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View application from Rajarshi Mukherjee

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Abstract

Title of Study	Is extra-pancreatic mitochondrial dysfunction a game changer in acute pancreatitis?
Abstract and methodological description	<p>Background:</p> <p>Severe acute pancreatitis (AP) has a mortality of >20% and lacks specific drug treatment. We have demonstrated that pancreatic mitochondrial injury, mediated through the mitochondrial permeability transition pore (MPTP), is a critical feature of severe AP. Whole body cyclophilin D (CypD, a key MPTP regulator) knockout mice (Ppif^{-/-} mice) are protected from mitochondrial injury, maintain cellular ATP far more effectively than their wild type counterparts, resulting in a markedly reduced severity of experimental AP (Mukherjee et al., 2016). This evidence has formed the basis of a translational drug discovery pipeline based on CypD inhibitors established in Liverpool (Shore et al., 2016), as well as an international multi-centre randomised trial (GOULASH) comparing high versus low energy administration in the early phase of AP (Marta et al., 2017). Whilst these strategies hold promise, much remains to be understood.</p>

Whilst injury starts in the pancreas, potentially leading to necrosis, organ failure may develop either early in the course of acute pancreatitis or later due to infected pancreatic necrosis-induced sepsis (Garg et al., 2019). Persistent inflammation and end organ failure may exist in the absence of pancreatic necrosis and vice-versa, indicating a range of cellular responses in a variety of cell types must exist. Novel transcriptomic analysis of experimental AP conducted in Liverpool indicates that mitochondrial injury predominates not only in the pancreas but also in leukocytes across the full range of clinically-relevant experimental AP models tested (Mukherjee et al., manuscript in press), but whether this extra-pancreatic mitochondrial injury is a bystander or driver of severity remains unclear. Thus, a crucial question for the development of AP drugs is to what extent does extra-pancreatic mitochondrial failure contribute to severity? If major, this challenges the concept of early treatment and the therapeutic window widens as do the requirements for bioavailability, whereas if minor, targeting is narrowed to an early time point and specifically to the pancreas.

To address this question, a novel cutting-edge transgenic mouse model will allow definitive assessment of the role of extra-pancreatic mitochondrial injury, opening up multiple new areas of investigation as well as facilitating ongoing translational drug discovery pipelines focussed on mitochondrial injury for human AP. Whilst previous CypD knockouts (globally protected from mitochondrial injury) have been generated, a whole body Ppif^{-/-} knockout with pancreas-specific CypD expression (extra-pancreatic protection from mitochondrial injury only) is proposed. This would be generated by the latest CRISPR/Cas9 transgenic techniques and would be an innovative and definitive approach to assess the importance of extra-pancreatic mitochondrial protection in AP.

Aims and Objectives:

The aim of this project is to establish the proposed model through the following objectives:

1. To generate an inducible pancreas-specific reporter-tagged CypD knock-in with a background of whole body constitutive Ppif^{-/-} knockout mouse.

2. To confirm CypD expression in the pancreas and absence in other organs.
3. To determine the severity of experimental AP in these mice using a range of clinically relevant models.

Methods:

Transgenic Mouse Generation:

An inducible pancreas-specific knockin mouse model of mouse CypD will be generated in C57BL/6 mice. Previous experiments using CRISPR/Cas9 and gRNA co-injection into fertilized eggs have successfully generated a transgenic mouse with a pancreatic acinar cell specific Ppif and tomato red reporter construct inserted into intron 1 at the ROSA26 locus (Elastase-1 promoter-Kozak-mouse Ppif CDS-2A-dTomato-polyA cassette), confirmed by genotyping and sequencing analysis (Taconic Biosciences Ltd.). As the mouse still constitutively expresses wildtype CypD and extra-pancreatic mitochondrial protection is our focus of interest, we propose to cross-breed this mouse with our whole-body Ppif^{-/-} colony to produce heterozygotes, then back-cross to develop both heterozygote and homozygote inducible pancreas-specific Ppif knockin mice on a whole body Ppif^{-/-} background as desired. All mice will be genotyped in a standard manner using tail snip samples at 10-15 days old, followed by PCR with primers specific to Ppif (NM_134084).

Confirmation of Specificity:

Confocal fluorescence microscopy (Zeiss LSM 710) will be used to detect tomato red fluorescence as a marker of specific CypD expression within pancreatic acinar cells, and absence in a range of other tissues eg. Blood, liver, kidney, lung. Western blot for CypD protein expression will be performed on a range of tissue lysates to further validate specificity.

