight of project, planning, negotiating, quality control, trouble shooting, manuscript preparation, reporting, communicating with DOD/ ACURO.
Post-Doctoral Fellows:
1. Dr. Cristiane de Oliveria, 12 person months from DOD. Role: IACUC protocol work, Execution of animal models, monitoring, harvesting, histological analyses, documentation, manuscript work, conference presentation.
2. Dr. Krutika Patel: 6 person months from DOD. Role: IACUC protocol work, Assisting in animal models, monitoring, biochemical analyses, the rest is intramural or NIDDK effort on other projects.
Technician: Judy Bradley, 1 person month from DOD, Role, Maintenance of animal housing, assist in sample collection, storage, monitoring of animals.

These individuals are supported via intramural/NIDDK funds for the remaining effort on other projects.

7.2 Has there been a change in active support of the PD/PI or senior/Key Personnel since the last reporting period? No

7.3 What other organization were involved as partners?

While it was originally planned that the balloons would be made at the University of Pittsburgh, these could be procured easily from a commercial vendor (Vention Medical, Marlborough, MA). This was also mentioned in the previous annual progress report. We are now attempting to make these balloons in house.

7: SPECIAL COLLABORATIVE REQUIREMENTS: Nothing to report.

8: REFERENCES:

1. Kovar JL, Simpson MA, Schutz-Geschwender A, Olive DM (2007) A systematic approach to the development of fluorescent contrast agents for optical imaging of mouse cancer models. Analytical biochemistry 367: 1-12.
2. Kovar JL, Volcheck W, Sevick-Muraca E, Simpson MA, Olive DM (2009) Characterization and performance of a near-infrared 2-deoxyglucose optical imaging agent for mouse cancer models. Analytical biochemistry 384: 254-262.
3. Zhou H, Luby-Phelps K, Mickey BE, Habib AA, Mason RP, et al. (2009) Dynamic near-infrared optical imaging of 2-deoxyglucose uptake by intracranial glioma of athymic mice. PloS one 4: e8051.
4. Wu JC, Nguyen PK (2011) Imaging atherosclerosis with F18-fluorodeoxyglucose positron emission tomography: What are we actually seeing? Journal of the American College of Cardiology 58: 615-617.
5. Folco EJ, Sheikine Y, Rocha VZ, Christen T, Shvartz E, et al. (2011) Hypoxia but not inflammation augments glucose uptake in human macrophages: Implications for imaging atherosclerosis with 18fluorine-labeled 2-deoxy-D-glucose positron emission tomography. Journal of the American College of Cardiology 58: 603-614.
6. Kovar JL, Xu X, Draney D, Cupp A, Simpson MA, et al. (2011) Near-infrared-labeled tetracycline derivative is an effective marker of bone deposition in mice. Analytical biochemistry 416: 167-173.
7. Durgampudi C, Noel P, Patel K, Cline R, Trivedi RN, et al. (2014) Acute Lipotoxicity Regulates Severity of Biliary Acute Pancreatitis without Affecting Its Initiation. The American journal of pathology 184: 1773-1784.

Animal Research Protocol

1. Title of Project: Transgastric hypothermia

Is this protocol submitted as a "replacement" for a previously approved IACUC protocol which has or will reach its 3-year approval limit? No

2. Mayo Foundation location(s) where the protocol will be conducted: Arizona

If more than one site will be used for this protocol, indicate the primary site:

3. Enter the Laboratory Head (The Mayo PI for this application). This person must be a Career Scientist, Collaborative Scientist or Clinician Investigator.

Name	Position	Viewed IACUC video in last 5 years	Experience > 1 yr.	Experience < 1 yr training.	Email	Access controlled drugs	Lock box currently audited	Lock box location	Disclosure Required?
Singh Vijay	PI	Yes	Yes		Yes	Yes	Yes	JRB 3-325/327	No conflict

List of all personnel who will perform procedures on live animals in this proposal:

Name	Position	Viewed IACUC video in last 5 years	Experience > 1 yr.	Experience < 1 yr training.	Email	Access controlled drugs	Disclosure Required?
de Oliveira Cristiane	RF	Yes	Yes		Yes	Yes	No
Noel Pawan	RF	Yes	Yes		Yes	Yes	No
Patel Krutika	RF	Yes	Yes		Yes	Yes	No
Stubblefield Tianna	RT	Yes	Yes		Yes	Yes	No
Trivedi Ram	RA	Yes	Yes		Yes	Yes	No

List of all personnel who will not perform animal procedures, but who will need access to the information on the IACUC web inquiry page:

Name	Position	Email	Access controlled drugs	Disclosure Required?
Beauvais Irene	SE	Yes	No	No
Boschma Anne	SE	Yes	No	No

4. Will this protocol require additional training in animal handling and procedures from personnel in the animal resource facility? No

5. List any personnel working with animals that are not affiliated with the Mayo Clinic. Describe experience with the animal model and confirm video viewing status.

Not Applicable.

6. Indicate funding information - Check any that apply, and complete subsequent fields as appropriate:

New/Renewal/Existing: Extramural grant funding (NIH, AHA, etc.)

Provide funding agency name (list only one): DOD

In whose name is the grant submitted? SINGH,V,P

Provide grant number (if available) DO NOT use fund number:

Submission Date (mm/dd/yyyy format): 07/01/2011

I certify that the detailed description of the proposed use of animals in the extramural funding application, including justification for the use of animals; choice of species; procedures for ensuring minimal discomfort, distress, pain and injury; and method of euthanasia, matches that described in this and/or other Animal Research Protocols. Yes, I Agree

Will an approval letter be required by the funding agency? Yes

7. Provide an NIH_style abstract that summarizes the overall scientific goals and specific objectives of the proposed work.

Acute pancreatitis or acute pancreatic necrosis is a sudden inflammation of the pancreas. It can have severe complications and high mortality. While mild cases are often successfully treated with conservative measures, such as NPO (nil per os, fasting) and aggressive intravenous fluid rehydration, severe cases may require admission to the intensive care unit or even surgery to deal with complications of the disease process. This study will examine and evaluate if cooling the pancreas via a cooling balloon in the stomach will improve outcomes of acute pancreatitis.

Previous Fluorine-18 deoxyglucose-positron emission tomography (FDG-PET) studies show the pancreas to be hypermetabolic during acute pancreatitis in humans. In our pilot study (A3114), We have showed efficacy of non radioactive fluorescent probes/dyes/tracers as an alternative for the radioactive 18F-2-fluoro-2-deoxy-D-glucose (FDG) in our experiments using the IVIS 200 Pre-Clinical In-vivo Imaging system. So, we propose to evaluate the base line non-radioactive tracer/probe uptake in the Pancreas of Control Rats, Mild and Severe Pancreatitis induced rats and the effect of hypothermia on the uptake.

8. Describe in non-scientific language (high-school level of understanding) how the proposed project will benefit human or animal health, the advancement of knowledge, or the good of society.

Determine if cooling the pancreas via a cooling balloon in the stomach will improve outcomes of acute pancreatitis, which currently has no treatment.
Previously, we have noted that the metabolic demand for glucose increases in the pancreas in diseased conditions. We want to determine if the cooling of pancreas will decrease the demand of glucose in diseased states. For determining that, we need to first test the uptake of glucose in control and diseased conditions in rodents, using the latest available imaging technique (IVIS) and then attempt to understand if cooling can be used as a method to manipulate glucose uptake in diseased states.

9. This section of the protocol is to verify that a literature search was performed to determine if alternatives exist and to determine whether the protocol unnecessarily duplicates previous research. As the principal investigator, I have determined, by means of the following sources, searches, or methods, that alternatives to the procedures which may cause animal pain or distress proposed in this protocol are not available and that this protocol does not unnecessarily duplicate previous experiments. PUBMED

Specify all **key words and concepts** important in the development of the search strategy e.g., general area of study, proposed animal species, systems or anatomy involved, drugs or compounds used, methods and procedures -- particularly alternatives to procedures causing pain or distress.

Pancreatitis, pain, rat, Zucker Diabetic Fatty, Wistar, analgesia, surgery, refinement, caerulein, taurocholate, inflammation, necrosis FDG, PET, Rats, Hypermetabolic, IVIS 200 preclinical in vivo imaging system, flurescent probes/dyes

What years were covered by the search? 1978-present

Provide the most recent date on which the search was performed. (m/d/yy format, must be with in 6 months) 09/20/2014

10. Is the proposed animal species a mouse, rat, bird, fish or amphibian? Yes

Select criteria explaining the appropriateness of the animal model.
Systemic interactions are needed
These studies would be dangerous to humans
No in vitro options are available
- Other

There is no non-animal model that replicates the local and systemic injury of acute pancreatitis and there is species phylogenetically lower than rodents that replicates the pathophysiology of pancreatitis.

11. Will live animal work be conducted as a part of this protocol at facilities or institutions outside of Mayo? No

12. Will this study be conducted in accordance with 21 CFR Part 58, Good Laboratory Practice for Nonclinical Laboratory Studies? No

13. Will any live animals be housed outside of the animal facility for continuous periods longer than 12 hours? Yes

Building: Johnson Research Building

Room Number: 325/327

Provide justification:
Due to the heavy instrumentation of the animals, it is not feasible to conduct this study in the animal facility, because animals require frequent observation.

14. Will procedures involving animals be performed in clinical areas that support patients? No

15. Will transgenic/knockout mice be generated at a Mayo core facility or will the Metabolic testing core in Arizona be used for this study? No

16. What species of animal(s) will be used in this protocol?

Rats

Rats Specific Information

1. Strain: Wistar, Zucker Diabetic Fatty Rats

Sex: Both

Weight: 150-500 gms

Age: 6-12 weeks

2. Rationale for using this species:

Precedent data available
Model a specific human condition or disease

3. General Procedures:

Provide a description of the specific experimental manipulations and treatments of animals in terms that are

intelligible to non-specialists. This description should be a chronological description that will allow IACUC reviewers to understand exactly what will be done to all animals from entry into the study to its endpoint.
The experiments will be in the three phases. Buprenorphine SR will be used as the analgesic for surgery and alleviation of pain induced by pancreatitis. This section describes the detail:

PHASE 1: CHOOSING METHOD OF BALLOON PLACEMENT: (See question #10 for surgical details.)
The surgeries involved are:
1. Placement of the gastric balloon, tunneling of the dual lumen perfusion catheter (which perfuses the gastric balloon) in the subcutaneous space to the interscaplular area. and letting the site heal. Additionally at this point we this will test the patency of the system by infusing and withdrawing a small amount (@20cc) of water. The healing time will be 1 day to 7 days. The catheter will remain buried in the subcutaneous space or be exteriorized and buried in a pocket in the Orednt jacket or attached to the tethering mechanism but not used till the healing period is deemed complete. Animals will be provided analgesia daily after this till sacrifice.
2. Placement of the Thermocouple probes or device in the pancreas and or the pelvis. The probes of the thermocouple may be buried in the subcutaneous space or be exteriorized and buried in a pocket in the Orednt jacket or attached to the tethering mechanism but not used till the healing period is deemed complete .
3. Induction of pancreatitis (including intraductal taurocholate or GTL), along with externalization of the buried catheter, and placement of a harness, tether, swivel, swivel mount and connecting the catheters to the perfusion system (Isotemp* Refrigerating Series Circulator). This will be done 1-7 days later depending on when the preliminary studies suggest optimal healing has occurred.

PHASE 2: POST BALLOON PLACEMENT OPTIMIZATION OF COOLING: 20 animals will be used for this. Once a balloon placement route is deemed successful, we will then determine the safety parameters for this. Animals will be kept warm and allowed to recover 1-7 days before starting the cooling infusion. The cooling reservoir's (and also fluid returning to the reservoir) height will be such that, a stomach volume sufficient to make contact with the pancreas (confirmed on ultrasound), is achieved. Both pelvic and pancreatic temperatures will be monitored and that of the pancreas adjusted by regulating the flow rate of the liquid from the reservoir, while avoiding generalized hypothermia (i.e. a 2 degree Centigrade drop in pelvic temperature). External heating with an infrared source will be used. Initially flow rates will be low (0.25 ml/minute), and adjusted till chosen temperature (as determined by previous invitro studies) is reached in the pancreas, while avoiding generalized hypothermia. The flow rates and height of reservoir will be noted and used for future reference. The animals will be monitored for the duration of the intended local hypothermia i.e. 2 weeks. This includes temperatures, weights, PO intake and distress. This will be initially done at 30 minute intervals till the animals are awake and ambulating and, after which it will be at 2 hour intervals for the first 8 hours and thereafter at 12 hour intervals. Blood glucose measurements may be done at 12 hour intervals. In the event of hypoglycermia (<60 mg/dl) the animals will be given 3 ml 50% dextrose Intraperitoneally, and the hydration solution for pancreatitis studies (please see below) can be changed to normal saline with 20% dextrose.

PHASE 3: STUDY EFFECT OF COOLING ON MODELS OF PANCREATITIS.

Section: Experimental Design and Endpoints

For the non-invasive models (CAERULIEN, L-ARGININE, please note that the L-arginine model may be replaced by intraductal glyceryl-tri-linoleate (GTL) injection, which we have noted to be more clinically relevant than L-arginine, since it replicates severe acute pancreatitis exacerbated by obesity in humans):

The abdomen will be cleaned with 70% ethanol and the agents will be administered with a single intraperitoneal injection to the appropriate animals at the appropriate dose. Along with this a subcutaneous injection of saline (4% body weight) will be given and repeated every 24 hours to prevent dehydration. Buprenorphine SR will be given and repeated every 2-3 days. Ear punching will be done for identification.

FOR the TAUROCHOLATE or glyceryl-tri-linoleate (GTL) models:
The overnight fasted male Wistar rats, anesthetized and placed on a heated table. After shaving, cleaning and draping, with sterile precautions, antibiotics (Cefazolin 100mg/kg IM), a 1 cm midline incision will be made in the skin of the epigastrium, and the duodenal loop identified. The pancreatic ampulla is visible through the serosa. This will be traced to the biliopancreatic duct and the biliary duct will be clamped using a bulldog clamp. the ampulla will be cannulated with a blunt 27 gauge needle mounted on a 1 cc tuberculin syringe containing the sodium taurocholate or GTL (0.05-0.1ml/100gm) to be injected after placing a 4.0-6.0 silk suture around the needle in the duct and knotting it loosely initially. The agent will be administered over 1 minute into the pancreatic duct. The needle will be withdrawn and the knot tightened around the pancreatic duct. The abdominal cavity will be closed in two layers (muscle and skin) using a 4.0 silk suture. Ear punching will be done for identification; the animals will be given the first dose of buprenorphine SR, kept warm and allowed to recover. On recovery these will be given saline (@ 4% bodyweight) subcutaneously to prevent dehydration . This will be repeated every 24 hours.

At what time point and/or how many days will experiments be completed on animals?
Caerulein: 6 hours
L-arginine: 3 days, 7 days
Taurocholate: 12 hours/overnight and 7 days.
GTL: 12 hours/overnight.
Rats will be euthanized with CO2 at the end of the study.

Fluorescent dye uptake in rats by IVIS imaging system (non-radioactive fluorescent probes/dyes)
We will use 3 different fluorescent probes/dyes:
- IRDye® 800CW 2-DG (Li-Cor), a fluorescent agent that targets cells with elevated rates of glycolysis. Dose range from 10-30nmol (100-300µl) per rat, depending upon the body weight.
- Superhance™ 680 (PerkinElmer), a fluorescent agent that enables imaging of vascular leak. Dose range from 4-8nmoles (150-300µl) per rat, depending upon the body weight.
- XenoLight RediJect 2-DeoxyGlucosone 750 (PerkinElmer), a fluorescent imaging probe that targets cells with elevated glucose uptake rate in comparison to surrounding tissues. Dose range from 10-30nmoles (100-300ul) per rat, depending upon the body weight.

The procedure for the Fluorescent dye uptake in rats by IVIS imaging system :
In rats with pre-implanted internal jugular catheter, after placement of Balloon and induction of acute pancreatitis, by the appropriate mild or severe model of pancreatitis induction;
1. In the sedated (with Ketamine and Xylazine) rats with internal jugular pre-implanted catheters, the port (which is externalized between the scapula) would be clamped, and the stainless steel pin removed from the catheter and set aside.
2. An empty syringe assembly (22 gauge blunt needle with a syringe) would be inserted into the port and the hemostat released.
3. The fill solution would be withdrawn gently and port clamped again.
4. A second syringe filled with IRDye® 800CW 2-DG, Superhance™ 680 or RediJect 2-DeoxyGlucosone 750 would be attached to the port, haemostat released, probe would be injected and port clamped again.
5. The hemostat would be released and the catheter would be slowly flushed with sterile saline (150 µl or more), port clamped;finally adding 50 µl of sterile lock solution [Heparinized Saline (500 IU/ml)] , clamping the port and replacing the stainless steel pin.
6. The rat would be placed in the light-tight chamber of the IVIS spectrum system (located in the JRB-C031) for fluorescent imaging for a period of 1-5 min, placing the rat in various positions - ventral, dorsal and laterally.
8. The rat would be placed in the chamber of the IVIS system for several fluorescent imaging taken along a period of 3 hours, placing the rat in various positions - ventral, dorsal and laterally.
9. After the imaging for fluorescent probe uptake, the rats would be humanely euthanized with appropriate measures and the pancreas will be harvested. The Pancreas will be scanned for an ex-vivo fluorescent imaging, by placing it in a weighing boat.

For the implantation of internal Jugular Catheterization
Rats will be anesthetized and subjected to internal jugular vein catheterization (similar to approved protocol A30014). The catheterization will be done with the placement of gastric balloon.
The skin on the ventral surface of the neck will be cleansed with an iodinated solution and incised. The left jugular vein will be exposed and a silicone catheter will be passed into the jugular vein secured in place with ligatures. The free end of the catheter will then be tunneled under the skin and exteriorized at the back of the neck. The animal will be allowed to recover for at least 3 days before the use on experiments. The catheter will be flushed with heparinized pyrogen-free saline on the day after the surgery and then every 3-4 days. The free end of the catheter will be connected to a tubing that allow us to perform I.V.. injections during the experiments.

4. Justify the number of animals required for the 3-year duration of the IACUC protocol:

Animals will be assigned to experimental groups.
Explanation:

Based on the sample size calculator at http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html; 8 animals will be necessary to have a 90% chance of coming up with statistical significant ($p<0.05$) 4 fold induction in a particular endpoint (e.g. serum amylase). The variation form animal to animal is 50% around the final mean for each value (previous experience). For e.g. a mean 4 fold increase (over saline controls) in serum amylase in the disease group, varies from 2 to 6 fold from experiment to experiment. A paired student's

T test will be done for each time point.
There are three stages of the study:

Section: Type of Protocol and Procedure Classification
For balloon placement and optimization a total of 65 rats are requested.

1. balloon placement: Approximately total 45 rats requested (since we are not sure how soon this will be successful). We will start with endoscopic route. We hope this will be successful, and if so we will go to step 2. If not we will use the laparotomy or orgoastic route.
2. optimization of cooling protocol after choosing placement route: Approximately 20 rats are requested for this. In this the infusion rates, requirement and settings for external heating will be optimized to safely achieve the Tt in the pancreas (a temperature at which deleterious phenomena including trypsinogen activation, cell death and chemokine up regulation are reduced by 80% of their magnitude at 37C. While achieving this Tt, we aim at preventing generalized hypothermia (i.e. a drop in pelvic/rectal temperatures by >2C), by externally heating the environment.
3: In vivo studies for pancreatitis.
For this there will be 5 models (4 lean and 1 obese):
1. caerulein
2. L-arginine or GTL
3. Taurocholate in lean rats 6 hours (non-lethal)
4. Taurocholate in lean rats (survival study up to 7 days)
5. Taurocholate in obese (ZDF) rats.

Therapeutic efficacy will be studied in 6 groups for each model of acute pancreatitis.
I)Controls
II)Pancreatitis with no balloon placement
III)Pancreatitis with balloon placement and local hypothermia
IV)Pancreatitis with balloon placement but no hypothermia
V)Pancreatitis with balloon placement (animals sacrificed at time of balloon placement to determine amount of injury prior to induction of local hypothermia)
VI)Pancreatitis with Generalized hypothermia
8 animals per group.
Therefore there will be (4 x 6 x 8)=192 lean rats.

There will be 1 x 6 x 8= 48 ZDF rats. Additionally there will be 16 ZDF rats sacrificed at 12 hours (8 with pancreatitis and without cooling, 8 with pancreatitis and cooling) to determine the effect of cooling on interim severity of the model. **Thus the total number of ZDF rats will be 64.**

5. Animal Numbers - Procedure Categories

Category A : No Pain	0
Category B : With Anesthesia	321
Category C : With Pain	0
A + B + C	321

6. The most commonly recognized complications or side effects of the specific drug, device and/or procedures that the animals may experience as a result of these experiments:

Caerulein, L-arginine: none.
Sodium Taurocholate, GTL models -
Infections: While all sterile precautions will be taken, these remain a possibility. the frequency would be <5% animals and we will look out for purulent discharge, reduced grooming, reduced activity, falling, staggering or a horizontal posture.
Wound dehiscence: This is a rare (<2%) event. We will inspect the abdominal wound daily looking for erythema, warmth, purulent discharge or breakdown.
Balloon placement:
Weight loss: Gastric balloons have previously been placed in rats- these have been rigid, 7 ml in volume. Rats have been monitored for 25 days with these with a 10% weight loss from baseline (Am J Physiol Regulatory Integrative Comp Physiol

251:794-797, 1986).
Decreased PO intake : In the above study, the PO intake was 22gm/day vs. 32 gm/day for rats without balloons. Esophageal perforation: This may happen during endoscopy If the endoscope is too big. We hope this does not happen (
In humans this is a rare event (<1:1000). 6mm balloons are tolerated by 400gm rats without perforation (Am J Physiol Regulatory Integrative Comp Physiol 274:1425-1435, 1998), and a 2 fold increase in balloon pressure compared to resting pressure.
Generalized hypothermia: This is a potential complication- that will be looked out for-with continuous monitoring of pelvic temperatures and prevented. The frequency cannot be predicted.
Infections: While all sterile precautions will be taken, these remain a possibility. the frequency would be <2 animals total and we will look out for purulent discharge, reduced grooming, reduced activity, falling, staggering or a horizontal posture.