Severity of Experimental AP Characterisation:

Following successful establishment of the proposed model, groups of control, heterozygous and homozygous knockin mice (n = 6/group) will be assessed for their severity responses to the induction of experimental AP using a range of clinically relevant experimental AP models: retrograde bile salt infusion (3 mM Tauroolithocholate sulphate) representing gallstone induced AP, alcohol metabolite fatty acid ethyl ester induced AP representing alcohol induced

AP and caerulein injection AP representing pancreatic hyperstimulation. Real-time recordings of heart rate, respiratory rate and arterial oxygen saturation in the mice will be made by novel pulse oximetry techniques (Mouse-ox system). Under terminal anaesthesia blood will be taken by cardiac puncture followed by exsanguination for termination. Collected blood will be maintained at room temperature for 2 h to allow clotting then centrifugation at 1,500 g and 4°C for 10 min. Serum will be separated and stored at -80°C until measurement of biomarkers (serum amylase, serum cytokines, serum DAMPs, serum alanine amino transferase, serum urea and creatinine) is undertaken. The right lung will be removed and snap frozen in dry ice for pulmonary myeloperoxidase (MPO) measurement, while the left lung will be injected with 0.5 mL 10% neutral formalin via the left bronchus and fixed in 10% neutral formalin for haematoxylin-eosin (H&E) staining. The pancreas will be removed via laparotomy and divided into three portions, the first for fixation in 10% neutral formalin and H&E staining, the second for MPO measurement and the third for pancreatic trypsin activity measurement. H&E sections of pancreas will be evaluated by two independent blinded investigators for severity of injury (pancreatic inflammatory infiltrate, oedema and necrosis) and mean lung field occupancy representative of alveolar thickening will be assessed by a novel method on H&E lung images obtained by Aperio Scanner (Leica) using ImageJ and CellProfiler software (Mukherjee et al., 2018).

Potential outcome and impact:

The proposed project provides crucial mechanistic understanding about mitochondrial injury in the pathogenesis of AP, a disease with increasing worldwide incidence, carrying a major detrimental impact to health and without targeted drug treatment. The project will provide novel insights applicable to ongoing drug development, to ultimately establish clinical trials to fill the unmet therapeutic need. Results from this research will be applicable to chronic pancreatitis and a range of other acute and chronic necrotic, inflammatory and degenerative conditions in which mitochondrial injury is implicated, with the focus of the project also having important wider relevance. Understanding the effects of MPTP induction in different cell types will have potential impact on the

fields of infection and immune biology, cardiovascular and neurological disease, all of which have already had a major role for the MPTP implicated.

The anticipated outcomes of this study are a high-impact publication and further research grant funding. Based on the crucial mechanistic insights into disease pathology gained by this project, we will be able to establish multiple new lines of investigation, contributing to a portfolio of planned studies for future Wellcome Trust and MRC grant applications. Generation of the mouse model proposed will provide an essential long-term foundation and resource, demonstrating RM's transition to independence as a clinical researcher and supporting RM's ongoing applications to clinician scientist programmes (Wellcome Trust Clinical Research Career Development, MRC Clinician Scientist, and UKRI Fellowship) by providing extremely precious pilot data.

Research Environment and People:

The Liverpool Pancreatitis Research Group, led by RS, has an outstanding track record in delivering an international standard of impactful research, focussing on pre-clinical mechanistic evaluation, drug discovery and clinical trials in acute pancreatitis. RM has been an active member of the group for the over ten years, having personally led, optimised and published on multiple experimental approaches described in this application. There already exists a tried and tested, well-established infrastructure for all experimental methods proposed. The whole-body Ppif^{-/-} colony is established in the University of Liverpool and the newly generated pancreas-specific knockin has just been successfully produced and shipped to Liverpool, setting the stage for the proposed breeding to develop the novel model proposed. The Zeiss LSM 710 confocal microscope that will be used to confirm pancreas-specificity of the CypD knockin tagged to a tomato red fluorescence reporter is already established and regularly used within the Liverpool Pancreatitis Research group.

During the funding request time period, RM will be overseeing transgenic mouse breeding that will primarily be conducted by personnel employed by the Biomedical Services Unit, University of Liverpool. On successful generation of the new mouse model, RM

will supervise a PhD student (Vincent Yip; China Scholarships Council) to perform all model characterisation experiments and test the severity of experimental AP in these mice.

Justification of Resources:

Funds requested cover the breeding and characterisation over a 12-month period of a novel knockin Ppif model. Cyclophilin D has been identified as a strong candidate for drug discovery, however, further mechanistic understanding is required to aid successful translation of these observations specifically pertaining to inflammatory mechanisms. Parallel 'in-silico' approaches as well as significant isolated pancreatic acinar cell in vitro experiments have been used prior to the experiments proposed to justify their use. This study is entirely novel and an important progression from RM's earlier research, which will now address the pivotal relationship between local pancreatic injury, systemic inflammation and distant organ injury in AP. Taconic Biosciences, the commercial partner used to generate the first part of the proposed model, have an established track record in CRISPR transgenic mouse generation with previous provision to the University of Liverpool. They have confirmed successful delivery of the initial Ppif ROSA knockin mouse with both detailed genotyping as well as CRISPR off-target analysis. RM personally has excellent previous experience in both generation and characterisation of transgenic mouse models (Athwal et al. 2014, Huang et al. 2015, Mukherjee et al. 2016). The Biomedical services unit (BSU) in the University of Liverpool has a wide-ranging proven track record in transgenic model breeding.

The strain proposed would be an entirely novel undertaking and would answer a crucial question related to disease mechanisms, not just of relevance to acute pancreatitis but also a wealth of other inflammatory conditions where systemic inflammation occurs, and the sequence of events remains to be elucidated. Without using disease models in living animals, there is little prospect of developing new diagnostics and therapeutics for AP, a disease that can result in very great human suffering, lifelong morbidity and untimely death. Murine pancreatic physiology at the molecular, organellar, cellular, organ and whole organism level is similar to that of humans.

For example, we have demonstrated that as in murine pancreatic acinar cells, human pancreatic acinar cells have high affinity cholecystokinin receptors that function in the same manner (Murphy et al., 2008). Similarly, pancreatic acinar cells that are hyperstimulated by supramaximal concentrations of cholecystokinin or exposed to clinically relevant concentrations of pancreatitis toxins (bile acids, FAEEs) release excessive quantities of calcium inside the cell, mitochondria are overloaded and adenosine triphosphate generation impaired. The resulting pathology, including premature digestive enzyme activation and necrotic cell death pathway activation, appears identical in murine and human cells (Mukherjee et al., 2016). Models of AP have been most thoroughly developed in the mouse and rat; the majority of preclinical studies have been undertaken in these species. Improvements resulting from this disease model will allow optimisation of an existing translational drug discovery pipeline focussed on cyclophilin D inhibitors, potentially reducing the number of mice required for future preclinical studies resulting from more appropriately targeted therapies.

RM has also been engaged in a parallel project optimising the latest single-cell transcriptomic assessment of experimental pancreatitis which is currently at the stage of bioinformatics analysis. We plan to apply these key approaches to the new model and Human AP in the future, setting the stage for future precision medicine strategies for AP, an area in which pancreas lags far behind other disease conditions (Mukherjee et al., 2019). The funds requested would also help inform an ongoing translational drug discovery programme focussed on CypD inhibitors, likely to secure larger future funding (MRC Developmental Pathway), paving the way to Phase 1 Human Clinical Trials.

Timetable

Name	Step one of breeding (2 pairs plus 15 offspring for 12 weeks)
Date	March-June 2020

Name	Step two of breeding (Backcross onto KO line: 4 pairs plus 25 offspring for 12 weeks)
Date	June-Aug 2020

Name	Step three of breeding (Cohort breeding to produce 10-12 experimental mice per month)
Date	Aug-Nov 2020

Name	Confirmation of specificity experiments
Date	September-November 2020

Name	Testing of experimental AP
Date	November 2020-January 2021

Funding

Name	Step one of breeding : 2 pairs plus 15 offspring for 12 weeks
Amount	981.24

Name	Step two of breeding : backcross onto KO line, 4 pairs plus 25 offspring for 12 weeks
Amount	1904.76

Name	Step three of breeding : cohort breeding to produce 10-12 experimental mice per month
Amount	5387.2

Name	Consumables for confirmation of specificity experiments: CypD antibody for western blotting
Amount	545.5

Name	Consumables for experimental AP: tauroolithocholate sulphate, cerulein, palmitoleic acid ethyl ester, histopathology, use of animal operating theatres for TLCS model, reagents for biochemical assays
Amount	1150.3

Details of ethical approval

All work proposed in this application will strictly adhere to the Animals (Scientific Procedures) Act 1986. Six animals per group will be used to ensure minimum numbers to identify meaningfully different measures between different groups, i.e. mean and standard error or median and inter-quartile range, as appropriate. Suffering will be reduced by best practice in animal management, in line with current guidelines and Home Office requirements, including full training and animal monitoring. Regimens to monitor animals will be followed with analgesia appropriate to the procedure, e.g. use of buprenorphine after induction of experimental AP. Animals expected to have recovered from any procedure but continuing to display marked abnormality will be humanely killed by a Schedule 1 method.

All personnel involved in the project (RS, RM, VY) already hold valid UK Home Office personal licenses, valid for the duration of the proposed experimental period. Work will be conducted under RS's Home Office animal project license (PDC14C46E), recently successfully renewed and valid until May 2024. Strong ties are already established with the excellent University of Liverpool Biomedical services unit (BSU) facilities, with ethical project design considering the three R's already verified by the head of the BSU.

Institutional approval information

This research proposal carries full approval and support from the Institute of Translational Medicine, University of Liverpool, fitting in with and complimentary to a number of existing research programmes within the University that already have institutional support. The Research Support office of the University of Liverpool has been notified of this submission. The proposed project has been reviewed in detail and approved by the Head of the BSU and lead transgenic officer (Dr. Lynn McLaughlin) as well as an independent researcher within the University, Prof. Bou-Gharrios (University of Liverpool), already holding an outstanding track-record in CRISPR transgenic mouse generation.