How will you manage the complications noted in the questions above?
Weight loss: this will be monitored daily. The weight of the animals with the cooling infusion will be compared to those with the distended balloon but without the cooling infusion. If the weight loss is unique to the group with the cooling-it will be attributed to the effect of cooling-since all other things are equal in both groups (and a cooling balloon may be used in future studies as a potential modality for inducing weight loss). If severe weight loss is a complication of the procedure (i.e. same extent in both groups), then if the animal becomes excessively cachectic (i.e. equal to 20% weight loss)- it will be euthanized.
Decreased PO intake- this will be monitored, if rats weight loss reaches 15% of original body weight, a consultation with Dr. Gades, from the Department of Comparative Medicine, will be done to determine a course of action regarding the animal. Weight loss of 20% is considered an indication for humane euthanasia.
Esophageal perforation: The animal will be sacrificed. We will attempt at seeking alternate smaller endoscopes. The smallest commercially available is 3.6 mm. We may also attempt at using a larger animal size in the future (e.g. 500gm) if the perforation occurs in a 350 gm rat.
Generalized hypothermia: We will reduce the infusion rate. The pelvic temperature will be continuously monitored by the implanted wireless thermometer. The flow will be started at the lowest rate (0.25ml/minute), and increased every 15 minutes by 0.25ml/minute, while monitoring the pelvic temperature while the flow rates are being standardized
Infections: we will treat this with repeat cefazoline if these are superficial. If the infection is deep, the animal will have to be euthanized.
The attending veterinarian, Dr. Gades, will be consulted if any of these conditions occur and are not resolved with the above treatments.

Other complications (due to Intravenous catheterisation) :
- Animals will be observed at least daily post surgery.
- To facilitate recovery, 5 ml of warm saline will be administered S.C.
- Possible complications are hypothermia due to anesthesia what will be solved keeping the animal on a heating pad during the surgery and after the surgery. Uncontrolled hemorrhage, vessel rupture and collateral tissue/nerve damage. Infection in the surgical site. In this case we will consult the veterinarian for treatment options.

7. List the clinical signs that will be used to remove an animal from the study before the planned experimental end point? Moribund animals must be removed from studies for humane purposes:

Esophageal perforation: The animal will be sacrificed.
Decreased PO intake: Weight loss of 20% is considered an indication for humane euthanasia.
If the animals appear to have a hunched posture, reduced activity levels, decreased food and water intake, excessive weight loss, uncontrolled or excessive bleeding; they will be euthanized humanely and removed from the study.

8. In what facility will the animals be housed?

Arizona

9. Describe any specialized husbandry requirements (metabolic cages, special diets, etc.).

Due to the heavy instrumentation of the rats, they will be housed in the metabolic/behavioral phenotyping laboratory for up to 14 days. Laboratory staff will be responsible for daily observation of the rats and complete a room log. The animals will be housed in

static microisolators on a bench top, ~4 rats per time. Temperature, humidity, air flow, and lighting have all been verified to within recommended *Guide* ranges.

10. Will a surgical procedure, either survival or terminal, or tissue harvest prior to death be performed on the animals?
Yes

Describe surgical or tissue harvest procedures performed on living animals in detail, including name of procedure, anatomical approach, tissue manipulation, and closure techniques including suture materials.

PHASE 1: CHOOSING METHOD OF BALLOON PLACEMENT:
A. ENDOSCOPIC:
After a 2 day period of acclimatization, 250-500 gm male Wister rats will be placed in a harness and acclimated to this for 2 days. They will then be fasted, anesthetized and will be placed on a heated table with a rectal temperature probe. After shaving, cleaning and draping, with sterile precautions, antibiotics (Cefazolin 100mg/kg IM), The 5mm endoscope (or smaller 3.8mm endoscope if necessary) will be passed through the mouth into the esophagus and stomach. The site of placement of the percutaneous endoscopic gastrostomy (PEG) incision will be confirmed by transillumination using the endoscope light source and indenting the anterior abdominal wall using a blunt tip device such as a forceps. A point will be marked and a 18-20G needle with a trocar and cannula will be passed percutaneously into the stomach. On confirming entry endoscopically, the trocar will be removed and a nylon thread loop (or suture) will be passed through the cannula into the stomach and grasped with a forceps coming through the working channel of the endoscope. The scope will then be removed along with the nylon thread through the mouth and a dual lumen peg tube (4mm) or 2 tubes with a balloon (7-8 ml capacity) will be attached to the nylon loop or suture. This will then be pulled through the abdominal PEG site, along with the tube, while retaining the balloon in the stomach. The skin incision of the PEG tube will be extended 1-2 cm each way-cephlad and caudally. A subcutaneous pocket and tunnel going towards the interscapular area will be created. The PEG tube will be disinfected again with Betadine and passed through this tunnel and externalized and anchored through the skin in the midback/ interscapular area- approximately at the location where the harness will attach.

THERMOCOUPLE PROBE PLACEMENT: We will then turn our attention to placement of the temperature monitoring device in the subcutaneous pocket created. The wireless temperature transmitter (TL11M3-F40-TT wireless Implantable transmitter, cat No. 270-0147-001, Data Sciences International, weight 7.5 grams, 3.5cc volume) will be placed in the pocket. It has 2 probes. The first one will be placed in the pancreatic bed through a point incision on the left side, just below the last rib about 2-3 cm from the spine. The second probe will be placed in the pelvis by extending the subcutaneous pocket caudally to 1 cm above the hip joint. A point incision will be made here and the second probe placed to monitor the distant temperature. The subcutaneous pocket will then be closed. Analgesic Buprenorphine SR will be given. The rats will be placed in a harness, and the tubing attached to the spring in the harness. The harness will be connected to a tether, which is connected to a 1-3 channel swivel (16-20G) and mount, fixed to the rat cage. The inflow tubing will be connected through a peristaltic pump (flow rate up to 0.25 to 25 ml/minute) to a cooling reservoir. The stomach outflow will be returned to the reservoir.

Alternately, for thermocouple probe placement after balloon placement; we will make an incision in the abdominal musculature in the left flank below the subcutaneous pocket. Through this, we will first turn our attention to placing the first flexible thermocouple probe (0.6 mm, 90-300 cm long, Harvard apparatus) in the distal body and tail of the pancreas. For this the stomach will be lifted, the spleen identified, the pancreas, whose tail lies at the tip of the spleen will be traced to the midbody, and the tip of the thermocouple will be placed here. We will then turn our attention to placement of the pelvic thermocouple probe. Both these thermocouples will be anchored to the incision in the flank musculature, and to the skin of the interscapular areas where these exit..

B: LAPOROTOMY: To be done in case of failure of endoscopic placement:
All procedures will be performed under sterile conditions. Surgery will be performed using sterile instruments, sterile surgical gloves, mask and aseptic procedures to reduce microbial contamination of exposed tissues.

THERMOCOUPLE PROBE PLACEMENT: After a 2 day period of acclimatization, 250-500 gm fasted (rats will be fasted overnight, to make sure there is not food in the stomach) male Wistar rats, will be anesthetized and placed on a heated table with a rectal temperature probe. After shaving, cleaning and draping, with sterile precautions, antibiotics (Cefazolin 100mg/kg IM), a 3 cm incision will be made in the skin of the left flank. A subcutaneous pocket will be created. The wireless temperature transmitter (TL11M3-F40-TT wireless Implantable transmitter, cat No. 270-0147-001, Data Sciences International, weight 7.5 grams, 3.5cc volume) will be placed in the pocket. It has 2 probes. The first one will be placed in the pancreatic bed through a point incision on the left side, just below the last rib about 2-3 cm from the spine. The second probe will be placed in the pelvis by extending the subcutaneous pocket caudally to 1 cm above the hip joint. A point incision will be made here and the second probe placed to monitor the distant temperature. The subcutaneous pocket will then be closed. With regards to closure, 3-0 silk suture will be used

to close the muscular layer and then the skin layer, a simple continuous pattern will be used for both layers.

Alternately, for thermocouple probe placement after balloon placement; we will make an incision in the abdominal musculature in the left flank below the subcutaneous pocket. Through this, we will first turn our attention to placing the first flexible thermocouple probe (0.6 mm, 90-300 cm long, Harvard apparatus) in the distal body and tail of the pancreas. For this the stomach will be lifted, the spleen identified, the pancreas, whose tail lies at the tip of the spleen will be traced to the midbody, and the tip of the thermocouple will be placed here. We will then turn our attention to placement of the pelvic thermocouple probe. Both these thermocouples will be anchored to the incision in the flank musculature, and to the skin of the interscapular areas where these exit using 3-0 silk suture..

An upper midline incision will then be made and a 5-7mm incision will be made on the anterior stomach wall. A purse string suture will be placed on the anterior stomach wall (5/0 silk), 5 mm from the incision and surrounding this. The stomach will be cleared of debris through this incision. Two sterile 16Fr. polyethylene catheters (inflow and outflow) passing through a 1cm long encasing leak proof polyethylene tube (6mm internal diameter) will be covered by a sterile latex balloon (7-8 ml volume at 30 cm water pressure). This balloon's attachment to the encasing tube will also be leak proof. The balloon, and the encasing tube will be inserted into the stomach and the purse string tightened in such a manner that the serosal surface is inwards and in contact with the encasing tube-making the opening leak-proof. Then we will place the wireless implantable transmitter in the pocket, pass its probes into the abdomen and place one of them on the posterior pancreatic body surface. The device has 2 probes and the second probe will be placed in the pelvis to monitor the temperature. The subcutaneous pocket will then be closed. Marlex mesh- a 20-mm-diameter circle with a slit for the 16Fr tubes will be placed on the stomach serosal surface with the 2, 16Fr tubes going through it. This mesh will increase adhesions between the stomach and parietal peritoneum and will be an additional measure to prevent leakage of stomach contents. The 2, 16Fr. tubes will then be passed through a small incision in the abdominal wall muscle to the subcutaneous space. The midline abdominal muscle incision will be closed. These catheters will then be tunneled subcutaneously to the midback. A point incision in the skin will be made to exteriorize these tubes and then these will be connected to the spring in the harness. Analgesic Buprenorphine SR will be given. The rats will be placed in a harness, with a tether connected to a swivel (16-20G) and mount, fixed to the rat cage. The inflow tubing will be connected through a peristaltic pump (flow rate up to 0.25 to 25 ml/minute) to a cooling reservoir. The stomach outflow will be returned to the reservoir.

C: OROGASTRIC: We have successfully done this for 6 hours in the past. This will be done in case of failure of the above 2 approaches.
All procedures will be performed under sterile conditions. All procedures will be done after a 2 day period of acclimatization, and overnight fasting. Surgery will be performed using sterile instruments, sterile surgical gloves, mask, and aseptic procedures to reduce microbial contamination of exposed tissues. In particular, surgical instruments will be sterilized using steam sterilization. For multiple surgeries, instruments will be disinfected between rats using a hot bead sterilizer. Surgeons will don surgical mask, a clean lab coat and wash hands before aseptically donning sterile surgical gloves. Before the procedure a blunt guide wire will be placed through the 6 french (2 mm diameter) dual lumen tube and will be passed into the balloon (3 cm long, 7-8 cc capacity) attached to it. The outer surface of the balloon and dual lumen tube will be lubricated with lubricating jelly. The rat will initially be anesthetized with the Ketamine and Xylazine. The distance from the incisors to the xiphistenum will be measured and noted (x cm). The collapsed soft polyurethane balloon (collapsed thickness 4-5mm) will then be placed in the stomach via the esophagus. Balloons up to 6 mm diameter have been placed in the esophagus previously (Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R1425–R1435, 1998). The distance this will be advanced to x+3 cm from the incisors. The guide wire will be withdrawn. Placement will be confirmed by distending the balloon with air and monitoring for stiffening of the anterior abdominal wall. The tube will be brought to the side of the mouth and will be anchored here with an anchoring suture on the skin at the side of the mouth.

THERMOCOUPLE PROBE PLACEMENT: after shaving, cleaning and draping, with sterile precautions, antibiotics (Cefazolin 100mg/kg IM), a 3 cm incision will be made in the skin of the left flank. A subcutaneous pocket will be created. The wireless temperature transmitter (TL11M3-F40-TT wireless Implantable transmitter, cat No. 270-0147-001, Data Sciences International, weight 7.5 grams, 3.5cc volume) will be placed in the pocket. It has 2 probes. The first one will be placed in the pancreatic bed through a point incision on the left side, just below the last rib about 2-3 cm from the spine.
The second probe will be placed in the pelvis by extending the subcutaneous pocket caudally to 1 cm above the hip joint. A point incision will be made here and the second probe placed to monitor the distant temperature. The subcutaneous pocket will then be closed. Alternately, the rat will be placed on heating pad in the chemical fume hood. We will then shave, clean and drape the anterior abdominal wall. A 2 cm midline incision will be made here. We will first turn our attention to placing the first flexible thermocouple probe (0.6 mm, 90-300 cm long, Harvard apparatus) in the distal body and tail of the pancreas. For this the stomach will be lifted, the spleen identified, the pancreas, whose tail lies at the tip of the spleen will be traced to the midbody, and the tip of the thermocouple will be placed here. We will then turn our attention to the head of the pancreas that lies in the duodenal loop. After identifying this, a second thermocouple probe will be placed here. Both of these thermocouples with anchored to the lower end of the incision.

Functioning of the balloon will be verified again by distending it with air. This will then be deflated, and the incision will be closed. A rectal thermometer will be inserted to record rectal temperatures manually every 5 minutes. Temperature monitoring will then begin. We will keep the animal warm on the heating pad and allow temperature to stabilize. We expect this to happen over 15-30 minutes. The heating pad will then be turned off. We will then start infusion of cold water at 4C though one channel of the tube at the rate of 25 cc/minute. the other lumen of the tube will passively drain the balloon. We will monitor the temperature every 5 minutes till the lowest temperature/s in the pancreatic bed is achieved. The animal will be sacrificed when rectal temperatures drop > 2 Centigrade from baseline. Intraoperatively, and during the post-operative monitoring period, we will look for any return of spontaneous movement or response to pinprick/ extended limb withdrawal reflex. If so we will repeat the ketamine/xylazine at half the dose subcutaneously. If this is ineffective, isoflurane anesthesia will be administered via a nose cone. A record of the rectal temperature will also be maintained. For anesthetized using isoflurane: A nose cone will be fashioned by cutting a 50 cc polypropylene falcon tube in half cross-sectionally and using the distal end. Before this several 5mm holes will be drilled in the conical part to allow passage of air. A 2 x 2 inch gauze will be soaked with isoflurane and placed in the base of the cone. The cone will then be placed on the nose of the rat taking care to avoid direct contact of the gauze with the animal. The gauze will be kept moist with isoflurane. Experiments using the orogastric route will be restricted to 7 hours, and this modality of cooling will only be resorted to in case of failure of the endoscopic and laparotomy routes.

Under a single I.P. injection of ketamine/xylazine cocktail or isoflurane inhalation anesthesia, each animal will be subjected to internal jugular vein catheterization: (similiar to approved protocol A30014)
Remove the hair from the ventral and dorsal surface of the surgical site. Clean surgical site with 70% alcohol followed by iodinated solution, then 70% alcohol and place a steile gauze on the dorsal prepared area to maintain sterility and place animal on a surgical board with the head facing the surgeon. Make a 1 cm incision, on the right side, 0.5 cm form the midline, cephalic to the sternum. Blunt dissect using a forceps to expose the right jugular vein. Ligate the cephalic end of the jugular vein with silk suture and loosely knot another piece of silk suture on the caudal end of the exposed vessel. Clamp vessel with a vessel clip. Use a iris scissor to make a small cut just below the ligated end and insert the catheter. Release the vessel clip and insert the catheter. Tie both ligatures and confirm that catheter is patent flusing with heparinized saline. Close the end of the catheter with metal pin to occlude the blood flow. Place gauze moistened with sterile saline over the incision site and turn animal over. Make a 0.3 cm incision between shoulder blades. Tunnel a blunted 13 gauge needle under skin through the incision on the back to meet the ventral incision point. This is done superficially as possible to prevent subcutaneous vessel damage. Thread catheters trough the blunted 13 guage needle to exteriorize them at the back of the animal. Close ventral incisions with 3-0 or 4-0 prolene/Surgilene/nylon suture or surgical staples in a simple interrupeted pattern. Clamp catheter with micro-serrefine at the incision site between shoulder blades and connect to MASA (trademarked name of the apparatus developed by Dr. Masakazu Shiota), a conection device constructed with tubing and silicon that the catheters are attached to via the lumen to create an external infusion point. MASA is to be placed under the skin of the back of the neck to secure the catheters to. Close the dorsal incision with 3-0 or 4-0 prolene/Surgilene/nylon suture or surgical staples in a simple interrupted pattern, confirm again the patency of the catheter flusing with heparinized saline saline and close with metal pins. Place animal in warmed clean cage for recovery. The animal will be allowed to recover for at least 3 days before the use on experiments. The catheter will be flushed with heparinized saline on the day after the surgery and then every 3-4 days.

Who will perform the surgical procedure or tissue harvest?
Singh, Vijay; Stubblefield, Tianna; Patel, Krutika; Noel, Pawan; Trivedi, Ram; de Oliveira, Cristiane

In what room will the procedure be done?
JRB 3-325/327 Metabolic/Behavioral Pheno Lab

Will the animals be allowed to recover from the anesthetic? Yes

I. How many days post-procedure will the animals survive? 14 days.

II. Will the surgical site be prepped including fur clipping and antiseptic skin scrub? Yes

III. Will a sterile drape be used for the surgical site? Yes

IV. Will sterile gloves be worn? Yes

V. Will surgeons wear a mask, cap, scrub suit and sterile gown? Yes

VI. How will the surgical instruments and/or implanted devices be sterilized initially? If a series of surgeries are planned between rodents, how will surgical instruments be sterilized between animals?

Note that a new sterile surgical pack must be used for every non-rodent surgery.

Initial sterilization with autoclaving, then will use a hot bead sterilizer so that we can rotate 3 sets of instruments, giving them time to cool between animals.

VII. How frequently will the animals be observed during surgery? What parameters (temperature, HR, BP, etc…) will be monitored to ensure the animal remains stable? How will you ensure that temperature remains as normothermic as possible?

Animals will be monitored during the surgery for respirations, rear toe pinch, and body temp with a temperature probe. Parameters will be assed every 15 minutes.

VIII. How frequently will the animals be observed post operatively?

Observation every 20 min postoperatively until recovery, then daily following the procedure.

IX. What is the post operative treatment plan, including fluid therapy, incision care, and antibiotics?

Rats will be given 20 ml warm saline and maintained on a heating pad until recovery.

X. When will skin sutures/clips be removed?

Sutures will not be removed due to the short duration of the experiment, i.e. 14 days.

XI. Will a single animal experience more than one major survival surgical procedure (penetrates and exposes a body cavity or produces substantial impairment of physiologic function; and the animal recovers from anesthesia)? Yes

How many surgeries will the animal experience? 2

What will be the time period between surgeries? 1-7 days

Provide a scientific rationale for the necessity of multiple major survival surgical procedures on the same animal:

To clarify, two surgeris will occur - (in one of these ie. balloon placement and thermocouple placement will be done at 1 time and, the second one ie. induction of pancreatitis at another time.)

The surgeries involved are: 1. Placement of the gastric balloon, tunneling of the dual lumen perfusion catheter (which perfuses the gastric balloon) in the subcutaneous space to the interscaplular area. and letting the site heal. Additionally at this point we this will test the patency of the system by infusing and withdrawing a small amount (@20cc) of water. The healing time will be 1 day to 7 days. The catheter will remain buried in the subcutaneous space. Animals will be given buprenorphine SR, after this till sacrifice. Placement of the Thermocouple probes or device in the pancreas and or the pelvis. The probes of the thermocouple may be buried in the subcutaneous space or be exteriorized and buried in a pocket in the Orednt jacket or attached to the tethering mechanism but not used till the healing period is deemed complete .

2. Induction of pancreatitis (including intraductal taurocholate or GTL), along with externalization of the buried catheter, and placement of a harness, tether, swivel, swivel mount and connecting the catheters to the perfusion system (Isotemp* Refrigerating Series Circulator). This will be done 1-7 days later depending on when the preliminary studies suggest optimal healing has occurred. It is necessary to separate the two surgeries, since combining a major surgery (e.g. balloon placement by laparotomy) with a mild model (e.g. caerulein pancreatitis) at the same time will seriously confound the results.

11. Will the animals be anesthetized? Yes

INDUCTION:

Agent	Dose(mg/kg)	Route	Length of time anesthesia session will last
Ketamine	100	IP	30 minutes till 7 hours (in case of orogastric balloon)
Xylazine	10	IP	30 minutes till 7 hours (for the orogastric ballon model)

MAINTENANCE:

Agent	Dose(mg/kg)	Route	Frequency of administration	Assess depth of anesthesia
Ketamine	50	IP	Repeated every 30 minutes	Withdrawal due to rear toe pinch
Xylazine	5.0	IP	Repeated every 30 minutes	Withdrawal due to rear toe pinch
Isoflurane	1-3%	Inhaled	Continuous	Withdrawal due to rear toe pinch

12. Will muscle relaxants (paralytics, neuromuscular blocking agents) be utilized? No

13. Will analgesics (pain relievers) be used? Yes

Agent	Dose(mg/kg)	Route	Frequency of administration	Duration
Buprenorphine SR	0.6	Subcutaneous	Up to 4 times (every 2-3 days)	14 days post-operatively

Specify the time point when analgesia will initially be given (e.g., prior to surgery [the preferred method], during surgery, immediately after surgery but before the animal wakes up).