Declaration

Confirm Declaration: Yes

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Abstract

Title of Study	Prospective evaluation of Patient Related Outcome Measures (PROMS) and Health Related Quality of life (HRQoL) in patients with pancreatic cancer
Abstract and methodological description	<p>ABSTRACT</p> <p>Pancreatic cancer is an aggressive cancer with poor survival. For those patients suitable for surgical intervention, the postoperative complication rates are high. The short survival and higher complication rate have a significant impact on quality of life. Patient related outcome measures are frequently used in cancer surgery due to often limited survival after morbid and complex treatments requiring careful appraisal and shared decision making. There is paucity of data in patients with pancreatic cancer regarding the impact of pancreatic cancer in general and postoperative complications on patient related outcome measures (PROMS). Furthermore, the effect of surgical treatment on PROMS is unknown. This prospective study aims to longitudinally assess PROMS using the core set of patient reported outcomes in pancreatic cancer (COPRAC)</p>

questionnaire and (EORTC) QLQ-C30 and the pancreatic cancer module EORTC QLQ-PAN26 for all patients undergoing pancreatic surgery for malignant pathology. The also aims to evaluate the association between postoperative complications and PROMS and HRQoL in the short-term (within 30 days) and long-term (after 3 and 6 months) in order to find out whether and to what extent complications (none/grade I vs. minor (grade II) vs. major (grade III-V)) influence the PROMs and HRQOL.

Introduction

Pancreatic cancer is one of the most aggressive cancers with a 5-year survival of around 20% in resected patients in spite of recent advances in chemotherapy. The short survival and higher complication rate after pancreatic surgery has significant impact on the quality of life¹. Whilst clinical trials have traditionally focussed on clinical and survival outcomes, patients related outcomes are infrequently assessed. In recent years, however, patient-reported outcomes (PROMs), referred to as 'any report coming directly from patients about a health condition and its treatment'², are of increasing importance in health care, in particular cancer care^{3,4}. PROMs can be used by clinicians and researchers to measure the impact of treatment on several domains of a patient's health status and might also enhance patient-provider communication. Patient related outcome measures are therefore paramount to ensure specific areas of treatment are addressed following diagnosis. PROMs in oncological trials can be classified into two main groups: PROMs for the assessment of cancer- and treatment-related symptoms and PROMs to measure health-related quality of life (HRQoL)⁵. A multicentre Delphi study among patients with pancreatic cancer (Curative- and palliative-setting) and health care practitioners in 6 pancreatic centres in the US (Baltimore, Boston), Europe (Amsterdam, Verona), and Asia (Mumbai, Seoul) identified 8 Core Set of Patient-reported Outcomes (COPRAC) in Pancreatic Cancer from 56 patient related outcomes after 2 rounds of Delphi survey⁶. The 8 COPRAC included general quality of life, general health, physical ability, ability to work/do usual activities, fear of recurrence, satisfaction with services/care organization, abdominal complaints (pain/discomfort), and relationship with partner/family.

Nevertheless, due to social-cultural differences, priorities and expectations of patients may differ between various regions and this might influence the selection of PROMS. The COPRAC measures were not validated in the UK cohort and only included 2 European centres (Amsterdam, Verona). Furthermore, PROMs may alter as patient's progress through the treatment pathway and PROMs considered important at the start of the treatment may not be relevant after completion of treatment and during follow up. A routine, longitudinal measurement in clinical practice, may allow identification of PROMs important to patient's which will allow further emphasis on patient centred care and general quality of care.

The second aspect of PROMS in cancer patients which is more frequently measured is HRQoL. HRQoL in pancreatic cancer patients is frequently assessed with the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 and the pancreatic cancer module EORTC QLQ-PAN26. Arguably the most important clinical outcome in surgical oncology from a patient's point of view is, apart from survival is the frequency and severity of complications. Although it seems intuitive that complications adversely affect HRQoL and symptoms, the extent to which HRQoL and symptoms are affected after complications is unknown. It is therefore also unclear whether complications considered more severe in the Clavien-Dindo classification system 7, i.e. those with a higher severity grade, are related to reduced HRQoL or symptoms. There is relative sparsity of data in pancreatic surgery assessing the impact of complications on short and long term QOL 8. Furthermore, development of complications following pancreatic surgery may affect PROMS and HRQOL and a longitudinal measurement of PROMS and QOL will allow to assess the impact of complications on these 2 domains.

Methods

Objectives and hypotheses

The primary aim of this prospective study to longitudinally assess PROMS using the COPRAC questionnaire and (EORTC) QLQ-C30 and the pancreatic cancer module EORTC QLQ-PAN26 for all patients undergoing pancreatic surgery for malignant

pathology. The secondary aim of the study is to evaluate associations between the clinical outcomes and the two PROMs in the short-term (within 30 days) and long-term (after 3 and 6 months) in order to find out whether and to what extent complications (none/grade I vs. minor (grade II) vs. major (grade III-V)) influence the PROMs and HRQOL.

Study population and location

The study population for the study will consist of patients undergoing elective pancreatic surgery for malignant tumours of the body and tail of the pancreas. The study will be performed at Freeman Hospital, a high volume HPB centre in the UK specialising in pancreatic surgery.

Inclusion criteria

Patients undergoing pancreatic surgery (pancreatoduodenectomy, distal pancreatectomy and total pancreatectomy) for a malignant pathology will be included.

The inclusion criteria are summarised in Table 1.

Sample size calculation

No sample size guidelines exist for assessment of PROMS and HRQOL in pancreatic cancer. Therefore, the aim is to include 100 patients being treated in a curative intent setting undergoing different pancreatic resections, so a heterogeneous group of patients is included in the study group.

Statistical analysis

Data will be analysed using SPSS for Windows version 21.0 (SPSS Inc., Chicago, IL, USA). Continuous variables will be expressed as median (interquartile range) unless specified otherwise. Categorical variables will be expressed as absolute number (percentage). Since this is an exploratory study, all analyses will be descriptive and p-values < 0.05 will be referred to as statistically significant in descriptive sense. The patient characteristics and outcomes will be described for the whole cohort and for the different subgroups divided according to type of pancreatic resection with appropriate descriptive statistics (mean, standard deviation, median, interquartile range, minimum, maximum in case of

continuous data and scores, or absolute and relative frequencies in case of categorical data). To analyse the association between perioperative complications (Clavien-Dindo grading) and the set of PROMs and HRQoL subscales in the short- and long-term, Spearman's rank-correlation coefficients (Spearman's rho) will be calculated and analysis of variance will be performed.

Ethics, study registration and consent

The study has been approved by the research department of Freeman hospital (reference no: NuTH823). The study will be conducted in the context of Good Clinical Practice and in accordance with the Declaration of Helsinki. All patients planned for pancreatic surgery at the Freeman hospital will be screened for eligibility at their clinic visit prior to the operation. The patients will be explained the rationale behind the study.

Study duration, schedule and data collection

Patient's with confirmed pancreatic cancer and planned for resection will be identified at the HPB MDT for recruitment into the study. Consenting for the study will be obtained at the first clinic appointment. All consented patients will be asked to complete the PROMs (appendix 1) and HRQOL questionnaire (appendix 2). The data collection will be led by a research nurse.

Patient time lines and description of study visits

Visit 1. Preoperatively at the clinic appointment following the MDT discussion.

After informed consent, the following data items will be collected during visit 1: a.) demographic data; b.) baseline clinical data c.) medical history; f.) PROMS g.) HRQOL according to EORTC QLQ-C30 and pancreatic cancer module EORTC QLQ-PAN26.

Visit 2: At the time of discharge.

a.) complications according to Clavien-Dindo b.) PROMS c.) HRQOL according to EORTC QLQ-C30 and pancreatic cancer module EORTC QLQ-PAN26.

Visit 3: First clinic appointment to discuss histology (3 weeks).

a.) PROMS c.) HRQOL according to EORTC QLQ-C30 and pancreatic cancer module EORTC QLQ-PAN26.

Visit 4 TEL: 3 months post-surgery

a.) PROMS b.) HRQOL according to EORTC QLQ-C30 and pancreatic cancer module EORTC QLQ-PAN26.

Visit 5 TEL 6 months post-surgery

a.) PROMS b.) HRQOL according to EORTC QLQ-C30 and pancreatic cancer module EORTC QLQ-PAN26.

The duration of the study for each patient is 6 months including follow-up. The duration of the overall study is expected to be 18 months.

Discussion

There is emerging evidence supporting the routine collection of PROMs enables improved and personalised care, especially in cancer settings 3,4. The studies have shown beneficial effects on patient centered care and general quality of care. However, the studies supporting these hypotheses, lack pancreatic cancer patients, which form a distinct subgroup of cancer patients given their short survival. Therefore, the impact of routine collection of PROMs in the pancreatic cancer setting needs yet to be determined. Furthermore, patients undergoing pancreatic surgery have a high risk of postoperative complications and there is relative paucity of data regarding the effect of complications on PROMs and HRQoL. One of the reasons for lack of PROMs in pancreatic cancer patients is the lack of universally accepted PROMs. The recently published COPRAC PROMs provide an opportunity to prospectively assess the PROMs in addition to assessing the effect of complications on PROMS. The present prospective study using a validated complication measurement tool (Clavien-Dindo classification) and two new PROMs would be the first of its kind in pancreatic

cancer patients. The results from this study may provide a valuable insight on PROMs and HRQoL during their treatment and identify patient reported outcomes for health care providers at various time points during their treatment.

References

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quality of life after pancreatic resection for malignancy in patients with and without severe postoperative complications. HPB (Oxford). 2018;20(2):188–95.

Table 1 Eligibility criteria

Inclusion criteria

Age> 18 years

Informed consent provided

Malignant tumours of the pancreatic body and tail planned for elective Whipples PD, Distal pancreatectomy or total pancreatectomy

Exclusion criteria

Lack of informed consent

Appendix 1

PROMs questionnaire based on Core Set of Patient-reported Outcomes in Pancreatic Cancer (COPRAC)

Please indicate how important you think it is that these topics are addressed in questionnaires for patients with pancreatic or periampullary cancer.