Prior to surgery and continued through completion of the pancreatitis model

List behavioral and/or clinical signs that will be utilized to evaluate for pain to determine duration of analgesic use OR to indicate need for continued administration of analgesics.

Reduced grooming, reduced level of spontaneous activity, piloerection, hunched posture, abdominal writhing, reduced food/H20 intake

14. Will tranquilizers be used? No

15. Will an overdose of a chemical (CO2, pentobarbital, etc.) be used as the means of euthanasia for the animals? Yes

Agent	Route (IV, IP, inhalation)	CO2 Training
Carbon Dioxide	Inhaled	Y

Will the animal be under anesthesia when the agent is administered? No

16. Will a physical means of euthanasia be used on the animals? No

17. Will the animals be immunized? No

18. Will blood be collected from the animals? Yes

Non-terminal Blood Withdrawal Guidelines:

Species	Maximum Blood Withdrawal
Mouse	0.1 ml/10 gm body wt
Rabbit	7 ml/kg body wt
Guinea Pig	6.5-7.7 ml/kg body wt every 3-4 weeks
Rat	0.7 ml/100 gm body wt
Goats/Large Animals	7 ml/kg body wt

NOTE: The maximum blood to be withdrawn is stated as a single bleed. At maximum, it can be repeated at two-week intervals. If more frequent bleeding is needed, the volume should be proportionally reduced. If more blood than this maximum volume is needed, the investigator must meet with a veterinarian to arrange for animal observation and hematocrit determination.

Describe the blood collection method(s). Note that anesthesia is required for a terminal bleed and/or cardiac puncture.

Blood glucose measurements may be done at 12 hour intervals during the study period. Nick the tail vein using a scapel to get a drop of blood for testing.

Tail vein to measure measure CBC, BUN, creatinine, amylase, lipase and cytokines. Rats will be placed briefly in a restrainer for blood collection.

Volume of blood to be obtained at each collection:

0.01 ml; 50-100ul

Frequency of blood collection:

every 12 h x 7 days; daily x 7 days

Total number of blood collections per animal:

14; 7

19. Will tumors be implanted or induced (including phenotypic expression) in animals? No

20. Will conscious animals be restrained for periods longer than two hours? No

21. Will conscious animals be subjected to aversive stimuli to elicit a response? No

22. Will food or water be withheld from the animals for over 24 hours during the study? No

23. Will machine sources of radiation (X-rays, UV, RF, Laser or CT) be used on animals in this study? No

24. Will the animals be irradiated using a gamma irradiator? No

25. Will radioisotopes be administered to the animals in the study? No

26. Will infectious agents, recombinant DNA molecules or toxins (Diphtheria, Pertussis, etc.) be administered to the animals? No

27. Will organisms that are infectious to other animals be administered to the animals in this protocol? No

28. Will a toxic substance, hazardous chemical, reproductive hazard (mutagen) or carcinogen be administered to the animals? Yes

Name	Route	Exposure	Housed	Excreted	Length of Excretion
Caerulein	IP (20 mcg/kg)	6 hours	JRB 3-325/327	N	
Arginine hydrochloride	IP (4 mg/kg)	Up to 7 days	JRB 3-325/327	N	
Glyceryl trilinoleate (GTL)	IP (0.1-0.32 ml/rat)	Up to 7 days	JRB 3-325/327	N	
Sodium taurocholate	Intraductal-Pancreatric Duct (0.1 ml/100 gm in 5% saline)	Up to 7 days	JRB 3-325/327	N	
IR Dye 800CW 2-DG	IV 10-30 nmol/dose, 1 dose per rat	6 hours	JRB 325/327	N	
XenoLight redijet 2-	IV 10-30 nmol/dose, 1	6 hours	JRB	N	

deoxyGlucosone 750	dose per rat		325/327		
SuperHance 680	IV 4-8 nmol/dose, 1 dose per rat	6 hours	JRB 327/325	N	

29. Does this protocol request an exemption to IACUC Policy or the Guide for the Care and Use of Laboratory Animals? No

30. Will animals require single housing at any point? Yes

A. List the experiments that will require single housing, the time point in the experiment at which single housing will occur and the length of time animals will be singly housed

the animals will require single housing after the placement of gastric balloon and jugular catheterization.

B. Provide a scientific justification for singly housing animals:

Due to the surgical manipulation of the animals, co-housed animals can remove the balloon tubes and jugular catheters of each other; requiring single housing

31. Will a piece of the tail be amputated or any other tissue be collected for DNA analysis or any other testing? No

32. Will toe clipping (amputation) be performed for identification purposes? No

33. Will animals be exposed to any biological product (cell line, tumors, serum, antibodies, embryonic stem cells) in this protocol? No

DEPARTMENT OF THE ARMY
HEADQUARTERS, US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
810 SCHREIDER STREET
FORT DETRICK, MD 21702-5000

REPLY TO
ATTENTION OF

November 26, 2014

Director, Office of Research Protections
Animal Care and Use Review Office

Subject: Review of USAMRMC Proposal Number PR110417entitled, "Transgastric Local Pancreatic Hypothermia: A Novel, Rapid Multimodal Therapy for Acute Pancreatitis"

Principal Investigator Vijay P. Singh
University of Pittsburgh
Pittsburgh, PA

Dear Dr. Singh:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Programs"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, an amendment dated 06 October 2014 to protocol PR110417.06 entitled, "Transgastric Hypothermia," IACUC protocol number A20813 , Protocol Principal Investigator Vijay Singh, is approved by the USAMRMC Animal Care and Use Review Office (ACURO) as of 25-NOV-2014 for the use of rats and will remain so until its modification, expiration or cancellation. This protocol was approved by the Mayo Clinic, Arizona IACUC on 06-OCT-2014.

Required Actions: When updates or changes occur, documentation of the following action or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)

- IACUC actions involving this protocol regarding
  a. any noncompliance;
  b. any deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or
  c. any suspension of this activity by the IACUC

- USDA or OLAW regulatory noncompliance evaluations of the animal facility or program

- AAALAC, International status change (gain or loss of accreditation only)

Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

- Species used (must be approved by this office)
- Number of each species used
- USDA Pain Category for all animals used

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

for 

Bryan K. Ketzenberger, DVM, DACLAM
Colonel, US Army
Director, Animal Care and Use
Review Office

Copies Furnished:
Mr. Ayi Ayayi, US Army Medical Research Acquisition Activity (USAMRAA)
CDR Alexis Mosquera, Congressionally Directed Medical Research Program (CDMRP)
Dr. Cherylann Gieseke, Mayo Clinic and Foundation, Rochester
Ms. Jennifer Walker, Mayo Clinic, Arizona

with pancreatic cancer was 15,023 pg/mL/μg protein (SEM = 5419). These values were significantly greater than the serum levels of healthy volunteers (4.32 pg/mL/μg protein, SEM = 1.77). Murine *in vivo* analyses indicate a three-fold increase in GRP78 mRNA expression of KPC tumor samples versus KPC pancreas without tumors. Additionally, the KPC tumors have increased GRP78 expression in the ducts as tumors progress from pre-cancerous lesions to pancreatic adenocarcinoma, detected by immunofluorescence. One possible route of chemoresistance in pancreatic cancer is through efflux of xenobiotics with ABC transporters. Our studies show that mRNA expression of NRF2, a transcription factor downstream of UPR activation, is increased in KPC tumors versus KPC pancreas without tumors. Further, mRNA of ABC-C transporters (transcriptionally regulated by NRF2) are also increased in KPC tumors versus that KPC pancreas without tumors. Based on our studies, UPR could provide multiple selective targets for future therapeutics, which may also decrease the chemoresistance in pancreatic cancer.

Targeting the Tumor-Associated Microenvironment: The Clinical Impact of Integrin Alpha 5 (ITGA5) in Patients With Pancreatic Ductal Adenocarcinoma

S.W.L. de Geus,[1] P.J.K. Kuppen,[1] H.A.J.M. Prevoo,[1] R.L. Vlierberghe,[1] R-J. Swijnenburg,[1] J.S. Mieog,[1] B.A. Bonsing,[1] H. Morreau,[2] C.J.H. van de Velde,[1] A.L. Vahrmeijer,[1] C.F.M. Sier.[1] *[1]Department of Surgery, University of Leiden, Leiden, The Netherlands; [2]Department of Pathology, University of Leiden, Leiden, The Netherlands.*

Background: There is cumulative evidence that the tumor-associated microenvironment is crucial to tumor development and progression. Integrin αv (ITGA5) and α-smooth muscle actin (α-SMA) are important players in the tumor-associated microenvironment. Therefore, the aim of this study is to examine the expression of ITGA5 and α-SMA in relation to their potential for tumor targeted therapy and imaging of pancreatic cancer patients.

Methods: Expression of α-smooth muscle actin (α-SMA) and integrin αv (ITGA5) was evaluated by immunohistological analyses in 137 patients with pancreatic ductal adenocarcinoma. In addition, the tumor-stroma ratio was assessed based on hematoxylin and eosin (H&E) stained tumor sections retrieved from the same patient population. Chi-square, fisher-exact and student t-tests were used to evaluate the association between clinical and pathological characteristics and biomarkers expression and survival analysis was performed using a Cox proportional hazard model.

Results: A high tumor-stroma ratio was found in 89.2% of the patients, α-SMA expression was over-expressed in 84.8%, membranous and stromal ITGA5 expression was abundant in 39.3% and 65.5% of the patients. High α-SMA expression was positively associated with up-regulated stromal ITGA5 expression ($p < 0.001$), but not with membranous ITGA5 expression ($p = 0.161$). ITGA5 expression of the tumor-associated microenvironment was also correlated with vascular invasion ($p = 0.023$). On multivariate analyses high α-SMA expression ($p = 0.041$) and combined stromal and membranous ITGA5 expression were of independent predictive value for OS ($p = 0.025$) and DFS ($p = 0.051$) in pancreatic ductal adenocarcinoma patients.

Conclusion: The results of this study suggest that the tumor-associated microenvironment and in particular ITGA5 play a critical role in the progression of pancreatic cancer. Therefore, ITGA5 holds great promise as target for targeted-therapy and personalized medicine in pancreatic cancer patients.

In Vivo Imaging of Non-Radioactive Near Infra-Red 2-Deoxyglucose During Mild and Severe Acute Pancreatitis (AP) in Rats

C. de Oliveira, K. Patel, P. Noel, R.N. Trivedi, A. Singh, V.P. Singh. *Department of Medicine, Mayo Clinic - Arizona, Scottsdale, AZ.*

Background: While 2-Deoxy-2-[18F] fluoroglucose (FDG) uptake is increased in human AP, its relevance to AP severity is unknown. We thus studied the uptake of the non-radioactive, near infrared fluorescence labelled 2-deoxyglucose analog, IRDye® 800CW 2-DG probe (2-DG; Li-Cor) during mild (cerulein; CER) and severe (intraductal glyceryl trilinoleate; ID-GTL) AP.

Methods: Wistar rats (300g; 3-6/group) were administered 2-DG (44mcg/kg; I.V.). CER AP (50μg/kg, I.V) and severe AP (ID-GTL 50μL/0.1kg) were induced within 10 minutes of giving 2-DG. DL group had only duct ligation and controls (CON) only received 2-DG. Imaging was done every 10-15 minutes over 2 hrs. Average Radiant Efficiency [p/s/cm^2/sr]/[μW/cm^2] was measured over the pancreas using the IVIS 200 *in-vivo* imaging system (PerkinElmer) using the Living Image® software and verified in *ex vivo* pancreata. Serum amylase, lipase and pancreatic edema measured after 6-8 hours of AP. Values are mean ± SEM and $p<0.05$ was considered significant.

Results: 2-DG uptake significantly increased over controls between 40-100 minutes in both AP models. The peak was 2-fold CON in the CER and 6-fold CON in ID-GTL groups ($p<0.05$), with a 1.2-fold increase in DL group. *Ex vivo* pancreas imaging confirmed the increased signal of ID-GTL (~4-fold) and CER (~1.7-fold) vs DL and control. ID-GTL but not CER rats were moribund and had hemorrhagic pancreatic necrosis when sacrificed. Both CER and ID-GTL AP increased serum amylase (4901±793 U/L; 9841±1530 U/L vs 2642±294 U/L in DL and 408±22 U/L in CON; $p<0.01$), lipase (3734±695 U/L; 6455±1684 U/L vs 486±71 U/L in DL and 23±3.4 U/L in CON; $p<0.05$) and pancreatic edema (87±2.1%; 88%±1.6% vs 79±1.4% in DL and 67±2.3% in CON; $p<0.05$).

Conclusion: *In-vivo* fluorescent imaging system using a non-radioactive 2-DG optical probe may be useful alternate to FGD and predict the AP severity early in the disease.

Role of Preoperative CA19-9 in Predicting Long and Short Term Progression Free Survival in Patients Undergoing Pancreatic Cancer Resection

S. Desai,[1] C. Langmead,[2] H. Zeh,[3] A. Zureikat,[3] N. Bahary,[1] R. Brand.[1] *[1]Department of Medicine, University of Pittsburgh, Pittsburgh, PA; [2]School of Computer Science, Carnegie Mellon University, Pittsburgh, PA; [3]Department of Surgery, University of Pittsburgh, Pittsburgh, PA.*

Background: A major advancement in the management of pancreatic adenocarcinoma (PC) patients would be identifying those patients who will not achieve significant benefit from attempted curative resection due to the presence of unrecognized metastatic disease at surgery or a short-time (<1yr) to PC recurrence. A pre-operative serum biomarker that could predict these patients would be of great benefit by avoiding unnecessary surgeries. Our aim was to determine if serum CA19-9 could predict long or short-term progression free survival (PFS) for those patients undergoing attempted curative resection.

Methods: We retrospectively reviewed 287 patients from our PC registry undergoing attempted curative resection from January 1, 2006 through February 28, 2014. Pre-surgical CA 19-9 levels, total bilirubin, neo-adjuvant or adjuvant chemotherapy, PFS and overall survival were collected. Analysis was limited to those patients who did not receive neoadjuvant therapy (50 patients). Long-term PFS was defined either as ≥3 yrs or ≥ 2 yrs without radiologic evidence of disease and short-term PFS was defined as <1 yr without radiologic evidence of disease. Statistical p-values were calculated with a Mann Whitney U test.

Results: Twenty-five of 50 patients had short-term PFS, 7 patients had PFS ≥ 3 yrs and 14 patients had PFS ≥ 2 yrs. There was no statistically significant difference in the serum CA19-9 between those with < 1 yr and either ≥ 2 ($p=0.77$) or 3 yrs PFS ($p=0.82$).

Conclusions: Pre-operative CA19-9 did not distinguish between long and short-term PFS. These results support the need for future studies aimed at evaluating different serum biomarkers alone, or in combination with CA 19-9 and/or pre-operative clinical factors, to predict those patients with a short (<1 yr) PFS.

Impact of the Anatomical Location of Necrosis on Outcome in Patients of Acute Pancreatitis

N. Dhaka,[1] A. Munit,[4] J. Samanta,[1] R. Prasada,[1] P. Gupta,[2] V. Gupta,[3] T.D. Yadav,[3] S.K. Sinha,[1] R. Kochhar.[1] *Departments of [1]Gastroenterology, [2]Radiodiagnosis, [3]Surgery, Postgraduate Institute of Medical Education And Research, Chandigarh, India; [4]John H Stroger Hospital of Cook, Chicago, IL.*

Aim: To evaluate the impact of anatomical location of necrosis on outcome in patients of acute nercrotizing pancreatitis (ANP).

Methods: 161 ANP patients were classified according to the site of necrosis [pancreatic (I), peripancreatic (II) or both (III)]. Patients with pancreatic necrosis were further divided in three groups based on anatomical location of necrosis [necrosis involving only head region (A), involving body with or without tail region (B), involving the whole pancreas (C)]. Site of necrosis was correlated with outcome (hospital stay, need for ICU care, need for ventilator, pigtail catheter drainage (PCD), surgery and mortality).

Results: Pancreatic necrosis was seen in 115 (71.5%) and peripancreatic necrosis alone in 46 (28.5%) patients. Out of 115 patients with pancreatic necrosis, necrosis at location A was seen in 35 (30.5%), at location B in 48 (41.7%) and at location C in 32 (27.8%) patients. Patients with location A and C necrosis

PLOS ONE

Characterization and predictive value of near infrared 2-deoxyglucose optical imaging in severe acute pancreatitis

--Manuscript Draft--

Manuscript Number:	PONE-D-15-51787R1
Article Type:	Research Article
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Corresponding Author:	Vijay P. Singh, M.D. Mayo Clinic Scottsdale, AZ UNITED STATES
Keywords:	Pancreas; pancreatitis; pancreatic necrosis; Severe pancreatitis; Animal models of disease; mortality; predictor; Prognosis; PET scan; 2-deoxyglucose; Near infrared imaging
Abstract:	Background: studying the uptake of 2-deoxy glucose (2-DG) analogs such as 2-Deoxy-2-[18F] fluoroglucose (FDG) is a common approach to identify and monitor malignancies and more recently chronic inflammation. While pancreatitis is a common cause for false positive results in human studies on pancreatic cancer using FDG, the relevance of these findings to acute pancreatitis (AP) is unknown. FDG has a short half-life. Thus, with an aim to accurately characterize the metabolic demand of the pancreas during AP in real-time, we studied the uptake of the non-radioactive, near infrared fluorescence labelled 2-deoxyglucose analog, IRDye® 800CW 2-DG probe (NIR 2-DG; Li-Cor) during mild and severe biliary AP. Methods: Wistar rats (300g; 8-12/group) were administered NIR 2-DG (44mcg/kg; I.V.). Mild and severe biliary AP were respectively induced by biliopancreatic duct ligation (DL) alone or along with infusing glyceryl trilinoleate (GTL; 50µL/100gm) within 10 minutes of giving NIR 2-DG. Controls (CON) only received NIR 2-DG. Imaging was done every 5-10 minutes over 3 hrs. Average Radiant Efficiency [p/s/cm²/sr]/[µW/cm²] was measured over the pancreas using the IVIS 200 in-vivo imaging system (PerkinElmer) using the Living Image® software and verified in ex vivo pancreata. Blood amylase, lipase and pancreatic edema, necrosis were measured over the course of AP. Results: NIR 2-DG uptake over the first hour was not influenced by AP induction. However while the signal declined in controls and rats with mild AP, there was significantly higher retention of NIR 2-DG in the pancreas after 1 hour in those with GTL pancreatitis. The increase was > 3 fold over controls in the GTL group and was verified to be in the pancreas ex vivo. In vitro, pancreatic acini exposed to GTL had a similar increase in NIR 2-DG uptake which was followed by progressively worse acinar necrosis. Greater retention of NIR 2-DG in vivo was associated with worse pancreatic necrosis, reduced ATP concentrations and mortality, which were not predicted by the blood parameters. Conclusion: In-vivo fluorescent imaging of a non-radioactive near infrared 2-DG optical probe can predict the AP severity early during the disease.
Order of Authors:	Cristiane de Oliveira
	Krutika Patel
	Vivek Mishra
	Ram Trivedi
	Pawan Noel
	Abhilasha Singh
	Jordan Yaron
	Vijay P. Singh, M.D.

Opposed Reviewers:	
Response to Reviewers:	Reviewer #1: In this manuscript the authors Oliviera et al studied the uptake of the non-radioactive, near infrared fluorescence labelled 2-deoxyglucose analog, IRDye® 800CW 2-DG probe (NIR 2-DG; Li-Cor) during mild and severe biliary AP. Their study shows that this method can be used for early diagnosis of the severe acute pancreatitis. This is a very attractive study. However, there are a couple of comments: 1. Since the diagnosis method is based on metabolic activity of the disease pancreas particularly the glucose uptake, are the authors implying that with the severity of AP, the glucose uptake in the pancreas increases? The authors need to discuss this a little. We thank the reviewer for this suggestion. Yes it does seem that the metabolic demand of the pancreas goes up due to the drop in ATP levels (Figure 5E) associated with inhibition of mitochondrial complexes I and V induced by the linoleate generated from GTL. This could result in increased glucose requirements. This is now discussed on page 13 line 19 onwards as: “We have previously shown that lipotoxicity from unsaturated fatty acids such as linoleic acid causes a reduction in ATP levels associated with acinar necrosis [22,33], which would be consistent with the large increase in necrosis (Fig 4A-C) and drop in ATP levels we note in GTL group (Fig 6E). These in combination with hypoxia could increase glycolysis dependence of the GTL treated pancreas and thus result in the increased NIR 2-DG uptake we note.” 2. AP primarily occurs due to dysfunctional acinar cells. Acinar cells are very active transcriptionally, with a great need for energy. Can the authors comment on why do they not see 2DG accumulation in the normal pancreas, but so when the pancreas is diseased? We believe there are 2 parts to addressing this question: A. Whether 2-DG uptake occurs in the normal pancreas: We do note an increase overlying the normal pancreas also. This occurs over the first hour (Fig 1) and is no different between animals with AP and controls. The competition between glucose and 2-DG becomes more apparent when a tissue needs more glucose as pancreatitis progresses. These animals were fasted prior to AP induction. How this influences NIR 2-DG uptake is unclear. On one hand it could reduce energy demand due to reduced protein synthesis during fasting. However fasting would also reduce/normalize blood glucose (which we note in all our groups prior to AP induction) so that the competition for glucose uptake against NIR 2-DG is reduced. Moreover the stoichiometry of how NIR 2-DG would equilibrate as its blood levels get lowered while it gets excreted remains to be explored. B. Occurrence/initiation of AP vs. severity of AP. While AP as defined by an increase in serum and histological parameters also occurred in the DL group, it was severe only in the GTL group. The relatively higher uptake later in the GTL group vs. controls (and despite lower plasma 2-DG levels due to clearance) suggests that that this is mediated the increase in glucose requirements due to necrosis, as mentioned in response to point 1. Reviewer #2: In this research paper de Oliveira et al. applies near infrared 2-deoxyglucose optical imaging for the prediction of acute pancreatitis outcome. These methods have been successfully applied in pancreatic cancer diagnostics, but not for the diagnosis of acute pancreatitis. The authors elegantly demonstrate that there is no significant difference in the uptake of 2-deoxyglucose in control, mild, or severe AP. However they were able to show a significantly higher retention in the severe group. This raises the possibility to use this imaging method to predict the outcome of AP in clinical patients in the early phase, which is an enormous clinical problem. The experiments are well designed and the interpretation of the data supports the conclusion and the topic is absolutely relevant. We thank the reviewer for the encouragement and have addressed the comments as below. Minor comments: 1. The authors shall check the text since spaces are missing at several places (especially where the references are inserted). Wherever missing, the spaces have been inserted.

2. The first sentence of the background shall be corrected.
The sentence has been changed to “While the utility of 2-deoxy glucose (2-DG) analogs is established in diagnosis and prognostication of malignant diseases, the relevance of such studies in inflammatory diseases is emerging”

3. On page 11. the concentration of GTL shall be corrected ("50µl/100 gm of body weight"), instead of gm use g or gramm.
We now use “g” instead of “gm”

4. On page 18. line 7. delete dot after in vitro
We have deleted the “.”

5. N numbers shall be highlighted for each individual experiments in the figure legend as well.
These are now mentioned in the legends.

6. On Fig.3. scale bar shall be added.
The scale bar is now added to the figure and described in the legend.

Reviewer #3: The authors present a manuscript describing the use of a labelled 2-deoxyglucose analog to characterize the metabolic demand of the pancreas in two models of experimental acute pancreatitis. They analyze the influence of pancreatitis induction, the changes in pancreas signal in the different models and the association with acinar necrosis and severity. Authors conclude that in-vivo fluorescent imaging of a nonradioactive near infrared 2-DG optical probe can predict the AP severity early during the disease.
This study is well conducted with appropriate levels of detail in the experimental design and clear results. The results are exciting, in particular taking into account the lack of early methods to differentiate mild and severe pancreatitis in the clinical practice. The discussion is complete and circumspect. I have only minor comments.

Since the GTL model is associated with hemorrhage, the possible effect of pancreatic hemorrhage in the increase observed in pancreas signal must be also discussed in addition to the increase in vascular permeability.

We thank the reviewer for this comment. The role of hemorrhage is now discussed on page 14 line 3 onwards as:
“Similarly, the increase in NIR 2-DG signal noted ex-vivo in the GTL group did not correlate with the amount or location of hemorrhages (Fig 2C, 6B, C), and while hemorrhages were sometimes noted within an hour of GTL injection (Fig 4C), these did not result in an increased of NIR 2-DG signal at 1 hour (Fig 1 J, K). These observations along with the in vitro findings in acini mentioned above make the contribution of hemorrhage to the in vivo NIR 2-DG signal also unlikely.”

In page 12, in the “pancreatic necrosis” section, it seems to have lost a reference
The references have now been added (refs 14, 31, 32)
Reviewer 4: Brief: The manuscript was an interesting evaluation of the use of a NIR 2DG optical imaging agent to monitor inflammation and specifically the metabolic demands on the pancreas during acute and severe pancreatitis in Control animals and AP and GLT mouse models. Additional criteria evaluated included in vitro acinar cell harvest and culture and assessment of LDH leakage, blood parameters (amylase, lipase, ALT and bilirubin), and tissue analyses. The authors concluded the data demonstrated a predictive assessment of AP severity early in the disease by a non-invasive imaging method and near infrared probe.

We thank the reviewer for the comments and suggestions. These are addressed point by point as below:
Comments and Suggestions:
1)Pg 5 L8 After pretreatment of cells with NIR 2-DG, cells were stimulated with GTL or saline. The time period was stated to be 1 to 4 h. This is extremely wide? What time was actually used and was it consistent across treatments?

This is now mentioned on page 5 in the methods section in more detail (please see response to point # 3) and remains as shown in figure 5. The time points after adding

GTL or saline for LDH assay were 0, 1, 2, 4 hours. The time point for NIR 2-DG reading was 1 hour.

2)Pg 5 L14It would appear a BCA protein Assay kit was used to determine protein concentration for normalization, but on what? This is not clear. If the protein level was determined for each individual well (or for # of cells in each well) then the reference to the 700nm autofluorescence detection and use for normalization is mute especially if the BCA protein quantification is being done as well? Please clarify to be a more defined description of the method of analysis and normalization?

As the reviewer states, the BCA assay was used to determine protein concentration for normalization of the 800 nm fluorescence signal/per well l. We have thus deleted to the reference to auto fluorescence at 700 nm. The results were similar if we normalized to the 700nm auto fluorescence. To avoid confusion we have omitted this portion. For sake of consistency with the previous text (page 6, line 2-3: “Ratios were calculated by normalizing to the saline treated control group”), we now show the data in figure 5A as a Fluorescence/protein (fold control) and mention the raw values in the text of the results (page 11, line 5-6; highlighted)

3)Pg 5 L15The LDL leakage description is also insufficient in detail. I assume you had an assay duration of 4 hours during which aliquots were taken for measurement. Where did the 1% triton x-100 lysates come from? What was the system used to make the measurement?

The protocol for LDH leakage is now detailed on page 5 lines 10-18, and previous publications where the same assay has been done by us (refs 20, 30, 31) are now mentioned on page 5 line 9.The instrument on which this was done i.e. the FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices)is now mentioned on page 7 under Biochemical Assays. The methods is detailed as below:
All studies were done in a shaking water bath (80 RPM) at 37°C at an ambient atmosphere. Cells were treated with either glyceryl trilinoleate (GTL, 300 μM) or saline as a control. Cell injury was quantified by measuring LDH leakage (Roche Diagnostics, Indianapolis, IN) into the medium as described previously [22,30,31]. This was done at 0, 1, 2, 4 hours by taking a 2% (40 l out of 2ml) aliquot of medium, centrifuging it (200g, 5 minutes) and measuring LDH in the supernatant. At the end of the incubation the cells were lyzed by incubating them for one hour in Triton x-100 at final concentration of 1%. LDH activity in this lysate was taken as representing total (100%). The activity in the aliquots calculated as a percentage of the total amount was depicted as % LDH leakage for each time point.

4)Pg 6 L15Authors mention animals were sacrificed at 1 or 3 h post AP induction. Later they mentioned animals become moribund after AP induction (between 5 and 8 hours in GTL group, median 6 hours) were sacrificed. This seems to imply that organs for imaging and analysis are not standardized across a set time line but on a time line associated with treatment, control, GTL or DL. I am assuming the animals all received the NIR 2-DG at the same time point. If animals were sacrificed or terminated at different time points then body clearance could have an effect on what level of NIR 2DG is detected at termination. Were there any ancillary studies done to confirm differences seen between treatments are due to the procedure and not just a clearance issue?

As previously mentioned and now further clarified and highlighted on page 6 line 19 and shown in figure 1; all NIR 2-DG imaging studies were done by collecting images every 5-10 minutes after administration of NIR 2-DG and over the 3 hours after inducing AP in the GTL or DL groups. All imaging time points are calculated by taking the time of NIR 2-DG at time zero. No imaging studies were done after 3 hours of inducing AP or 3.5 hours after NIR 2-DG administration in controls. So the clearance of the agent is matched across all groups for each time point after NIR 2-DG administration. Noting that the fluorescence increases in the pancreas but not the duodenum or spleen in the GTL group after 2 hours of NIR 2-DG administration (Figure 6C); it is unlikely that reduced clearance in the GTL group accounts for the increase in intensity over the pancreas (otherwise it should also have increased in the duodenum or spleen).

As mentioned (page 7 line 2) parameters of AP induction, severity and survival were however assessed over the 8 hour time course when the study was terminated. Data for these is shown in figures 3, 4 and was collected from animals that were electively sacrificed at 1, 3, 8 hours after AP induction or those becoming moribund during this time.

5)Pg 7 L5Biochemical assays: This section should provide clear definition for which instrument is being used with which assay? It is unclear.

The instruments on which the respective biochemical assays were done are now clearly mentioned.

6)Pg 7 L10Pancreas water content:The equation provided is an assessment of % tissue moisture by weight, correct? You use a “fresh” weight in the formula which most likely is “wet”?
The term “fresh” is now replaced with “wet”

7)Pg 7 L18Pancreatic necrosis: “morphologist” ??? “pathologist” Please clarify.

The blinded morphologist was Dr. Krutika Patel. Dr. Patel has extensive experience in morphometric analysis of the pancreas as noted in previous publications (refs 14, 31, 32). Her initials (KP) are now mentioned.

8)Pg 7 L18 (REF)Missing reference?
These have now been added.

9)Pg 7 L22Is this procedure being done on a tissue “section”? It isn’t clear. Please clarify. If so, please add how sections were prepared, thickness, etc.
Paraffin sections of a 5 micron thickness were used for morphometric analyses. This is now mentioned.

10)Pg 7 L20The manufacturer of the PathScan Enabler IV slide scanner should be noted. Not the distributor for the instrument?
The manufacturer (Meyer Instruments, Huston, TX) is now mentioned.

11)Pg 8 L3Please provide information on Statistical Package used for any and all analyses.

This has been extensively reworded as mentioned below:
“This was done using the SigmaStat statistical package integrated into the graphic program SigmaPlot 11 (Systat Software Inc, San Jose, CA). All values, unless otherwise specified, are reported as mean ± SEM. Groups were compared by one way analysis of variance (ANOVA) versus controls. Pairs were compared using a two tailed Students t-test when the distribution was normal (Shaprio-Wilk test) or using a Mann-Whitney test when failing the Shapiro-Wilk test. Differences were considered significant at a p value <0.05”.

12)Pg 8 L22Text references a missing Fig 1L ? Please eliminate from text or amend figures and legend to reflect decision.

This was a typographical error and has been corrected to figure 1K.

13)Pg 9 14In explaining the comparison of ARE authors have provide description and what appears to be an equation. Please present more clearly as it is very hard to follow in current form.

The equation is an example of how to calculate fold change in ARE over controls. For example:
Fold change in ARE in the GTL group over controls after “n” minutes of NIR 2-DG administration =
{ARE in GTL at n min. - ARE in GTL at 5 min.} ÷ {ARE in control at n min. - ARE in control at 5 min.}.
The text has been modified to reflect this.

14)Pg 11 L18At one point authors discuss observation and preliminary studies … These do not appear to be clearly referenced. Please clarify.
The preliminary studies were done by comparing the uptake of 2-DG give immediately before and after AP induction. The reference to preliminary studies is now deleted and the logic of doing so is discussed under point 18. Please see below.
General queries:
15)In general, the language in this section seems very “casual”. Suggest being more definitive in your narrative and what points you wish to stress to the reader.
There are points where we can be definite. These include our experimental design, results, and our conclusions. The data are shown in the figures and the definite results are:
1. NIR 2-DG uptake over the first hour was not influenced by AP induction
2. There is significantly higher retention of NIR 2-DG in severe GTL pancreatitis vs. mild AP or controls.
3. Higher NIR 2-DG uptake in vivo was associated with worse pancreatic necrosis, reduced ATP concentrations and higher AP mortality
4. A higher NIR 2-DG uptake in AP vs. controls predicted severe AP better than serum amylase, lipase, ALT or bilirubin.
5. NIR 2-DG imaging can be done in rats.
These are stated in the abstract and the results section (opening paragraph and sub-titles). We do not wish to over stress these.
However, there are areas we cannot be definite, and only hypothesize. These include:
1. The mechanisms underlying the increased uptake of NIR 2-DG (e.g. developing necrosis vs. permeability vs. reduced clearance vs. upregulation of glucose transporters etc.,)
2. Whether our findings will be replicated in other acute inflammatory conditions or in humans with AP in whom FDG is imaged using PET.
3. Whether the results obtained on cross sectional imaging will give better results than the planar imaging used in our study.
The interpretation and relevance of our work with respect to these points is left up to the reader.

16)After imaging animals following AP induction you saw no change in fluorescence and suggested there was no effect on uptake of the NIR 2-DG. However, when the NIR 2DG is given clearance begins. To some extent this will be variable with each animal. How do you address this issue?
It is unlikely that clearance confounded the NIR 2-DG signal over the pancreas. This is discussed now on page 14, lines 8-12. The detailed logic is as follows:
1. The control group was imaged at the same time intervals after NIR 2-DG administration as the DL, GTL groups. Since we see no difference in pancreatic NIR 2-DG signal between the three groups over the first hour after NIR 2-DG administration irrespective of AP (Fig 1); the clearance over the first hour does not account for the differences we note later.
2. If differences in clearance were responsible for the differences we note later, then we should also see an increase in other organs. However, we do not see a higher signal over the duodenum or spleen in the GTL group (Fig 6B). The increase in signal is just over the pancreas.
3. The study was conducted and analyzed with 8-12 animals per group (and not as single animals) to exclude the several variables other than AP severity (such as clearance) that could bring in minor variations if analyzed for each individual animal and influence the results. Therefore the statistically significant differences we note between the GTL vs. DL vs. control groups are despite these variations (mentioned in the next paragraph) and imply the low likelihood of these contributing to the results.

Clearance is unlikely to be a confounder. Apart from clearance, other potential confounders include i) variations in age and weight within the range chosen, ii) biological variation in size and shape of organs around the pancreas that could affect the penetration of near infra-red light. These include the stomach, spleen and liver size along with their exact location, iii) skin thickness, iv) pressure applied when shaving the hair resulting in variation in hair length, v) bruising of skin result in bleeding while shaving the hair, vi) how much the animal is turned to the right, vii) the degree of interference from overlying ribs among several other variables. The statistically significant differences we note between the GTL vs. DL vs. control groups are despite

	these variations and imply the low likelihood of these or clearance contributing to the results. 17)Why was the probe, NIR 2DG, given prior to establishment of the AP and GTL models? Would you expect a greater effect if the disease model was established before the injection of NIR 2DG? Please clarify. There are several reasons for giving NIR 2-DG before AP induction: 1. A basic requirement of our study would be to know whether AP induction and manipulation of the pancreas affects the NIR 2-DG signal over the pancreas. If this had happened, we would not be able to use this approach. Giving NIR 2-DG prior to AP induction was the only way to exclude this. 2. It allows a direct comparison to controls since all groups of animals have the same baseline parameters prior to AP induction, which might otherwise influence NIR 2-DG uptake. i.e. they have the same glucose, pancreatic perfusion, and baseline parameters of inflammation etc. at the time of giving NIR 2-DG 3. With no previous data on the kinetics of NIR 2-DG uptake, it would be risky to choose a time for NIR 2-DG administration after AP induction. For example if NIR 2-DG is given 3 hour after AP induction, when significant necrosis (@ 45-50%) has developed, then it may not be taken up by the necrotic pancreas or there may be thrombosed vessels that prevent its uptake. 4. Administration of NIR 2-DG after 2 hours of AP would also make it loose prognostic value, since the necrosis is already established. 5. NIR 2-DG administration prior to AP induction ensured that the blood glucose was the same among all groups. If NIR 2-DG is given 30 minutes aster AP induction then the uptake equilibration of the dye over the next 60-90 minutes may not be uniform and glucose homeostasis may be disrupted due to the ongoing AP resulting in variation in glucose levels being a confounder of the results, and the time taken in correction of the blood glucose would result in inhomogeneity and delay in the time when NIR 2-DG is administered to animals with AP and have questionable prognostic value. 18)Was there an effort to assess the presence or absence of possible interactions between NIR 2DG and GTL treatment injections? There was no difference in any parameter at the 3 hour time point in the rats with NIR 2-DG vs. those without. For example, edema, (88±1.7% vs 90±0.8%), plasma parameters (e.g. amylase 7461± 2572 U/L vs. 11931±3424U/L) and necrosis (50±7% vs 48±9% %) were the same in those give NIR 2-DG VS. those without. As the reviewer would have noted in the graphs of figures 3 and 4, 6D, E the plasma parameters, necrosis, mortality, edema and ATP data in the GTL and DL groups flows smoothly within the respective groups from 3 to 8 hours, which further supports that the NIR 2-DG did not affect the parameters of pancreatitis. Majority of the animals at the 3 hour time points had received NIR 2-DG, but none of the rats which were sacrificed at the time points after that.
Additional Information:	
Question	**Response**
Financial Disclosure Please describe all sources of funding that have supported your work. A complete funding statement should do the following: Include **grant numbers and the URLs** of any funder's website. Use the full name, not acronyms, of funding institutions, and use initials to identify authors who received the funding. **Describe the role** of any sponsors or funders in the study design, data collection and analysis, decision to	Supported by Grant number PR110417 Award # W81XWH-12-1-0327 from the Department of Army (DOA) (VPS), award number R01DK092460, R01DK100358 (VPS) from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The contents of the manuscript are solely the responsibility of the authors and do not necessarily represent the official view of DOA, NIDDK. Also supported by a startup package from the Mayo Clinic Arizona, Department of Medicine (VPS).

publish, or preparation of the manuscript. If they had no role in any of the above, include this sentence at the end of your statement: "*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*" If the study was **unfunded**, provide a statement that clearly indicates this, for example: "*The author(s) received no specific funding for this work.*" * typeset	
Competing Interests You are responsible for recognizing and disclosing on behalf of all authors any competing interest that could be perceived to bias their work, acknowledging all financial support and any other relevant financial or non-financial competing interests. Do any authors of this manuscript have competing interests (as described in the PLOS Policy on Declaration and Evaluation of Competing Interests)? **If yes**, please provide details about any and all competing interests in the box below. Your response should begin with this statement: *I have read the journal's policy and the authors of this manuscript have the following competing interests:* **If no** authors have any competing interests to declare, please enter this statement in the box: "*The authors have declared that no competing interests exist.*" * typeset	The authors have declared that no competing interests exist
Ethics Statement You must provide an ethics statement if your study involved human participants, specimens or tissue samples, or vertebrate animals, embryos or tissues. All information entered here should **also**	All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (Pittsburgh, PA) and the Mayo Clinic (Scottsdale, AZ) and the Animal Care and Use Review Office (ACURO), a component of the USAMRMC Office of Research Protections.

be included in the Methods section of your manuscript. Please write "N/A" if your study does not require an ethics statement.

Human Subject Research (involved human participants and/or tissue)

All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or an equivalent committee, and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. Informed consent, written or oral, should also have been obtained from the participants. If no consent was given, the reason must be explained (e.g. the data were analyzed anonymously) and reported. The form of consent (written/oral), or reason for lack of consent, should be indicated in the Methods section of your manuscript.

Please enter the name of the IRB or Ethics Committee that approved this study in the space below. Include the approval number and/or a statement indicating approval of this research.

Animal Research (involved vertebrate animals, embryos or tissues)

All animal work must have been conducted according to relevant national and international guidelines. If your study involved non-human primates, you must provide details regarding animal welfare and steps taken to ameliorate suffering; this is in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research." The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement.

If anesthesia, euthanasia or any kind of animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied.

Please enter the name of your Institutional

Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained. **Field Permit** Please indicate the name of the institution or the relevant body that granted permission.	
Data Availability PLOS journals require authors to make all data underlying the findings described in their manuscript fully available, without restriction and from the time of publication, with only rare exceptions to address legal and ethical concerns (see the PLOS Data Policy and FAQ for further details). When submitting a manuscript, authors must provide a Data Availability Statement that describes where the data underlying their manuscript can be found. Your answers to the following constitute your statement about data availability and will be included with the article in the event of publication. **Please note that simply stating 'data available on request from the author' is not acceptable.** ***If, however, your data are only available upon request from the author(s), you must answer "No" to the first question below, and explain your exceptional situation in the text box provided.*** Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?	Yes - all data are fully available without restriction
Please describe where your data may be found, writing in full sentences. **Your answers should be entered into the box below and will be published in the form you provide them, if your manuscript is accepted.** If you are copying our sample text below, please ensure you replace any instances of XXX with the appropriate details. If your data are all contained within the paper and/or Supporting Information files, please state this in your answer below. For example, "All relevant data are within the paper and its Supporting Information	All relevant data are within the paper and its Supporting Information files

files." If your data are held or will be held in a public repository, include URLs, accession numbers or DOIs. For example, "All XXX files are available from the XXX database (accession number(s) XXX, XXX)." If this information will only be available after acceptance, please indicate this by ticking the box below. If neither of these applies but you are able to provide details of access elsewhere, with or without limitations, please do so in the box below. For example: "Data are available from the XXX Institutional Data Access / Ethics Committee for researchers who meet the criteria for access to confidential data." "Data are from the XXX study whose authors may be contacted at XXX." * typeset	
Additional data availability information:	

January 7th, 2016

To,
Dr. Zoltan Rakonczay MD, PhD, DSc
Academic Editor
PLOS ONE

Re: Manuscript PONE-D-15-51787 entitled "Characterization and predictive value of near infrared 2-deoxyglucose optical imaging in severe acute pancreatitis"

Dear Dr. Rakonczay,

Happy New Year!

On 12/29/2015 we had received your letter stating the need for minor revisions in our manuscript along with the reviewer's comments. I am glad to say that we have been able to successfully address all the issues mentioned.

Your note had specifically mentioned that the original manuscript was in draft format and needed more methodological detail. We have removed the red highlights (previously put in response to Dr. Alvino's comments), and the uncited references have now been inserted. We have also formatted the manuscript in PLOS ONE style, amended the funding statement that Mayo Clinic did not have any role in the study apart from supporting our salaries, and state that no competing interest affects our adhere to PLOS ONE policies on sharing data and materials.

Our point by point responses to each of the reviewer comments are mentioned in italicized font under each of the comments in the attached "Response to Reviewers" file. We hope that the changes made make our manuscript suitable for publication in PLOS ONE and eagerly await your decision in this regards.