Please mark the number that reflects your opinion the best. 1=not very important 9=very important

- 1. General quality of life 1 2 3 4 5 6 7 8 9
- 2. General health 1 2 3 4 5 6 7 8 9
- 3. Physical ability 1 2 3 4 5 6 7 8 9

4. Ability to work/do usual activities 1 2 3 4 5 6 7 8 9
5. Medication 1 2 3 4 5 6 7 8 9
6. Side effects 1 2 3 4 5 6 7 8 9
7. Weight changes 1 2 3 4 5 6 7 8 9
8. Appetite 1 2 3 4 5 6 7 8 9
9. Change in taste of food 1 2 3 4 5 6 7 8 9
10. Limitation in type of food 1 2 3 4 5 6 7 8 9
11. Limitation in amount of food 1 2 3 4 5 6 7 8 9
12. Enteral nutrition 1 2 3 4 5 6 7 8 9
13. Nutritional supplements 1 2 3 4 5 6 7 8 9
14. Dry mouth 1 2 3 4 5 6 7 8 9
15. Shortness of breath 1 2 3 4 5 6 7 8 9
16. Coughing 1 2 3 4 5 6 7 8 9
17. Nausea 1 2 3 4 5 6 7 8 9
18. Vomiting 1 2 3 4 5 6 7 8 9
19. Dysphagia (difficulty in swallowing) 1 2 3 4 5 6 7 8 9
20. Heartburn/acid reflux 1 2 3 4 5 6 7 8 9
21. Abdominal complaints (pain/discomfort) 1 2 3 4 5 6 7 8 9
22. Flatulence 1 2 3 4 5 6 7 8 9
23. Belching 1 2 3 4 5 6 7 8 9
24. Bloating 1 2 3 4 5 6 7 8 9
25. Defecation (diarrhoea, obstipation) 1 2 3 4 5 6 7 8 9
26. Jaundice (yellowish pigmentation of skin or eyes) 1 2 3 4 5 6 7 8 9
27. Itching 1 2 3 4 5 6 7 8 9
28. Hair loss 1 2 3 4 5 6 7 8 9
29. Eye problems 1 2 3 4 5 6 7 8 9
30. Headache 1 2 3 4 5 6 7 8 9
31. Dizziness 1 2 3 4 5 6 7 8 9
32. Cognition (attention, memory) 1 2 3 4 5 6 7 8 9
33. Sexuality 1 2 3 4 5 6 7 8 9
34. Sleeping 1 2 3 4 5 6 7 8 9
35. Fatigue 1 2 3 4 5 6 7 8 9
36. Weakness 1 2 3 4 5 6 7 8 9
37. Pain 1 2 3 4 5 6 7 8 9
38. Skin problems 1 2 3 4 5 6 7 8 9
39. Fever/chills 1 2 3 4 5 6 7 8 9
40. Tingling extremities 1 2 3 4 5 6 7 8 9
41. Negative feelings 1 2 3 4 5 6 7 8 9
42. Positive feelings 1 2 3 4 5 6 7 8 9
43. Anxiety 1 2 3 4 5 6 7 8 9
44. Change of appearance 1 2 3 4 5 6 7 8 9
45. Body image 1 2 3 4 5 6 7 8 9
46. Coping 1 2 3 4 5 6 7 8 9
47. Worrying 1 2 3 4 5 6 7 8 9
48. Fear of recurrence 1 2 3 4 5 6 7 8 9

- 49. Relationship with partner/family 1 2 3 4 5 6 7 8 9
- 50. Relationship with friends/relatives 1 2 3 4 5 6 7 8 9
- 51. Financial problems 1 2 3 4 5 6 7 8 9
- 52. Satisfaction with caregivers 1 2 3 4 5 6 7 8 9
- 53. Satisfaction with services/care organization 1 2 3 4 5 6 7 8 9
- 54. Pancreatic enzyme replacement therapy 1 2 3 4 5 6 7 8 9
- 55. Contact between patients 1 2 3 4 5 6 7 8 9
- 56. Diabetes 1 2 3 4 5 6 7 8 9

Appendix 2

EORTC QLQ-C30 questionnaire

1= Not all 2= A little 3=Quite a bit 4=Very much

- 1. Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase? 1 2 3 4
- 2. Do you have any trouble taking a long walk? 1 2 3 4
- 3. Do you have any trouble taking a short walk outside of the house? 1 2 3 4
- 4. Do you need to stay in bed or a chair during the day? 1 2 3 4
- 5. Do you need help with eating, dressing, washing yourself or using the toilet? 1 2 3 4

During the past week:

- 6. Were you limited in doing either your work or other daily activities? 1 2 3 4
- 7. Were you limited in pursuing your hobbies or other leisure time activities? 1 2 3 4
- 8. Were you short of breath? 1 2 3 4
- 9. Have you had pain? 1 2 3 4
- 10. Did you need to rest? 1 2 3 4
- 11. Have you had trouble sleeping? 1 2 3 4
- 12. Have you felt weak? 1 2 3 4
- 13. Have you lacked appetite? 1 2 3 4
- 14. Have you felt nauseated? 1 2 3 4
- 15. Have you vomited? 1 2 3 4
- 16. Have you been constipated? 1 2 3 4

During the past week

- 17. Have you had diarrhoea? 1 2 3 4

18. Were you tired? 1 2 3 4
19. Did pain interfere with your daily activities? 1 2 3 4
20. Have you had difficulty in concentrating on things, like reading a newspaper or watching television? 1 2 3 4
21. Did you feel tense? 1 2 3 4
22. Did you worry? 1 2 3 4
23. Did you feel irritable? 1 2 3 4
24. Did you feel depressed? 1 2 3 4
25. Have you had difficulty remembering things? 1 2 3 4
26. Has your physical condition or medical treatment interfered with your family life? 1 2 3 4
27. Has your physical condition or medical treatment interfered with your social activities? 1 2 3 4
28. Has your physical condition or medical treatment caused you financial difficulties? 1 2 3 4
- For the following questions please circle the number between 1 and 7 that best applies to you 1= very poor 7= excellent
29. How would you rate your overall health during the past week? 1 2 3 4 5 6 7
30. How would you rate your overall quality of life during the past week?
1 2 3 4 5 6 7