Sincerely,

Vijay P. Singh M.D.,
Corresponding author:
Vijay P Singh. MD,
Associate Professor,
3rd floor Collaborative Research Building,
Mayo Clinic Arizona,
13400 Shea Boulevard, Scottsdale, AZ, 85259.
Phone: 480-301-4286
Fax: 480-301-7017

Characterization and predictive value of near infrared 2-deoxyglucose optical imaging in severe acute pancreatitis

Cristiane de Oliveira[1], Krutika Patel[1], Vivek Mishra[2], Ram N. Trivedi[1], Pawan Noel[1], Abhilasha Singh[1], Jordan R. Yaron[1], Vijay P. Singh[1]

[1]Department of Medicine, Mayo Clinic, Scottsdale, AZ, [2]Department of Medicine, University of Pittsburgh, Pittsburgh, PA.

Corresponding author:
Vijay P Singh. MD,
Associate Professor,
3rd floor Collaborative Research Building,
Mayo Clinic Arizona,
13400 Shea Boulevard, Scottsdale, AZ, 85259.
Phone: 480-301-4286
Fax: 480-301-7017

ABSTRACT:

Background: studying the uptake of 2-deoxy glucose (2-DG) analogs such as 2-Deoxy-2-[18F] fluoroglucose (FDG) is a common approach to identify and monitor malignancies and more recently chronic inflammation. While pancreatitis is a common cause for false positive results in human studies on pancreatic cancer using FDG, the relevance of these findings to acute pancreatitis (AP) is unknown. FDG has a short half-life. Thus, with an aim to accurately characterize the metabolic demand of the pancreas during AP in real-time, we studied the uptake of the non-radioactive, near infrared fluorescence labelled 2-deoxyglucose analog, IRDye® 800CW 2-DG probe (NIR 2-DG; Li-Cor) during mild and severe biliary AP. **Methods:** Wistar rats (300g; 8-12/group) were administered NIR 2-DG (10 nanomoles; I.V.). Mild and severe biliary AP were respectively induced by biliopancreatic duct ligation (DL) alone or along with infusing glyceryl trilinoleate (GTL; 50µL/100gm) within 10 minutes of giving NIR 2-DG. Controls (CON) only received NIR 2-DG. Imaging was done every 5-10 minutes over 3 hrs. Average Radiant Efficiency [p/s/cm²/sr]/[µW/cm²] was measured over the pancreas using the IVIS 200 *in-vivo* imaging system (PerkinElmer) using the Living Image® software and verified in *ex vivo* pancreata. Blood amylase, lipase and pancreatic edema, necrosis were measured over the course of AP. **Results:** NIR 2-DG uptake over the first hour was not influenced by AP induction. However while the signal declined in controls and rats with mild AP, there was significantly higher retention of NIR 2-DG in the pancreas after 1 hour in those with GTL pancreatitis. The increase was > 3 fold over controls in the GTL group and was verified to be in the pancreas *ex vivo*. *In vitro*, pancreatic acini exposed to GTL had a similar increase in NIR 2-DG uptake which was followed by progressively worse acinar necrosis. Greater retention of NIR 2-DG *in vivo* was associated with worse pancreatic necrosis, reduced ATP concentrations and mortality, which were not predicted by the blood parameters. **Conclusion:** *In-vivo* fluorescent imaging of a non-radioactive near infrared 2-DG optical probe can predict the AP severity early during the disease.

BACKGROUND:

While the utility of 2-deoxy glucose (2-DG) analogs is established in diagnosis and prognostication of malignant diseases, the relevance of such studies in inflammatory diseases is emerging [1,2,3,4,5]. Acute Inflammatory diseases such as acute pancreatitis (AP) have a sudden onset with a rapid, variable and unpredictable course ranging from resolution with minimal care over a few days, to a severe course (severe acute pancreatitis; SAP) progressing to extensive pancreatic necrosis, requiring intensive care, a prolonged hospitalization with high costs and sometimes resulting in death. However we currently lack tools which can reliably predict the course of AP takes early on in the disease.

There are reports of increased 2-Deoxy-2-[18F] fluoroglucose (FDG) uptake in human pancreatitis detected by positron emission tomography (PET) imaging [6,7,8,9,10,11]. These are typically done with intent to study pancreatic cancer, and pancreatitis is an incidental finding contributing to the "false positives" seen. However, the relevance of the uptake of 2-DG tracers in predicting the AP severity early in the course of the disease is unknown and may need further exploration. Such evidence, if present in preclinical studies, can potentially guide studies in human pancreatitis.

The current project was designed to explore the behavior of, and study whether a 2-DG probe can be used to predict the severity of AP early in its course. For this we chose the non-radioactive, near-infrared probe (IRDye® 800CW 2-DG; NIR 2-DG; Li-Cor) and studied its uptake into the pancreas during mild AP and SAP. NIR 2-DG has been previously used as a 2-DG mimetic for both *in vivo* and *in vitro* studies [12,13]. Compared to FDG it offers the additional advantage of not being limited by a short half-life, and since it is non-radioactive, it is amenable to high throughput imaging requiring less specialized instrumentation. The emission spectra of NIR probes range between 650-900 nm which allows for their *in vivo* use without interference by either absorbance or non-specific emission from neighboring tissue.

The severe and mild AP models used in this study were chosen based on their relevance to human disease [14]. The mild model involves bilio-pancreatic duct ligation, which is sufficient to fulfil the criteria of biliary acute pancreatitis, but does not result in severe necrosis as shown recently [15], this is similar to mild biliary pancreatitis as may occur in humans due to a mass in the head of the pancreas causing duct obstruction. The severe model simulates severe biliary pancreatitis complicated by fat necrosis, as may occur in humans [16,17,18,19,20,21].In this, the triglyceride precursor of the second most abundant unsaturated fatty acid in pancreatic fat (linoleic acid), i.e. glyceryl trilinoleate (GTL) is infused into the pancreatic duct prior to the ligation. The amount of GTL is equivalent or lower than the percentage of adipocytes in the obese human pancreas [14,22,23,24,25,26]. Using the above tools we set out to study whether the course of NIR 2-DG uptake in the pancreas in controls, mild and severe AP can be used to predict AP outcomes.

MATERIALS AND METHODS

Reagents: IRDye 800CW 2-DG (NIR 2-DG, Li-Cor Biosciences) was dissolved in 1 ml sterile saline at a final concentration of 100 nM. Glyceryl trilinoleate (GTL; TCI America) for in vitro studies was dissolved and sonicated in HEPES containing buffer; for *in vivo* studies was directly injected into the pancreatic duct of the rats. Ketamine Hydrochloride (Ketaset, Fort Dodge Animal health), Xylazine (Anased, Lloyd Laboratories) and Isoflurane USP (Piramal Healthcare).

Animal work: All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (Pittsburgh, PA), the Mayo Clinic (Scottsdale, AZ) and the Animal Care and Use Review Office (ACURO), a component of the USAMRMC Office of Research Protections. Mice: 6-8 week old male CD-1/ICR mice (Charles River Laboratories, Wilmington, MA) housed with a 12-h light/dark cycle at temperatures ranging from 21-25°C, were fed standard laboratory

chow and allowed to drink ad libitum. Rats: 250-350g male Wistar rats (Charles Rivers Laboratories, Wilmington, MA) were housed with a 12 hour light-dark cycle, fed standard laboratory chow and were allowed to drink ad libitum. A Jugular venous catheter was placed in all rats under anesthesia (ketamine-Xylazine, 90mg/kg, 8 mg/kg respectively, IP; or isoflurane, 3% + 0.8-1 L/min induction; 1.5% + 0.8-1 L/min maintenance). The rats were left to recover for at least 3 days prior experiments. Catheters were flushed with 4U/ mL heparin/saline solution every other day and the patency of catheter verified.

Acinar Harvest and 2-DG *in vitro assay*: For NIR 2-DG *in vitro* studies, pancreatic acini were harvested from mice and used in oxygenated HEPES buffer pH 7.4 as described previously [27,28,29]. Viability was confirmed by trypan blue exclusion (>95%) at the start of each experiment. All studies were done in a shaking water bath (80 RPM) at 37°C at an ambient atmosphere. Cells were treated with either glyceryl trilinoleate (GTL, 300 µM) or saline as a control. Cell injury was quantified by measuring LDH leakage (Roche Diagnostics, Indianapolis, IN) into the medium as described previously [22,30,31]. This was done at 0, 1, 2, 4 hours by taking a 2% (40 µl out of 2ml) aliquot of medium, centrifuging it (200g, 5 minutes) and measuring LDH in the supernatant. At the end of the incubation the cells were lyzed by incubating them for one hour in Triton x-100 at final concentration of 1%. LDH activity in this lysate was taken as representing total (100%). The activity in the aliquots calculated as a percentage of the total amount was depicted as % LDH leakage for each time point. For measurement of NIR 2-DG uptake, IRDye 800CW 2-DG (NIR 2-DG) was added at a final concentration of 1µM to the medium containing the acini. These were then stimulated with GTL or saline. The reaction was terminated at 1 hour by putting cells on ice followed by 3 washes in ice cold HEPES buffer to remove unbound dye. The pellet was suspended in HEPES buffer and platted in triplicate in 96-well black plate. The plate was scanned at 800 nm for the targeted NIR 2-DG fluorescence signal (arbitrary units; AU) using the Odyssey Imaging System (Li-Cor) and analyzed using the Image Studio Software. Each condition in an experiment had a minimum of 3 replicates. Signals were averaged and the relative

fluorescence was determined by normalizing to protein levels (mg/ml) per well measured using the BCA Protein Assay Kit (Thermo Fisher Scientific). Ratios were calculated by normalizing to the saline treated control group. 4 or more experiments were performed for each parameter.

***In vivo* 2-DG uptake and AP studies:** In vivo 2-DG fluorescence imaging was performed with an IVIS Spectrum in vivo imaging system (PerkinElmer). All fluorescent images were acquired using identical illumination settings (EX/EM ICG filter, f/stop 2, field of views 22, binning 8), using 0.5 second of exposure time and analyzed using the Living Image® software. Fluorescence emission was normalized to photons per second per centimeter square per steradian and expressed as average radiant efficiency [p/s/cm²/sr] / [µW/cm²]. All NIR fluorescent images were displayed in the same scale of fluorescent intensity. At the day of experiments, rats under ketamine-Xylazine anesthesia had the fur overlying the abdomen and back completely shaved, the catheter patency checked and intravenous bolus injected with 100 µl of 2-DG (10 nanomoles) followed by 100 µl of saline to wash the catheter. Preliminary studies were done to study early intensity changes. In the abdomen, the only changes noted were rapid accumulation in the bladder after 1 minute in the supine position and after 10 minutes as a diffuse uptake in the posterior superior abdomen in the right lateral position. After 10 minutes of 2-DG signal stabilization, severe AP was induced by intra pancreatic duct injection of GTL (50µl/100 g of body weight) followed by ligating of the bilio-pancreatic duct just proximal to its entry into the duodenum (GTL group). Mild AP was induced by bilio-pancreatic duct ligation alone (DL group). Control groups just received 2-DG. For imaging, sedated rats were placed inside the 37^0C warmed IVIS chamber and images were taken every 5-10 min after NIR 2-DG administration before AP induction and over the 3 hours following this. When rats were euthanized and pancreas harvested for *ex vivo* imaging or gauging the severity of pancreatitis. Animals were treated in a humane fashion with close attention to pain control. This was via keeping them anesthetized with ketamine-Xylazine, (90mg/kg, 8 mg/kg respectively, repeated as needed when animal could be aroused by toe pinch) over the period imaging studies were

done and electively sacrificing them 1 or 3 hours post AP induction. The sedation prevented interference from movement artefact. For post-AP induction survival studies, animals were administered cefazolin (WG critical care, NJ, 100 mg/kg, intramuscular) for infection prevention, buprenorphine SR (ZooPharm, at 0.6 mg/Kg, s.c.) for pain management and normal saline (10ml, subcutaneously) immediately after AP induction and allowed to recover. These animals were monitored continuously for signs of distress. Those noted to become moribund after AP induction (between 5 and 8 hours in GTL group, median 6 hours) were sacrificed with carbon dioxide anesthesia. Animals were sacrificed electively at 8 hours in the DL group. Serum, tissue samples were collected to study severity. Blood and pancreas tissue were harvested to analyze plasma lipase, amylase, alanine aminotransferase (ALT), bilirubin, and pancreas tissue edema and ATP levels and for pancreatic necrosis analysis as described previously [14,22,32]. Blood glucose levels were measured before and after 2-DG injection. There were 8-12 rats in each group for all quantified data.

Biochemical assays: LDH (Roche Diagnostics, Indianapolis, IN), plasma Amylase, Lipase, ALT (Pointe Scientific Inc) were measured following the manufacturer's instructions [14,22,32] on the FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices) and total bilirubin (Pointe Scientific Inc) on the Eppendorf Biophotometer (Eppendorf). Blood glucose concentrations were measured by the glucose oxidase method using a FreeStyle glucose meter (Abbott Laboratories).

Pancreas water content: Pancreata was weighed on an analytic balance (wet weight) and dehydrated by heating at 37ºC overnight (dry weight). Water content was calculated according to the formula: [(wet weight - dry weight)/wet weight] x 100 and expressed in total weight percentage.

Pancreatic tissue ATP level determination: This was done as described previously [31,33]. A bioluminescent kit was used to measure ATP levels (Sigma-Aldrich, Saint Louis, MO) following manufacturer's instructions. Briefly, pancreatic tissue was disrupted in tri-chloroacetic acid and EDTA

containing buffer followed by appropriate dilution in Tris-EDTA buffer and application of a luminescent substrate. Luminescence was measured on a Promega Glomax 20/20 Luminometer and was normalized per milligram of protein determined using the BCA Protein Assay Kit (Thermo Fisher Scientific).

Pancreatic necrosis: Whole pancreas paraffin section (5 micron) slides stained by hematoxylin & eosin were examined by a trained morphologist (KP) blinded to the sample as described previously [14,22,31,32,33]. In brief, all pancreatic parenchymal area was imaged using the PathScan Enabler IV slide scanner (Meyer Instruments, Huston, TX) and images were evaluated for acinar necrosis as described previously[14,22,31,32,33]. Necrotic area and total acinar area were measured in pixels for each pancreas. Percentage necrosis was reported as a percentage of total area for each pancreas.

Statistical analysis: This was done using the SigmaStat statistical package integrated into the graphic program SigmaPlot 11 (Systat Software Inc, San Jose, CA). All values, unless otherwise specified, are reported as mean ± SEM. Groups were compared by one way analysis of variance (ANOVA) versus controls. Pairs were compared using a two tailed Students t-test when the distribution was normal (Shaprio-Wilk test) or a Mann-Whitney test when failing the Shapiro-Wilk test. Differences were considered significant at a p value <0.05

RESULTS:

NIR 2-DG uptake in the pancreas is not affected early in AP induction:

We started with intent to understand the dynamics of NIR 2-DG uptake into the pancreas and whether this could reliably discriminate between mild and severe AP. Since rats are commonly used in surgical models of biliary pancreatitis, we first chose to learn if NIR 2-DG could be reliably visualized in an area overlying the pancreas in 250-350 gram rats during the initial 3 hours over which AP progressively gets worse. The blood glucose in these rats after overnight fasting and prior to infusion of NIR 2-DG was

177±9.1 mg/dl and did not change over the duration of the study in controls (182±25 mg/dl). 3 groups were studied: controls, severe AP; i.e. rats with GTL infusion which develop severe pancreatic necrosis [14], and mild AP; with duct ligation alone who have a much milder course [15]. We chose a lateral position since the stomach overlies the pancreas and to avoid the confounding effect of a variable amount of food present in the stomach of rats despite fasting. A right side down position was chosen since the pancreas tail extends to the left side and thus would not be covered by the liver.

Prior to NIR 2-DG administration, there was no fluorescence detectable in any group of sedated rats (Fig 1A). NIR 2-DG administration without AP resulted in an initial generalized increase in fluorescence which was similar in all groups (Fig 1B, K). This synchronously increased over the pancreas (dashed polygon fig 1A-I) and plateaued by 30-40 minutes to values that were no different in the control (black dots Fig 1J, K), GTL and DL groups (red and green dots). This increase remained similar in all three groups for the hour following AP induction (Fig 1K) for a total of 90 minutes after NIR 2-DG administration. These results show that manipulation of the pancreas or induction of AP does not affect the initial uptake or equilibration of the NIR 2-DG.

NIR 2-DG is retained in the pancreas during severe AP:

We then analyzed the pattern of NIR 2-DG retention during the subsequent 2 hours. It was notable that during this time the control and DL groups progressively showed lesser retention of NIR 2-DG (Fig 1J). We then averaged the values for every 30 minute interval (i.e. time ± 15 minutes) and normalized it to the first 30 minute value when NIR 2-DG accumulation peaks or starts to plateau. As seen in figure 2A the NIR 2-DG uptake in the GTL group remained elevated and equivalent to the peak 30 minute value for the subsequent 2 hours. This was significantly elevated over controls after 90 minutes ($p<0.05$, *, on ANOVA) and higher than the DL group at or after 120 minutes ($P<0.03$, #, Students t-test). To compare the fold increase in average radiance efficiency (ARE) in an AP group over the controls at any particular

time point, the baseline ARE values i.e. those measured immediately after NIR-2DG administration (average 5 minutes) were subtracted from the ARE at that time point, and divided by the ARE change in controls. For e.g. the fold change in ARE at n minutes after NIR 2-DG in the GTL group was calculated as: {ARE in GTL at n min. - ARE in GTL at 5 min.} ÷ {ARE in control at n min. - ARE in control at 5 min.}. As can be seen in Figure 2B, this progressively increased, peaking at 3-14 folds between 2-3 hours of AP induction, while the increase in the DL group was insignificant. To verify that the increase was indeed in the pancreas, we removed the pancreas at 3 hours and measured the ARE overlying it. As seen in figure 2C and quantified in 2D, there was a large increase in the NIR-2DG accumulation in the pancreas of the GTL group compared to the DL group.

Higher retention of NIR 2-DG may be helpful in predicting severe AP early on in the disease: We then compared the uptake of NIR 2-DG to parameters of AP commonly measured in blood. As can be seen in figures 3A, B the parameters of AP induction i.e. plasma amylase and lipase were similar in the GTL and DL groups whether measured early or later in the disease course. Similarly the markers of a biliary etiology, i.e. plasma ALT and bilirubin were not higher in the in the GTL group. These findings correlate well with human data showing that criteria used to diagnose AP or its etiology have no bearing on its severity [35].

We then turned our attention to parameters of pancreatitis severity, focusing on local injury and mortality. Pancreatic necrosis in the GTL groups progressively increased from a median of 18% at 1 hour (Fig 4A-C), when the necrosis in DL group was insignificant to a median of 54% (range 40-70%) at 8 hours with associated 100% mortality (Fig 4D). Duct ligation alone caused mild pancreatic necrosis at the time of sacrifice i.e. 8 hours (median 10%), at which time the pancreas had a yellowish hue, consistent with bile staining induced by the duct ligation. The worsening necrosis in the GTL group compared to the DL

group correlates well with the fluorescence in the GTL group starting to increase between 60 and 90 minutes of AP induction (Figure 2A, B) and remaining increased over 3 hours. The *in vivo* findings of increased NIR 2-DG signal over the pancreas early in the course of AP were paralleled by increased NIR 2-DG binding to acinar cells within 1 hour of exposure to 300 micromolar GTL *in vitro* (Fig 5A, B). The fluorescence (arbitrary units/ well) /protein (mg/ml) was 54323 ± 18317 (AU/mg) in controls vs. 313265 ± 104180 AU/mg (p<0.01) in the GTL group. There was an insignificant increase in LDH leakage in the GTL group at this this point (4.6 ±1.7 vs. 1.8 ±0.4 In controls, p=0.12), followed by a large increase in LDH leakage over 4 hours (Fig 5C). It is notable that the increase in NIR 2-DG uptake was associated with a trend of decreasing pancreatic ATP levels in the GTL group which became significant only in the group that was moribund prior to sacrifice (Fig 6E).

DISCUSSION:

In this study we find that severe biliary AP is associated with an early and sustained retention of the near-infrared 2-DG probe (IRDye® 800CW 2-DG; NIR 2-DG; Li-Cor) in the pancreas in rats. This retention is associated with worse pancreatic necrosis and mortality. The study is unique for the following reasons: **1)** It uses a rapidly evolving inflammatory disease model which is unlike the much longer duration cancer [12,13,36] or chronic inflammation [5,37] models commonly employing 2-DG probes. **2)** it shows the feasibility of detecting NIR signals in large (250-350 gm) rats in contrast to mice which are commonly used for such imaging studies [12,13,38]. **3)** It shows that the increased retention of NIR 2-DG between 1 and 3 hours of AP induction has a stronger relation with severe outcomes than commonly used blood parameters used to determine AP severity in animal models **.**

Preliminary studies helped us decide the positioning of the rat and the timing of the imaging. While previous studies have shown a rapid first pass effect in mice with fluorescence overlying the kidneys to increase within 5 seconds of IRDye® 800CW 2-DG administration [36], we did not note such an increase

during the time fluorescence over the pancreas became intense (Fig 6A). We did note a rapid increase over the bladder to begin after the first minute and become intense by 10 minutes on imaging in the supine position. However we could not visualize the increase over the pancreas in this position, perhaps due to the stomach which overlies it. Placing the rat in a right lateral position allowed imaging the fluorescence over the pancreas (Fig 1A, dashed outline). The pancreatic signal was more cephalic and posterior than the bladder in the right lateral position. The right lateral position also resulted in the signal from the bladder to be largely masked by the pelvis and thigh, thus preventing it from interfering with the signal from the pancreas. In some cases we did note a distinct small signal from the bladder (seen in some images of figure 1A-I) which is anterior and inferior to the dashed outline corresponding to the signal from the pancreas. AP induction took 10-15 minutes and was executed within the 30 minutes that the signal peaked in controls (Fig 1K, 2A).This observation and the similarity in baseline glucose and other parameters between the 3 groups prior to AP induction prompted us to design the experiments to study 2 end points: **1)** whether AP induction affects the increase in signal from NIR 2-DG over the pancreas**. 2)** Whether the pattern of NIR 2-DG signal over time can be used to predict the severity of AP.

As seen in Fig 1, 2A, we note that AP induction does not interfere with the increase in ARE overlying the pancreas, which is similar in the controls and AP groups immediately after induction. After 30 minutes the trend changes with controls depicting a clear decline, whereas the GTL group shows a sustained increase over the course of the 3 hours these animals were imaged. The generalized increase over the posterior abdomen corresponding to where the kidney, pancreas, duodenum are located is at least partly contributed by the duodenum irrespective of AP as shown in the white outlines (Fig 6B, C). While bowel fluorescence could be easily excluded *ex-vivo* after removing the pancreas (Fig 2C, D) this could not be done *in vivo* since the imaging was planar. This uptake by the bowel likely contributed to the *in*

vivo background signal in controls, making the signal from the GTL group comparatively less impressive. It remains to be seen whether such interference can be reduced by cross sectional imaging.

Lipolysis of GTL by pancreatic lipases results in the release of the unsaturated fatty acid linoleic acid. Human pancreatic necrosis collections have high concentrations of linoleic acid [14,31,39], which can result in necrotic cell death [40,41] via inhibition of mitochondrial complexes I and V [22]. This would also be consistent with the decrease in ATP levels we note in the damaged pancreas (Fig 6E). The amounts of GTL used in this study are less than the adipocyte mass in the obese human pancreas [14,22,23,24,25,26]. Further proof validating the GTL model as being representative of human severe biliary AP is discussed in the manuscript in which this was originally described[14]. Additionally, GTL was preferred over the bile salt infusion model since the concentrations of bile salts used (20-100mM) are >10 times their critical micellar concentrations and thus have a detergent effect [42,43] on cell membranes. Moreover these high bile acid concentrations are in stark contrast to those in human pancreatic necrosis collections which are < 200 micromolar (Data not shown, separate manuscript on this issue is under review).

There are several factors that could be associated with increased NIR 2-DG retention during the progression of SAP. These include increased vascular permeability [44]measured as an increase in percentage water content of the pancreas during pancreatitis (Fig 6D), hemorrhage into the pancreas resulting in persistent NIR 2-DG accumulation, reduced clearance of NIR 2-DG in the GTL group, and hypoxia resulting in increased glucose requirement [5]. We have previously shown that lipotoxicity from unsaturated fatty acids such as linoleic acid causes a reduction in ATP levels associated with acinar necrosis [22,33], which would be consistent with the large increase in necrosis (Fig 4A-C) and drop in ATP levels we note in GTL group (Fig 6E). These in combination with hypoxia could increase glycolysis dependence of the GTL treated pancreas and thus result in the increased NIR 2-DG uptake we note. The

contribution of vascular permeability to the increased *in vivo* signal noted in the GTL group while possible is unlikely, since the fluorescence increase during pancreatitis *in vivo* (Fig 2) is similar to that in acini exposed to GTL *in vitro* (Fig 5A, B). Similarly, the increase in NIR 2-DG signal noted *ex-vivo* in the GTL group did not correlate with the amount or location of hemorrhages (Fig 2C, 6B, C), and while hemorrhages were sometimes noted within an hour of GTL injection (Fig 4C), these did not result in an increased of NIR 2-DG signal at 1 hour (Fig 1 J, K). These observations along with the *in vitro* findings in acini mentioned above make the contribution of hemorrhage to the *in vivo* NIR 2-DG signal also unlikely. The possibility of variable clearance in affecting the NIR 2-DG signal is also unlikely since all animals were imaged at 5-10 minute intervals after its administration, and the signal increase was similar in all groups up to 90 minutes after administration. A selective reduction in clearance of NiR 2-DG the GTL group should have resulted in a generalized increase in NIR signal, but the increase noted *ex-vivo* was only over the pancreas and not the duodenum or spleen (Fig 2C, 6B, C). We have not looked at the contribution of increased expression of glucose transporters, hexokinase or the direct effect of inflammatory mediators to the increased NIR 2-DG retention noted in the GTL group. However the *in vitro* data in isolated acinar cells showing increased NIR 2-DG retention within an hour of GTL exposure, in the absence of exogenous inflammatory mediators (Fig 5 A, B) makes the contribution of these to the *in vivo* signal unlikely.

These studies make a case that measuring the increase in metabolic demand of the pancreas helps predict the subsequent severity of local injury. This is important since most clinical cases of AP are mild, and currently there are no reliable early predictors to distinguish these mild ones from the ones that subsequently develop severe necrosis. In the clinical setting necrosis typically develops after the first several days of AP, and an early predictor of necrosis may allow early changes in management and help focus therapeutic approaches to patients with predicted severe AP.

In summary, the current studies show increased and persistent accumulation of the near-infrared 2-DG probe, IRDye® 800CW 2-DG, over the pancreas during the initial hours of AP to be a more reliable predictor of severity than conventional serum markers of AP. This binding of the 2-DG probe precedes the drop in ATP levels and necrosis that eventually results in severe AP. Based on the utility demonstrated in this rapidly evolving inflammatory disease model in rats, this study opens the scope of imaging 2-DG analogs as early predictors of inflammatory disease severity in humans.

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LEGENDS:

Figure 1: NIR 2-DG signal increase over the pancreas is unaffected by AP induction, but persists in severe AP unlike in mild AP or controls: A-I : Series of

thoracic and abdominal images in the right lateral position acquired after different times in minutes (m) after administration of NIR 2-DF (2-DG) in controls, or after AP induction with duct ligation (DL) or GTL administration(GTL). The dashed polygon denotes the region of interest overlying the pancreas in which the fluorescence was measured. **J:** Average radiant efficiency as measured over time from when NIR 2-DG was administered in controls (Con, black circles), and in the duct ligation (DL, green circles) and GTL groups (red circles). There were 9-12 animals per group. * indicate the values that were significantly different from the corresponding controls at that time **K:** An enlarged view of the first 90 minutes showing the time when acute pancreatitis was induced.

Figure 2: Severe AP is associated with significantly more retention of NIR 2-DG in the pancreas early during AP compared to controls: A: Time course showing means of

the Average radiant efficiency for the values obtained ±15 minutes for each time point within each group (i.e. control; Con, duct ligation; DL, and GTL) after normalizing to the 30 minute peak (100%) for that group. * depicts a significant ($p<0.05$) increase in the GTL vs. control and # depicts significance vs. DL on ANOVA at each time point. **B:** Time course showing Average radiant efficiency (ARE) normalized to controls. The initial mean AREs for each group prior to AP induction, were subtracted from those at each time point. The difference in the DL or GTL pancreatitis groups was divided by the corresponding difference in the control group, and this ratio is shown as fold change over control. Please note, that with the decline in the ARE values in controls over time (A), there is a significant increase in this ratio in the GTL group vs. DL group (#). There were 9-12 animals per group. **C:** Fluorescence images (upper

panel) and gross appearance (Lower panel) of the pancreas *ex vivo* in the control, DL and GTL groups. **D:** Bar graphs showing the quantification of the ARE for each group. "*" depicts a significant ($p<0.05$) increase in the GTL vs. control and "#" depicts significance vs. DL on ANOVA.

Figure 3: The increase of AP parameters in the blood is similar in both mild and severe disease. Time course of the activities of amylase (A), Lipase (B), alanine amino transferase (ALT; C) and amount of bilirubin (D) measured in heparinized plasma samples after 1, 3 hours of AP induction, at euthanasia (≤ 8 hours), and as baseline control value without the procedure (0 Hours). Values at 1 hour, 3 hours and at the time of euthanasia for each group (DL- green, GTL-red) were compared to each other and the controls by ANOVA. "*" depicts a significant ($p<0.05$) increase vs. control and "#" depicts significance in GTL vs. DL. There were 8-12 animals per group.

Figure 4: Severe AP is associated with a progressive increase in necrosis and mortality: A: time course of percentage of pancreatic parenchyma what was necrotic in the GTL (red) and DL (green) groups measured when electively sacrificed at 1, 3 hours, at euthanasia (≤ 8 hours). Values at 1 hour, 3 hours and at the time of euthanasia for each group were compared to each other and the controls by ANOVA. "*" depicts a significant ($p<0.05$) increase vs. control and "#" depicts significant differences in GTL vs. DL. Representative microscopic (5x, B) and gross (C) images are shown. D: Kaplan-Meyer curve showing % survival in the different study groups. There was 100% mortality in the GTL group by 8 hours, but none in the duct ligation group. There were 8-12 animals per group. Scale bars are 1mm in length.

Figure 5: NIR 2-DG uptake *in vitro* occurs before the induction of GTL induced injury: Pancreatic acinar cells were exposed to 300 μM GTL and binding of NIR 2-DG (A) was measured at 1 hour, with results being depicted as fold control (arbitrary units fluorescence/mg protein). B shows

an image of the bound fluorescence in control and GTL exposed acini as visualized on the Odyssey Imaging System. C: shows the time course of LDH leakage induced by 300 μM GTL (red) vs. controls (black). The "*" depict $p<0.01$ vs. corresponding controls. Note, that the increased NIR 2-DG binding noted at 1 hour precedes LDH leakage. Data is representative of a minimum of 4 experiments.

Figure 6: Parameters potentially affecting the NIR 2-DG signal measured in our system. A: Images of a rat administered NIR 2-DG collected on the IVIS imaging system in the supine position over the first 10 minutes (First five images) and in the right lateral position (last image). Note the increase over the bladder but the lack of signal over the pancreas in the supine position. This pattern is reversed in the right lateral position. Fluorescent (B) and black and white(C) Images of the duodenum, pancreas and spleen removed *en block* after 2 hours of NIR 2-DG administration in controls or pancreatitis in the DL and GTL groups. Please note an increased uptake in the duodenum (white outlines) especially in controls. **D:** Data from groups of 8-12 animals showing the time course of the increase in pancreatic edema (measured as % water content or pancreatic ATP concentrations (**E**) in the DL and GTL groups. The "*" depict $p<0.05$ vs. corresponding controls "#" depicts significant differences in GTL vs. DL.

Characterization and predictive value of near infrared 2-deoxyglucose optical imaging in severe acute pancreatitis

Cristiane de Oliveira[1], Krutika Patel[1], Vivek Mishra[2], Ram N. Trivedi[1], Pawan Noel[1], Abhilasha Singh[1], Jordan R. Yaron[1], Vijay P. Singh[1]

[1]Department of Medicine, Mayo Clinic, Scottsdale, AZ, [2]Department of Medicine, University of Pittsburgh, Pittsburgh, PA.

Corresponding author:
Vijay P Singh. MD,
Associate Professor,
3rd floor Collaborative Research Building,
Mayo Clinic Arizona,
13400 Shea Boulevard, Scottsdale, AZ, 85259.
Phone: 480-301-4286
Fax: 480-301-7017

ABSTRACT:

Background: studying the uptake of 2-deoxy glucose (2-DG) analogs such as 2-Deoxy-2-[18F] fluoroglucose (FDG) is a common approach to identify and monitor malignancies and more recently chronic inflammation. While pancreatitis is a common cause for false positive results in human studies on pancreatic cancer using FDG, the relevance of these findings to acute pancreatitis (AP) is unknown. FDG has a short half-life. Thus, with an aim to accurately characterize the metabolic demand of the pancreas during AP in real-time, we studied the uptake of the non-radioactive, near infrared fluorescence labelled 2-deoxyglucose analog, IRDye® 800CW 2-DG probe (NIR 2-DG; Li-Cor) during mild and severe biliary AP. **Methods:** Wistar rats (300g; 8-12/group) were administered NIR 2-DG (10 nanomoles; I.V.). Mild and severe biliary AP were respectively induced by biliopancreatic duct ligation (DL) alone or along with infusing glyceryl trilinoleate (GTL; 50µL/100gm) within 10 minutes of giving NIR 2-DG. Controls (CON) only received NIR 2-DG. Imaging was done every 5-10 minutes over 3 hrs. Average Radiant Efficiency [p/s/cm²/sr]/[µW/cm²] was measured over the pancreas using the IVIS 200 *in-vivo* imaging system (PerkinElmer) using the Living Image® software and verified in *ex vivo* pancreata. Blood amylase, lipase and pancreatic edema, necrosis were measured over the course of AP. **Results:** NIR 2-DG uptake over the first hour was not influenced by AP induction. However while the signal declined in controls and rats with mild AP, there was significantly higher retention of NIR 2-DG in the pancreas after 1 hour in those with GTL pancreatitis. The increase was > 3 fold over controls in the GTL group and was verified to be in the pancreas *ex vivo*. *In vitro*, pancreatic acini exposed to GTL had a similar increase in NIR 2-DG uptake which was followed by progressively worse acinar necrosis. Greater retention of NIR 2-DG *in vivo* was associated with worse pancreatic necrosis, reduced ATP concentrations and mortality, which were not predicted by the blood parameters. **Conclusion:** *In-vivo* fluorescent imaging of a non-radioactive near infrared 2-DG optical probe can predict the AP severity early during the disease.

BACKGROUND:

While the utility of 2-deoxy glucose (2-DG) analogs is established in diagnosis and prognostication of malignant diseases, the relevance of such studies in inflammatory diseases is emerging [1,2,3,4,5]. Acute Inflammatory diseases such as acute pancreatitis (AP) have a sudden onset with a rapid, variable and unpredictable course ranging from resolution with minimal care over a few days, to a severe course (severe acute pancreatitis; SAP) progressing to extensive pancreatic necrosis, requiring intensive care, a prolonged hospitalization with high costs and sometimes resulting in death. However we currently lack tools which can reliably predict the course of AP takes early on in the disease.

There are reports of increased 2-Deoxy-2-[18F] fluoroglucose (FDG) uptake in human pancreatitis detected by positron emission tomography (PET) imaging [6,7,8,9,10,11]. These are typically done with intent to study pancreatic cancer, and pancreatitis is an incidental finding contributing to the “false positives” seen. However, the relevance of the uptake of 2-DG tracers in predicting the AP severity early in the course of the disease is unknown and may need further exploration. Such evidence, if present in preclinical studies, can potentially guide studies in human pancreatitis.

The current project was designed to explore the behavior of, and study whether a 2-DG probe can be used to predict the severity of AP early in its course. For this we chose the non-radioactive, near-infrared probe (IRDye® 800CW 2-DG; NIR 2-DG; Li-Cor) and studied its uptake into the pancreas during mild AP and SAP. NIR 2-DG has been previously used as a 2-DG mimetic for both *in vivo* and *in vitro* studies [12,13]. Compared to FDG it offers the additional advantage of not being limited by a short half-life, and since it is non-radioactive, it is amenable to high throughput imaging requiring less specialized instrumentation. The emission spectra of NIR probes range between 650-900 nm which allows for their *in vivo* use without interference by either absorbance or non-specific emission from neighboring tissue.

The severe and mild AP models used in this study were chosen based on their relevance to human disease [14]. The mild model involves bilio-pancreatic duct ligation, which is sufficient to fulfil the criteria of biliary acute pancreatitis, but does not result in severe necrosis as shown recently [15], this is similar to mild biliary pancreatitis as may occur in humans due to a mass in the head of the pancreas causing duct obstruction. The severe model simulates severe biliary pancreatitis complicated by fat necrosis, as may occur in humans [16,17,18,19,20,21].In this, the triglyceride precursor of the second most abundant unsaturated fatty acid in pancreatic fat (linoleic acid), i.e. glyceryl trilinoleate (GTL) is infused into the pancreatic duct prior to the ligation. The amount of GTL is equivalent or lower than the percentage of adipocytes in the obese human pancreas [14,22,23,24,25,26]. Using the above tools we set out to study whether the course of NIR 2-DG uptake in the pancreas in controls, mild and severe AP can be used to predict AP outcomes.

MATERIALS AND METHODS

Reagents: IRDye 800CW 2-DG (NIR 2-DG, Li-Cor Biosciences) was dissolved in 1 ml sterile saline at a final concentration of 100 nM. Glyceryl trilinoleate (GTL; TCI America) for in vitro studies was dissolved and sonicated in HEPES containing buffer; for *in vivo* studies was directly injected into the pancreatic duct of the rats. Ketamine Hydrochloride (Ketaset, Fort Dodge Animal health), Xylazine (Anased, Lloyd Laboratories) and Isoflurane USP (Piramal Healthcare).

Animal work: All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (Pittsburgh, PA), the Mayo Clinic (Scottsdale, AZ) and the Animal Care and Use Review Office (ACURO), a component of the USAMRMC Office of Research Protections. Mice: 6-8 week old male CD-1/ICR mice (Charles River Laboratories, Wilmington, MA) housed with a 12-h light/dark cycle at temperatures ranging from 21-25°C, were fed standard laboratory

chow and allowed to drink ad libitum. Rats: 250-350g male Wistar rats (Charles Rivers Laboratories, Wilmington, MA) were housed with a 12 hour light-dark cycle, fed standard laboratory chow and were allowed to drink ad libitum. A Jugular venous catheter was placed in all rats under anesthesia (ketamine-Xylazine, 90mg/kg, 8 mg/kg respectively, IP; or isoflurane, 3% + 0.8-1 L/min induction; 1.5% + 0.8-1 L/min maintenance). The rats were left to recover for at least 3 days prior experiments. Catheters were flushed with 4U/ mL heparin/saline solution every other day and the patency of catheter verified.

Acinar Harvest and 2-DG *in vitro assay*: For NIR 2-DG *in vitro* studies, pancreatic acini were harvested from mice and used in oxygenated HEPES buffer pH 7.4 as described previously [27,28,29]. Viability was confirmed by trypan blue exclusion (>95%) at the start of each experiment. All studies were done in a shaking water bath (80 RPM) at 37°C at an ambient atmosphere. Cells were treated with either glyceryl trilinoleate (GTL, 300 µM) or saline as a control. Cell injury was quantified by measuring LDH leakage (Roche Diagnostics, Indianapolis, IN) into the medium as described previously [22,30,31]. This was done at 0, 1, 2, 4 hours by taking a 2% (40 µl out of 2ml) aliquot of medium, centrifuging it (200g, 5 minutes) and measuring LDH in the supernatant. At the end of the incubation the cells were lyzed by incubating them for one hour in Triton x-100 at final concentration of 1%. LDH activity in this lysate was taken as representing total (100%). The activity in the aliquots calculated as a percentage of the total amount was depicted as % LDH leakage for each time point. For measurement of NIR 2-DG uptake, IRDye 800CW 2-DG (NIR 2-DG) was added at a final concentration of 1µM to the medium containing the acini. These were then stimulated with GTL or saline. The reaction was terminated at 1 hour by putting cells on ice followed by 3 washes in ice cold HEPES buffer to remove unbound dye. The pellet was suspended in HEPES buffer and platted in triplicate in 96-well black plate. The plate was scanned at 800 nm for the targeted NIR 2-DG fluorescence signal (arbitrary units; AU) using the Odyssey Imaging System (Li-Cor) and analyzed using the Image Studio Software. Each condition in an experiment had a minimum of 3 replicates. Signals were averaged and the relative

fluorescence was determined by normalizing to protein levels (mg/ml) per well measured using the BCA Protein Assay Kit (Thermo Fisher Scientific). Ratios were calculated by normalizing to the saline treated control group. 4 or more experiments were performed for each parameter.

***In vivo* 2-DG uptake and AP studies:** In vivo 2-DG fluorescence imaging was performed with an IVIS Spectrum in vivo imaging system (PerkinElmer). All fluorescent images were acquired using identical illumination settings (EX/EM ICG filter, f/stop 2, field of views 22, binning 8), using 0.5 second of exposure time and analyzed using the Living Image® software. Fluorescence emission was normalized to photons per second per centimeter square per steradian and expressed as average radiant efficiency [p/s/cm²/sr] / [μW/cm²]. All NIR fluorescent images were displayed in the same scale of fluorescent intensity. At the day of experiments, rats under ketamine-Xylazine anesthesia had the fur overlying the abdomen and back completely shaved, the catheter patency checked and intravenous bolus injected with 100 μl of 2-DG (10 nanomoles) followed by 100 μl of saline to wash the catheter. Preliminary studies were done to study early intensity changes. In the abdomen, the only changes noted were rapid accumulation in the bladder after 1 minute in the supine position and after 10 minutes as a diffuse uptake in the posterior superior abdomen in the right lateral position. After 10 minutes of 2-DG signal stabilization, severe AP was induced by intra pancreatic duct injection of GTL (50μl/100 g of body weight) followed by ligating of the bilio-pancreatic duct just proximal to its entry into the duodenum (GTL group). Mild AP was induced by bilio-pancreatic duct ligation alone (DL group). Control groups just received 2-DG. For imaging, sedated rats were placed inside the 37^0C warmed IVIS chamber and images were taken every 5-10 min after NIR 2-DG administration before AP induction and over the 3 hours following this. When rats were euthanized and pancreas harvested for *ex vivo* imaging or gauging the severity of pancreatitis. Animals were treated in a humane fashion with close attention to pain control. This was via keeping them anesthetized with ketamine-Xylazine, (90mg/kg, 8 mg/kg respectively, repeated as needed when animal could be aroused by toe pinch) over the period imaging studies were

done and electively sacrificing them 1 or 3 hours post AP induction. The sedation prevented interference from movement artefact. For post-AP induction survival studies, animals were administered cefazolin (WG critical care, NJ, 100 mg/kg, intramuscular) for infection prevention, buprenorphine SR (ZooPharm, at 0.6 mg/Kg, s.c.) for pain management and normal saline (10ml, subcutaneously) immediately after AP induction and allowed to recover. These animals were monitored continuously for signs of distress. Those noted to become moribund after AP induction (between 5 and 8 hours in GTL group, median 6 hours) were sacrificed with carbon dioxide anesthesia. Animals were sacrificed electively at 8 hours in the DL group. Serum, tissue samples were collected to study severity. Blood and pancreas tissue were harvested to analyze plasma lipase, amylase, alanine aminotransferase (ALT), bilirubin, and pancreas tissue edema and ATP levels and for pancreatic necrosis analysis as described previously [14,22,32]. Blood glucose levels were measured before and after 2-DG injection. There were 8-12 rats in each group for all quantified data.