EORTC QLQ - PAN26 component

- 31 Have you had abdominal discomfort? 1 2 3 4
32. Did you have a bloated feeling in your abdomen?
1 2 3 4
33. Have you had back pain? 1 2 3 4
34. Did you have pain during the night? 1 2 3 4
35. Did you find it uncomfortable in certain positions (e.g. lying down)? 1 2 3 4
36. Were you restricted in the types of food you can eat as a result of your disease or treatment? 1 2 3 4
37. Were you restricted in the amounts of food you could eat as a result of your disease or treatment? 1 2 3 4
38. Did food and drink taste different from usual? 1 2 3 4
39. Have you had indigestion? 1 2 3 4
40. Were you bothered by gas (flatulence)? 1 2 3 4
41. Have you worried about your weight being too low? 1 2 3 4
42. Did you feel weak in your arms and legs? 1 2 3 4
43. Did you have a dry mouth? 1 2 3 4
44. Have you had itching? 1 2 3 4

	<p>45. To what extent was your skin yellow? 1 2 3 4</p> <p>46. Did you have frequent bowel movements? 1 2 3 4</p> <p>47. Did you feel the urge to move your bowels quickly? 1 2 3 4</p> <p>48. Have you felt physically less attractive as a result of your disease and treatment? 1 2 3 4</p> <p>49. Have you been dissatisfied with your body? 1 2 3 4</p> <p>50. To what extent have you been troubled with side-effects from your treatment? 1 2 3 4</p> <p>51. Were you worried about your health in the future? 1 2 3 4</p> <p>52. Were you limited in planning activities, for example meeting friends, in advance? 1 2 3 4</p> <p>53. Have you received adequate support from your health care professionals? 1 2 3 4</p> <p>54. Has the information given about your physical condition and treatment been adequate? 1 2 3 4</p> <p>55. Have you felt less interest in sex? 1 2 3 4</p> <p>56. Have you felt less sexual enjoyment? 1 2 3 4</p>
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Timetable

Name	The proposed time table is summarised in the study protocol. The study aims to recruit 100 patients over a 18 month period
Date	The recruitment is expected to start on 01.03.2020

Funding

Name	The costing for the study was approved by the research design office. The costing tool was based on the Attributing the costs of health and social care research (AcoRD) document
Amount	10698.0

Details of ethical approval

This is a questionnaire based study and is registered as a Quality Improvement Study and does not require ethics approval.

Institutional approval information

The Newcastle upon tyne research office has approved the study (Study ID NuTH 823)

Declaration

Confirm Declaration: Yes

Head of Department

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View application from Peter Szatmary

Created: 30 Jan 2020, 5:13 p.m.

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Abstract

Title of Study	Development, optimisation and validation of a pancreatic spheroid and organoid culture system as a new model for the investigation of acute pancreatitis therapies.
Abstract and methodological description	<p>Title: Development, optimisation and validation of a pancreatic spheroid and organoid culture system as a new model for the investigation of acute pancreatitis therapies.</p> <p>Abstract: Acute pancreatitis is a common cause for hospital admission due to a digestive disease and causes significant morbidity, mortality, and health care costs. The study of acute pancreatitis, including the development of new therapies, is largely dependent on modelling the disease in rodent systems. Differences between mice and men, as well as limitations of the agents used to induce pancreatitis experimentally, have contributed to a translational gap with many therapies showing promise in early trials not producing benefit in patients. Due to difficulties in maintaining pancreatic acinar cells in culture, novel therapeutic agents are mostly tested in whole-animal models using a single pancreatitis toxin. Advances in cell culture, including 3D culture systems with polarised cells and culture under flow conditions, offer the opportunity to rapidly test multiple new therapies using multiple pancreatitis toxins simultaneously. This project will develop and optimise the co-culture of primary murine exocrine pancreatic ductal and acinar cells in spheroids and polarised sandwich culture and validate the system over a 7-day period by comparing response to common toxins to freshly isolated acinar cells. The system will further be developed using primary human exocrine pancreatic cells to further reduce the reliance on murine models and allow rapid testing of new therapies on human tissue.</p>

Background:

Acute pancreatitis is the commonest cause for admission to hospital for a gastro-intestinal illness in the United States of America and a considerable health care burden world-wide [1]. Its anatomical location and high vascularity renders sampling of human pancreatic tissue for experimental purposes impractical and unsafe. While human pancreatic tissue can be obtained from patients undergoing pancreatic resection, acinar cells are difficult to maintain in culture and rapidly undergo either loss of secretory phenotype or apoptosis [2]. The investigation of the pathogenesis and/or therapy for acute pancreatitis is, therefore, heavily reliant on experimental models in rodents, which are of variable relevance to human pancreatitis [3]. Moreover, different models of rodent pancreatitis often produce different results with respect to efficacy of novel therapeutic agents. These and other challenges have led to a translational gap, with many therapeutic agents showing promise in rodents proving ineffective in human trials.