Biochemical assays: LDH (Roche Diagnostics, Indianapolis, IN), plasma Amylase, Lipase, ALT (Pointe Scientific Inc) were measured following the manufacturer's instructions [14,22,32] on the FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices) and total bilirubin (Pointe Scientific Inc) on the Eppendorf Biophotometer (Eppendorf). Blood glucose concentrations were measured by the glucose oxidase method using a FreeStyle glucose meter (Abbott Laboratories).

Pancreas water content: Pancreata was weighed on an analytic balance (wet weight) and dehydrated by heating at 37ºC overnight (dry weight). Water content was calculated according to the formula: [(wet weight - dry weight)/wet weight] x 100 and expressed in total weight percentage.

Pancreatic tissue ATP level determination: This was done as described previously [31,33]. A bioluminescent kit was used to measure ATP levels (Sigma-Aldrich, Saint Louis, MO) following manufacturer's instructions. Briefly, pancreatic tissue was disrupted in tri-chloroacetic acid and EDTA

containing buffer followed by appropriate dilution in Tris-EDTA buffer and application of a luminescent substrate. Luminescence was measured on a Promega Glomax 20/20 Luminometer and was normalized per milligram of protein determined using the BCA Protein Assay Kit (Thermo Fisher Scientific).

Pancreatic necrosis: Whole pancreas paraffin section (5 micron) slides stained by hematoxylin & eosin were examined by a trained morphologist (KP) blinded to the sample as described previously [14,22,31,32,33]. In brief, all pancreatic parenchymal area was imaged using the PathScan Enabler IV slide scanner (Meyer Instruments, Huston, TX) and images were evaluated for acinar necrosis as described previously[14,22,31,32,33]. Necrotic area and total acinar area were measured in pixels for each pancreas. Percentage necrosis was reported as a percentage of total area for each pancreas.

Statistical analysis: This was done using the SigmaStat statistical package integrated into the graphic program SigmaPlot 11 (Systat Software Inc, San Jose, CA). All values, unless otherwise specified, are reported as mean ± SEM. Groups were compared by one way analysis of variance (ANOVA) versus controls. Pairs were compared using a two tailed Students t-test when the distribution was normal (Shaprio-Wilk test) or a Mann-Whitney test when failing the Shapiro-Wilk test. Differences were considered significant at a p value <0.05

RESULTS:

NIR 2-DG uptake in the pancreas is not affected early in AP induction:

We started with intent to understand the dynamics of NIR 2-DG uptake into the pancreas and whether this could reliably discriminate between mild and severe AP. Since rats are commonly used in surgical models of biliary pancreatitis, we first chose to learn if NIR 2-DG could be reliably visualized in an area overlying the pancreas in 250-350 gram rats during the initial 3 hours over which AP progressively gets worse. The blood glucose in these rats after overnight fasting and prior to infusion of NIR 2-DG was

177±9.1 mg/dl and did not change over the duration of the study in controls (182±25 mg/dl). 3 groups were studied: controls, severe AP; i.e. rats with GTL infusion which develop severe pancreatic necrosis [14], and mild AP; with duct ligation alone who have a much milder course [15]. We chose a lateral position since the stomach overlies the pancreas and to avoid the confounding effect of a variable amount of food present in the stomach of rats despite fasting. A right side down position was chosen since the pancreas tail extends to the left side and thus would not be covered by the liver.

Prior to NIR 2-DG administration, there was no fluorescence detectable in any group of sedated rats (Fig 1A). NIR 2-DG administration without AP resulted in an initial generalized increase in fluorescence which was similar in all groups (Fig 1B, K). This synchronously increased over the pancreas (dashed polygon fig 1A-I) and plateaued by 30-40 minutes to values that were no different in the control (black dots Fig 1J, K), GTL and DL groups (red and green dots). This increase remained similar in all three groups for the hour following AP induction (Fig 1K) for a total of 90 minutes after NIR 2-DG administration. These results show that manipulation of the pancreas or induction of AP does not affect the initial uptake or equilibration of the NIR 2-DG.

NIR 2-DG is retained in the pancreas during severe AP:

We then analyzed the pattern of NIR 2-DG retention during the subsequent 2 hours. It was notable that during this time the control and DL groups progressively showed lesser retention of NIR 2-DG (Fig 1J). We then averaged the values for every 30 minute interval (i.e. time ± 15 minutes) and normalized it to the first 30 minute value when NIR 2-DG accumulation peaks or starts to plateau. As seen in figure 2A the NIR 2-DG uptake in the GTL group remained elevated and equivalent to the peak 30 minute value for the subsequent 2 hours. This was significantly elevated over controls after 90 minutes ($p<0.05$, *, on ANOVA) and higher than the DL group at or after 120 minutes ($P<0.03$, #, Students t-test). To compare the fold increase in average radiance efficiency (ARE) in an AP group over the controls at any particular

time point, the baseline ARE values i.e. those measured immediately after NIR-2DG administration (average 5 minutes) were subtracted from the ARE at that time point, and divided by the ARE change in controls. For e.g. the fold change in ARE at n minutes after NIR 2-DG in the GTL group was calculated as: {ARE in GTL at n min. - ARE in GTL at 5 min.} ÷ {ARE in control at n min. - ARE in control at 5 min.}.
As can be seen in Figure 2B, this progressively increased, peaking at 3-14 folds between 2-3 hours of AP induction, while the increase in the DL group was insignificant. To verify that the increase was indeed in the pancreas, we removed the pancreas at 3 hours and measured the ARE overlying it. As seen in figure 2C and quantified in 2D, there was a large increase in the NIR-2DG accumulation in the pancreas of the GTL group compared to the DL group.

Higher retention of NIR 2-DG may be helpful in predicting severe AP early on in the disease: We then compared the uptake of NIR 2-DG to parameters of AP commonly measured in blood. As can be seen in figures 3A, B the parameters of AP induction i.e. plasma amylase and lipase were similar in the GTL and DL groups whether measured early or later in the disease course. Similarly the markers of a biliary etiology, i.e. plasma ALT and bilirubin were not higher in the in the GTL group. These findings correlate well with human data showing that criteria used to diagnose AP or its etiology have no bearing on its severity [35].

We then turned our attention to parameters of pancreatitis severity, focusing on local injury and mortality. Pancreatic necrosis in the GTL groups progressively increased from a median of 18% at 1 hour (Fig 4A-C), when the necrosis in DL group was insignificant to a median of 54% (range 40-70%) at 8 hours with associated 100% mortality (Fig 4D). Duct ligation alone caused mild pancreatic necrosis at the time of sacrifice i.e. 8 hours (median 10%), at which time the pancreas had a yellowish hue, consistent with bile staining induced by the duct ligation. The worsening necrosis in the GTL group compared to the DL

group correlates well with the fluorescence in the GTL group starting to increase between 60 and 90 minutes of AP induction (Figure 2A, B) and remaining increased over 3 hours. The *in vivo* findings of increased NIR 2-DG signal over the pancreas early in the course of AP were paralleled by increased NIR 2-DG binding to acinar cells within 1 hour of exposure to 300 micromolar GTL *in vitro* (Fig 5A, B). The fluorescence (arbitrary units/ well) /protein (mg/ml) was 54323 ± 18317 (AU/mg) in controls vs. 313265 ± 104180 AU/mg (p<0.01) in the GTL group. There was an insignificant increase in LDH leakage in the GTL group at this this point (4.6 ±1.7 vs. 1.8 ±0.4 In controls, p=0.12), followed by a large increase in LDH leakage over 4 hours (Fig 5C). It is notable that the increase in NIR 2-DG uptake was associated with a trend of decreasing pancreatic ATP levels in the GTL group which became significant only in the group that was moribund prior to sacrifice (Fig 6E).

DISCUSSION:

In this study we find that severe biliary AP is associated with an early and sustained retention of the near-infrared 2-DG probe (IRDye® 800CW 2-DG; NIR 2-DG; Li-Cor) in the pancreas in rats. This retention is associated with worse pancreatic necrosis and mortality. The study is unique for the following reasons: **1)** It uses a rapidly evolving inflammatory disease model which is unlike the much longer duration cancer [12,13,36] or chronic inflammation [5,37] models commonly employing 2-DG probes. **2)** it shows the feasibility of detecting NIR signals in large (250-350 gm) rats in contrast to mice which are commonly used for such imaging studies [12,13,38]. **3)** It shows that the increased retention of NIR 2-DG between 1 and 3 hours of AP induction has a stronger relation with severe outcomes than commonly used blood parameters used to determine AP severity in animal models **.**

Preliminary studies helped us decide the positioning of the rat and the timing of the imaging. While previous studies have shown a rapid first pass effect in mice with fluorescence overlying the kidneys to increase within 5 seconds of IRDye® 800CW 2-DG administration [36], we did not note such an increase

during the time fluorescence over the pancreas became intense (Fig 6A). We did note a rapid increase over the bladder to begin after the first minute and become intense by 10 minutes on imaging in the supine position. However we could not visualize the increase over the pancreas in this position, perhaps due to the stomach which overlies it. Placing the rat in a right lateral position allowed imaging the fluorescence over the pancreas (Fig 1A, dashed outline). The pancreatic signal was more cephalic and posterior than the bladder in the right lateral position. The right lateral position also resulted in the signal from the bladder to be largely masked by the pelvis and thigh, thus preventing it from interfering with the signal from the pancreas. In some cases we did note a distinct small signal from the bladder (seen in some images of figure 1A-I) which is anterior and inferior to the dashed outline corresponding to the signal from the pancreas. AP induction took 10-15 minutes and was executed within the 30 minutes that the signal peaked in controls (Fig 1K, 2A).This observation and the similarity in baseline glucose and other parameters between the 3 groups prior to AP induction prompted us to design the experiments to study 2 end points: **1)** whether AP induction affects the increase in signal from NIR 2-DG over the pancreas**. 2)** Whether the pattern of NIR 2-DG signal over time can be used to predict the severity of AP.

As seen in Fig 1, 2A, we note that AP induction does not interfere with the increase in ARE overlying the pancreas, which is similar in the controls and AP groups immediately after induction. After 30 minutes the trend changes with controls depicting a clear decline, whereas the GTL group shows a sustained increase over the course of the 3 hours these animals were imaged. The generalized increase over the posterior abdomen corresponding to where the kidney, pancreas, duodenum are located is at least partly contributed by the duodenum irrespective of AP as shown in the white outlines (Fig 6B, C). While bowel fluorescence could be easily excluded *ex-vivo* after removing the pancreas (Fig 2C, D) this could not be done *in vivo* since the imaging was planar. This uptake by the bowel likely contributed to the *in*

vivo background signal in controls, making the signal from the GTL group comparatively less impressive. It remains to be seen whether such interference can be reduced by cross sectional imaging.

Lipolysis of GTL by pancreatic lipases results in the release of the unsaturated fatty acid linoleic acid. Human pancreatic necrosis collections have high concentrations of linoleic acid [14,31,39], which can result in necrotic cell death [40,41] via inhibition of mitochondrial complexes I and V [22]. This would also be consistent with the decrease in ATP levels we note in the damaged pancreas (Fig 6E). The amounts of GTL used in this study are less than the adipocyte mass in the obese human pancreas [14,22,23,24,25,26]. Further proof validating the GTL model as being representative of human severe biliary AP is discussed in the manuscript in which this was originally described[14]. Additionally, GTL was preferred over the bile salt infusion model since the concentrations of bile salts used (20-100mM) are >10 times their critical micellar concentrations and thus have a detergent effect [42,43] on cell membranes. Moreover these high bile acid concentrations are in stark contrast to those in human pancreatic necrosis collections which are < 200 micromolar (Data not shown, separate manuscript on this issue is under review).

There are several factors that could be associated with increased NIR 2-DG retention during the progression of SAP. These include increased vascular permeability [44]measured as an increase in percentage water content of the pancreas during pancreatitis (Fig 6D), hemorrhage into the pancreas resulting in persistent NIR 2-DG accumulation, reduced clearance of NIR 2-DG in the GTL group, and hypoxia resulting in increased glucose requirement [5]. We have previously shown that lipotoxicity from unsaturated fatty acids such as linoleic acid causes a reduction in ATP levels associated with acinar necrosis [22,33], which would be consistent with the large increase in necrosis (Fig 4A-C) and drop in ATP levels we note in GTL group (Fig 6E). These in combination with hypoxia could increase glycolysis dependence of the GTL treated pancreas and thus result in the increased NIR 2-DG uptake we note. The

contribution of vascular permeability to the increased *in vivo* signal noted in the GTL group while possible is unlikely, since the fluorescence increase during pancreatitis *in vivo* (Fig 2) is similar to that in acini exposed to GTL *in vitro* (Fig 5A, B). Similarly, the increase in NIR 2-DG signal noted *ex-vivo* in the GTL group did not correlate with the amount or location of hemorrhages (Fig 2C, 6B, C), and while hemorrhages were sometimes noted within an hour of GTL injection (Fig 4C), these did not result in an increased of NIR 2-DG signal at 1 hour (Fig 1 J, K). These observations along with the *in vitro* findings in acini mentioned above make the contribution of hemorrhage to the *in vivo* NIR 2-DG signal also unlikely. The possibility of variable clearance in affecting the NIR 2-DG signal is also unlikely since all animals were imaged at 5-10 minute intervals after its administration, and the signal increase was similar in all groups up to 90 minutes after administration. A selective reduction in clearance of NiR 2-DG the GTL group should have resulted in a generalized increase in NIR signal, but the increase noted *ex-vivo* was only over the pancreas and not the duodenum or spleen (Fig 2C, 6B, C). We have not looked at the contribution of increased expression of glucose transporters, hexokinase or the direct effect of inflammatory mediators to the increased NIR 2-DG retention noted in the GTL group. However the *in vitro* data in isolated acinar cells showing increased NIR 2-DG retention within an hour of GTL exposure, in the absence of exogenous inflammatory mediators (Fig 5 A, B) makes the contribution of these to the *in vivo* signal unlikely.

These studies make a case that measuring the increase in metabolic demand of the pancreas helps predict the subsequent severity of local injury. This is important since most clinical cases of AP are mild, and currently there are no reliable early predictors to distinguish these mild ones from the ones that subsequently develop severe necrosis. In the clinical setting necrosis typically develops after the first several days of AP, and an early predictor of necrosis may allow early changes in management and help focus therapeutic approaches to patients with predicted severe AP.

In summary, the current studies show increased and persistent accumulation of the near-infrared 2-DG probe, IRDye® 800CW 2-DG, over the pancreas during the initial hours of AP to be a more reliable predictor of severity than conventional serum markers of AP. This binding of the 2-DG probe precedes the drop in ATP levels and necrosis that eventually results in severe AP. Based on the utility demonstrated in this rapidly evolving inflammatory disease model in rats, this study opens the scope of imaging 2-DG analogs as early predictors of inflammatory disease severity in humans.

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LEGENDS:

Figure 1: NIR 2-DG signal increase over the pancreas is unaffected by AP induction, but persists in severe AP unlike in mild AP or controls: A-I : Series of

thoracic and abdominal images in the right lateral position acquired after different times in minutes (m) after administration of NIR 2-DF (2-DG) in controls, or after AP induction with duct ligation (DL) or GTL administration(GTL). The dashed polygon denotes the region of interest overlying the pancreas in which the fluorescence was measured. **J:** Average radiant efficiency as measured over time from when NIR 2-DG was administered in controls (Con, black circles), and in the duct ligation (DL, green circles) and GTL groups (red circles). There were 9-12 animals per group. * indicate the values that were significantly different from the corresponding controls at that time **K:** An enlarged view of the first 90 minutes showing the time when acute pancreatitis was induced.

Figure 2: Severe AP is associated with significantly more retention of NIR 2-DG in the pancreas early during AP compared to controls: **A:** Time course showing means of

the Average radiant efficiency for the values obtained ±15 minutes for each time point within each group (i.e. control; Con, duct ligation; DL, and GTL) after normalizing to the 30 minute peak (100%) for that group. * depicts a significant ($p<0.05$) increase in the GTL vs. control and # depicts significance vs. DL on ANOVA at each time point. **B:** Time course showing Average radiant efficiency (ARE) normalized to controls. The initial mean AREs for each group prior to AP induction, were subtracted from those at each time point. The difference in the DL or GTL pancreatitis groups was divided by the corresponding difference in the control group, and this ratio is shown as fold change over control. Please note, that with the decline in the ARE values in controls over time (A), there is a significant increase in this ratio in the GTL group vs. DL group (#). There were 9-12 animals per group. **C:** Fluorescence images (upper

panel) and gross appearance (Lower panel) of the pancreas *ex vivo* in the control, DL and GTL groups. **D:** Bar graphs showing the quantification of the ARE for each group. "*" depicts a significant ($p<0.05$) increase in the GTL vs. control and "#" depicts significance vs. DL on ANOVA.

Figure 3: The increase of AP parameters in the blood is similar in both mild and severe disease. Time course of the activities of amylase (A), Lipase (B), alanine amino transferase (ALT; C) and amount of bilirubin (D) measured in heparinized plasma samples after 1, 3 hours of AP induction, at euthanasia (≤ 8 hours), and as baseline control value without the procedure (0 Hours). Values at 1 hour, 3 hours and at the time of euthanasia for each group (DL- green, GTL-red) were compared to each other and the controls by ANOVA. "*" depicts a significant ($p<0.05$) increase vs. control and "#" depicts significance in GTL vs. DL. There were 8-12 animals per group.

Figure 4: Severe AP is associated with a progressive increase in necrosis and mortality: A: time course of percentage of pancreatic parenchyma what was necrotic in the GTL (red) and DL (green) groups measured when electively sacrificed at 1, 3 hours, at euthanasia (≤ 8 hours). Values at 1 hour, 3 hours and at the time of euthanasia for each group were compared to each other and the controls by ANOVA. "*" depicts a significant ($p<0.05$) increase vs. control and "#" depicts significant differences in GTL vs. DL. Representative microscopic (5x, B) and gross (C) images are shown. D: Kaplan-Meyer curve showing % survival in the different study groups. There was 100% mortality in the GTL group by 8 hours, but none in the duct ligation group. There were 8-12 animals per group. Scale bars are 1mm in length.

Figure 5: NIR 2-DG uptake *in vitro* occurs before the induction of GTL induced injury: Pancreatic acinar cells were exposed to 300 μM GTL and binding of NIR 2-DG (A) was measured at 1 hour, with results being depicted as fold control (arbitrary units fluorescence/mg protein). B shows

an image of the bound fluorescence in control and GTL exposed acini as visualized on the Odyssey Imaging System. C: shows the time course of LDH leakage induced by 300 μM GTL (red) vs. controls (black). The "*" depict $p<0.01$ vs. corresponding controls. Note, that the increased NIR 2-DG binding noted at 1 hour precedes LDH leakage. Data is representative of a minimum of 4 experiments.

Figure 6: Parameters potentially affecting the NIR 2-DG signal measured in our system. A: Images of a rat administered NIR 2-DG collected on the IVIS imaging system in the supine position over the first 10 minutes (First five images) and in the right lateral position (last image). Note the increase over the bladder but the lack of signal over the pancreas in the supine position. This pattern is reversed in the right lateral position. Fluorescent (B) and black and white(C) Images of the duodenum, pancreas and spleen removed *en block* after 2 hours of NIR 2-DG administration in controls or pancreatitis in the DL and GTL groups. Please note an increased uptake in the duodenum (white outlines) especially in controls. **D:** Data from groups of 8-12 animals showing the time course of the increase in pancreatic edema (measured as % water content or pancreatic ATP concentrations (**E**) in the DL and GTL groups. The "*" depict $p<0.05$ vs. corresponding controls "#" depicts significant differences in GTL vs. DL.

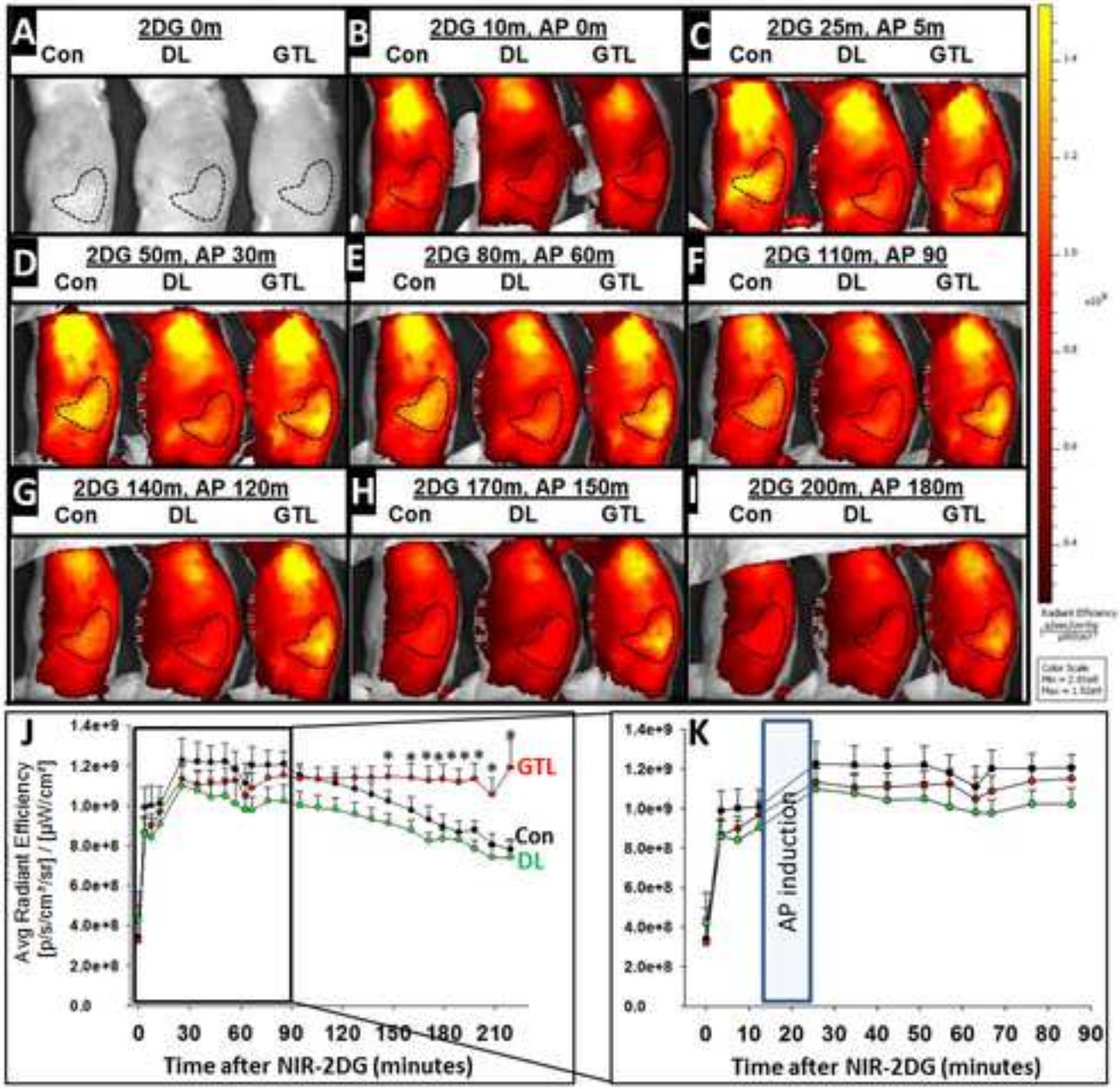

Figure 1

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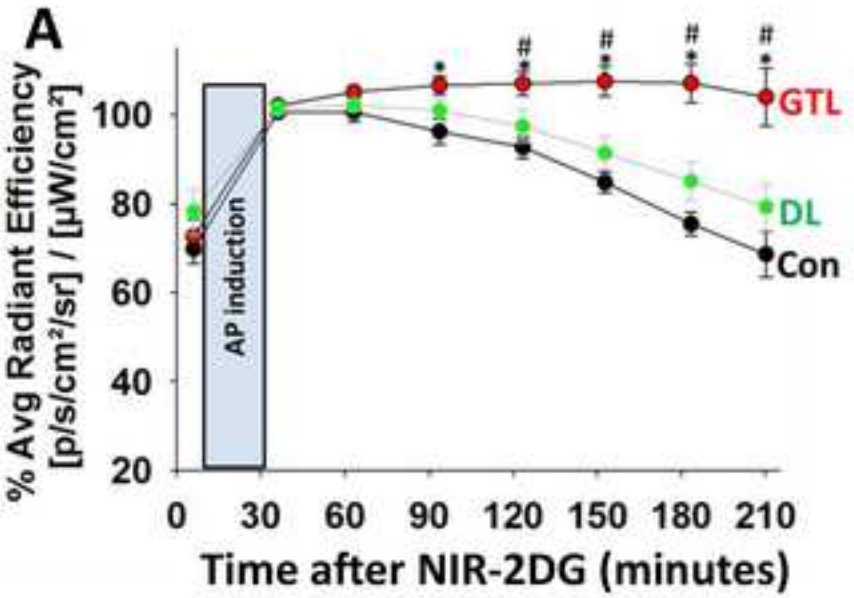
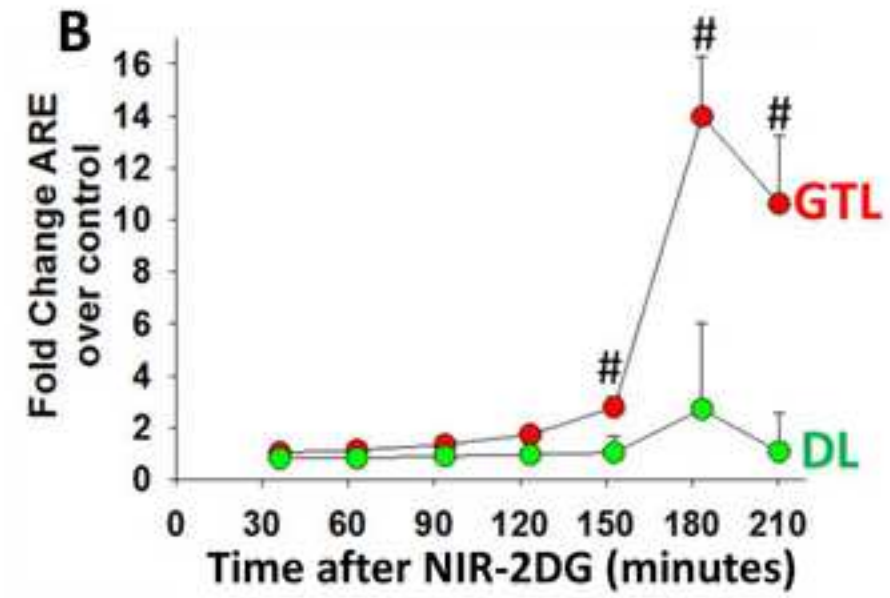
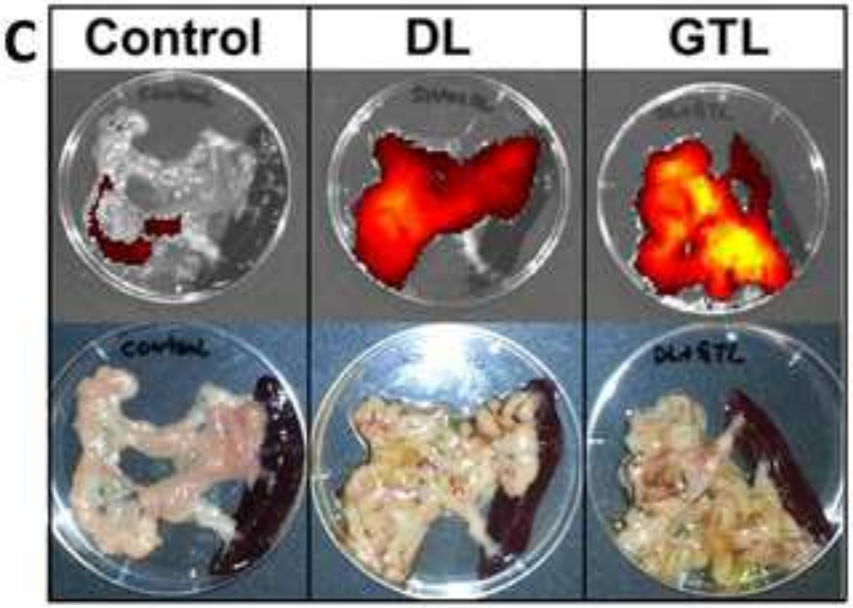






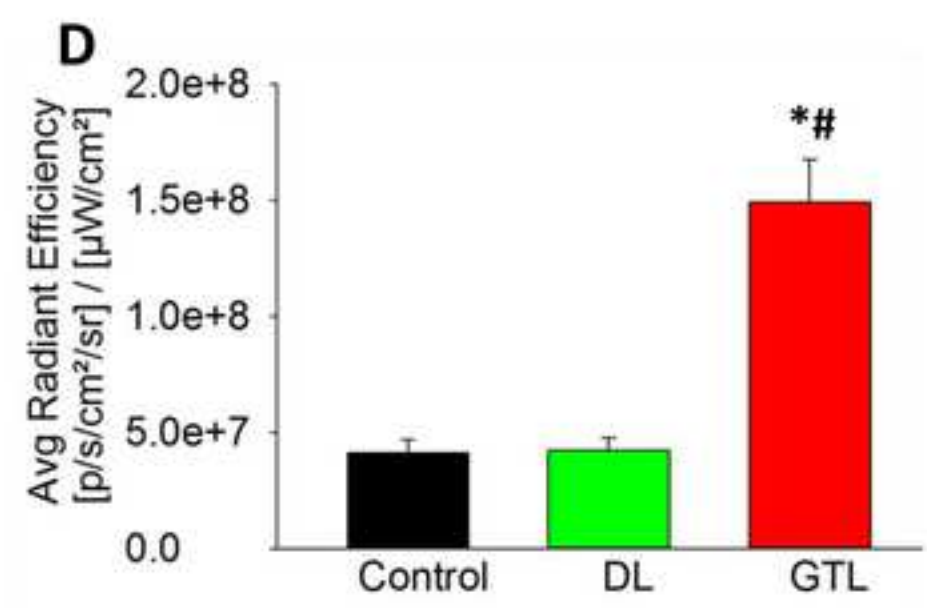


Figure 2

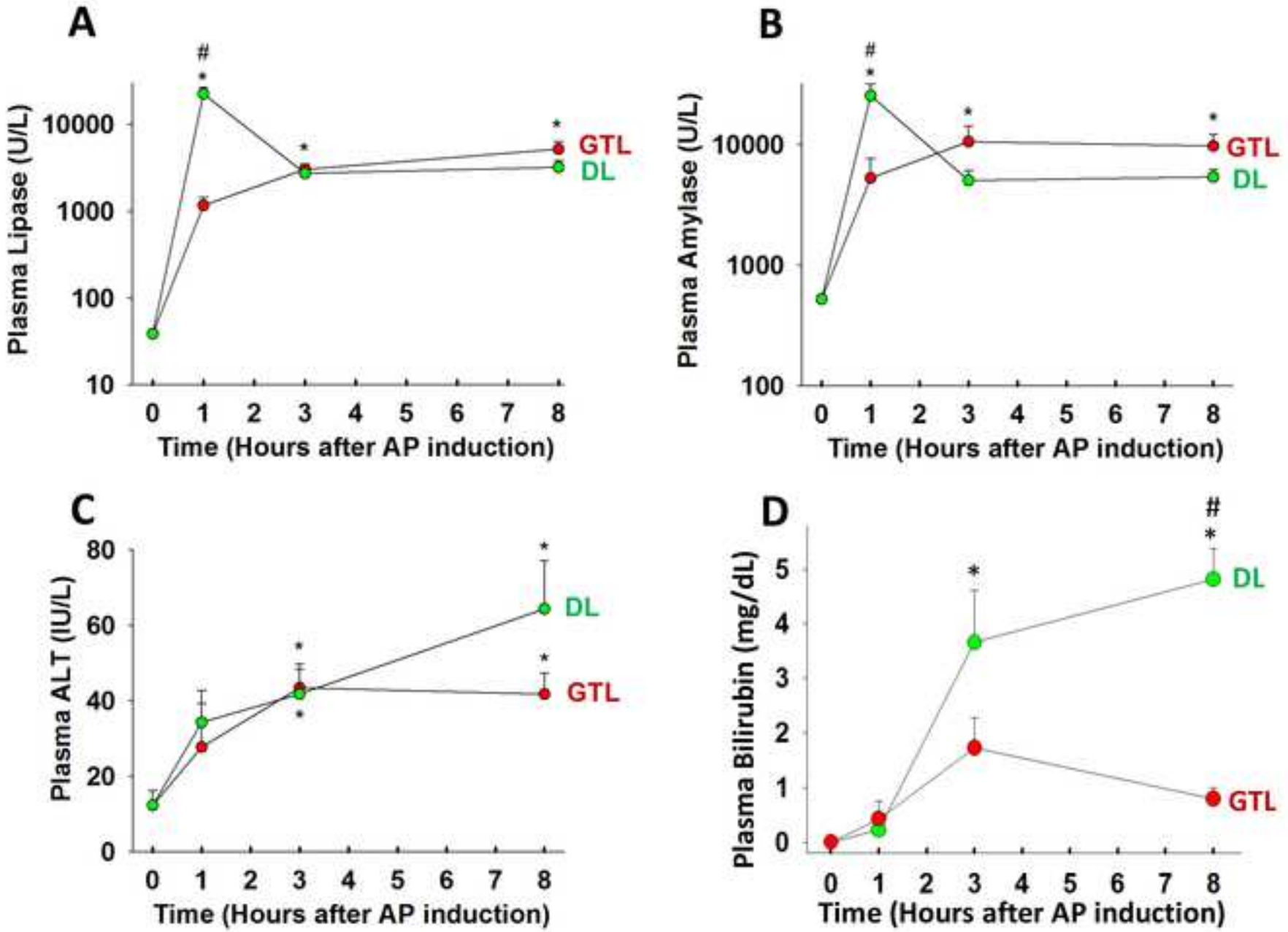
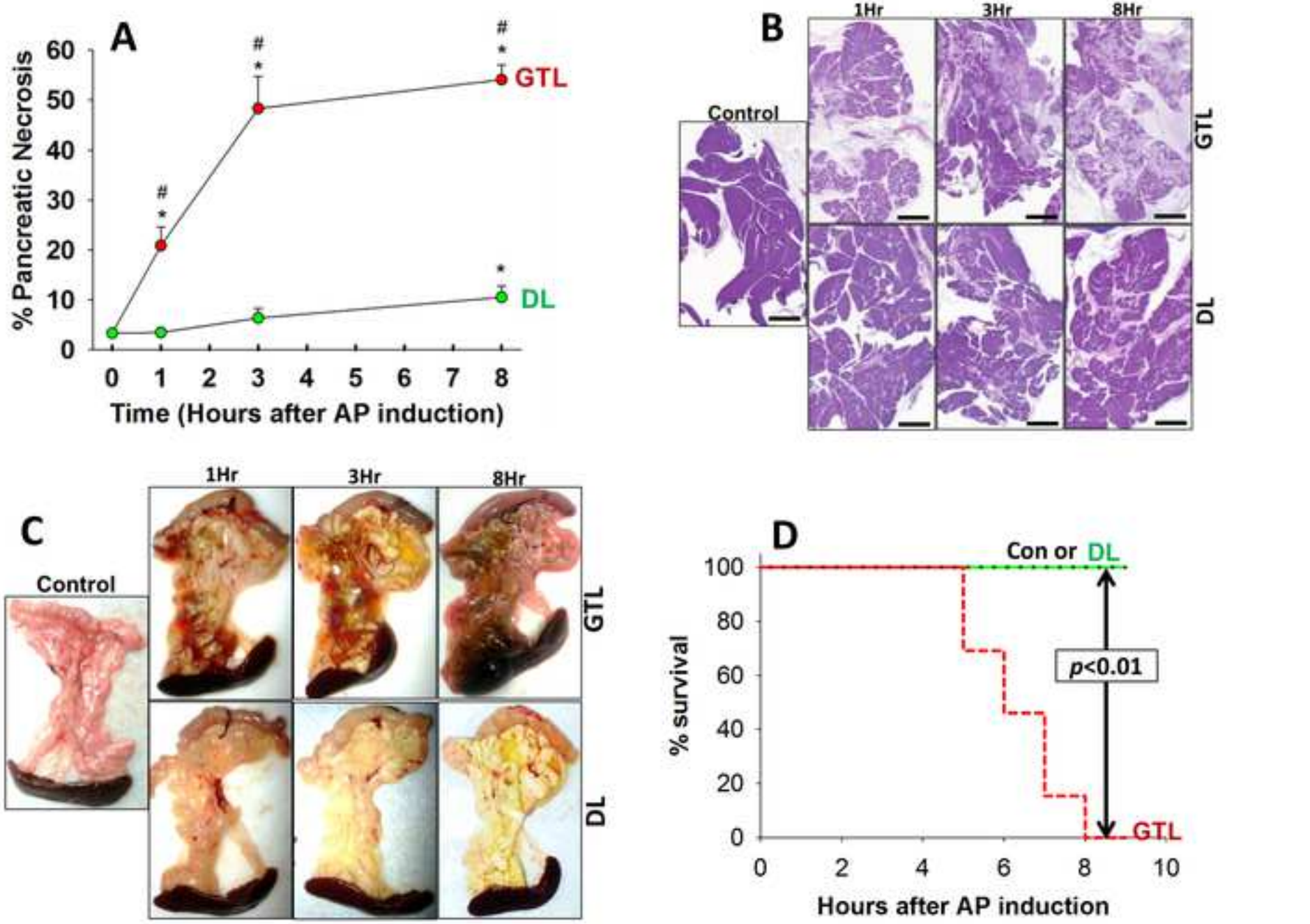


Figure 3



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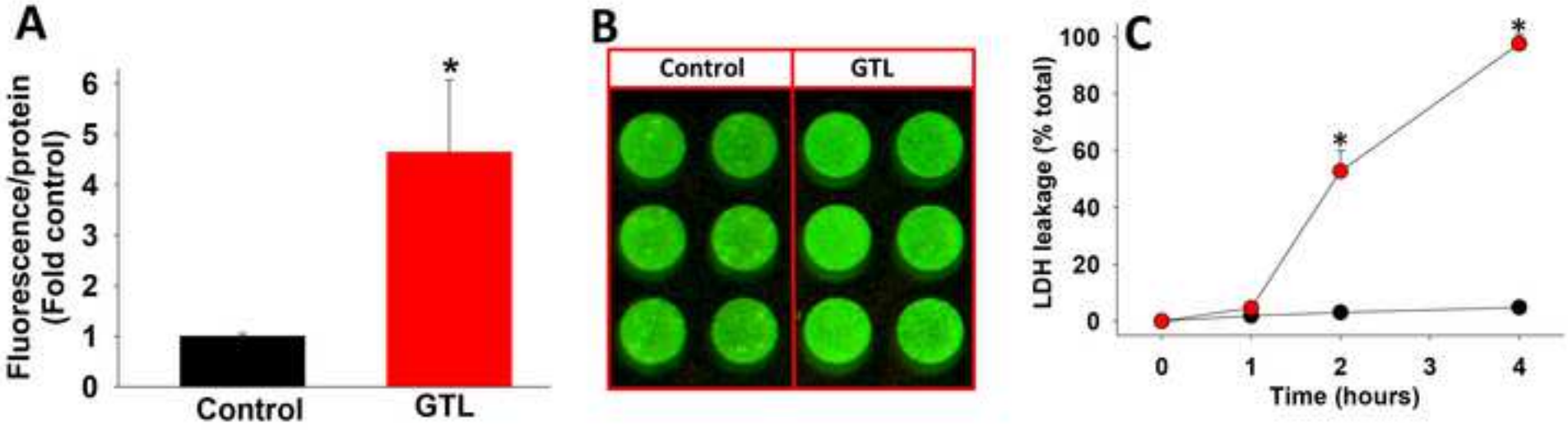

Figure 5

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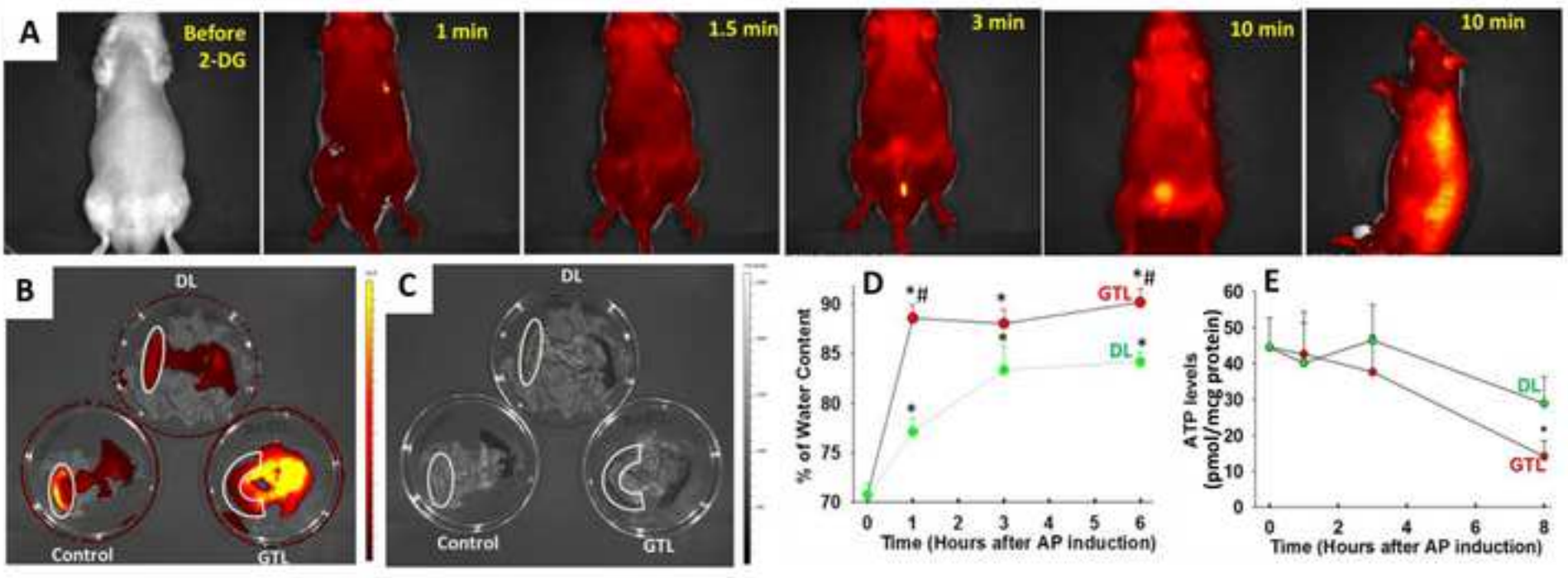

Figure 6