The development of novel culture methods, such as 3D culture or organ-on-a-chip systems, has revolutionised drug and toxicity screening by using primary mouse or human cells to model an increasing number of organs, including liver, kidney and intestine [4]. 3D culture promotes spatial polarisation of cells, a critical feature of pancreatic acinar cells which secrete zymogens across the apical membrane. Indeed, maintenance of primary pancreatic acinar cells in culture has been difficult due to their propensity to autodigest [5] or change towards a ductal phenotype. The perfusion state of organ-on-a-chip systems would be optimally placed to reduce autodigestion by continuous washout of activated proteases. It further permits application of substances without disturbing the target tissue, followed by sampling of culture media to assess tissue response.

This project aims to develop, optimise and evaluate a novel in vitro system of 3D co-culture and compare responses to physiological and pathological stimuli to those seen in freshly isolated cells, enabling future high-throughput preclinical study of novel therapeutic agents.

Hypotheses:

The central hypotheses for this study are:

1. The interaction of matrix-producing stellate cells, ductal cells and acinar cells is essential for the longer-term culture of functional exocrine pancreatic tissue.
2. Pancreatic organoids and/or spheroids in culture for up to 7 days are as effective as freshly isolated acinar cells in modelling cellular mechanisms seen in acute pancreatitis.
3. Culture of pancreatic organoids and/or spheroids under flow conditions promotes polarisation of acinar cells and helps promote long-term culture through washout of zymogens and reduced autodigestion.

Experimental Design, Methods and Timeline:

Work will commence on murine tissue and is to be expanded to include human exocrine pancreatic tissue when available. Specific methods are detailed in the following timeline.

Apr 2020 – May 2020:

Harvest of murine pancreas from naïve CD1 Swiss mice (male and female). Previous experiments have shown significant variability depending on sex of the animal and this setup permits rapid and effective investigation of any true variability.

Isolation and purification of pancreatic ductal (incl lgr5+ stem cells), acinar and stellate cells by fluorescence-activated cell sorting. Maintenance of stellate and ductal cells in monoculture.

May 2020 – Aug 2020:

Growth of pancreatic organoids using lgr5+ ductal stem cells and growth-factor (EGF) enriched culture conditions according to established protocols. This method will lead to the growth of ductal organoids.

Milestone 1: Established monoculture of purified primary pancreatic stellate and ductal cells as well as ductal organoids.

Aug 2020 – Nov 2020:

Optimisation of acinar cell co-culture conditions, including sandwich culture (acinar cells with

ductal/stellate cells), magnetic 3D spheroid culture, perfusion culture. Apoptosis/necrosis (Annexin V/propidium iodide assays) and phenotype (blebbing, loss of secretory granules) will be checked daily.

For sandwich culture, freshly isolated pancreatic acinar cells will be selectively seeded onto a matrix-embedded (Collagen; Matrigel) mixture of ductal and stellate cells in different ratios, or onto the surface of stem cell-derived ductal organoids.

Magnetic spheroids will be generated using the Greiner Mag3D culture system by coating isolated cells in inert iron oxide nanoparticles and culturing on top of a solid-state magnet.

Perfusion culture will use the Mimetas OrganoPlate system which maintains a gravity-assisted perfusion by rocking a specially etched culture plate containing two reservoirs per well (40 wells per plate) connected by a microfluidic channel. It has 2 further channels in continuity with the culture chamber, allowing simultaneous application of drugs/toxins and sampling of media.

Milestone 2: Established culture system, which maintains murine pancreatic acinar cells for up to 7 days with visual characteristics consistent with healthy cells and secretory phenotype.

Nov 2020 – Mar 2021:

Characterisation of the response to physiological and pathological stimuli (acetyl choline, cholecystokinin, tauro lithocholate-sulphate, fatty acid ethyl ester) using established toxicity assays at each of the first seven days in culture, comparing findings to freshly isolated murine pancreatic acinar cells.

This will include measurements of physiological and pathological calcium signalling in response to stimuli using confocal microscopy, cell death and apoptosis assays in response to lethal doses of pancreatitis toxin and measurement of activity of secreted proteases (amylase, lipase, trypsin/chymotrypsin) on each of the first 7 days of culture and comparison of findings to fresh pancreatic acinar cells isolated in the standard manner of collagenase digestion. Proportion of ductal metaplasia will be quantified using immunocytochemistry with anti-amylase and cytokeratin19

antibodies.

Milestone 3: Acquisition of data for the first 7 days in culture for the physiological and pathological behaviour of murine pancreatic acinar cells in the optimal culture system defined in milestone 2 compared to freshly isolated acinar cells.

Uniquely in Liverpool we have intermittent access to human pancreas tissue from patients undergoing pancreatic resection. This will be incorporated and assayed in parallel with murine tissues during experiments for milestones 2 and 3.

Risks and assumptions:

Reaching milestone 1 depends on replication of established procedures from other units as well as common techniques in cell biology. Pancreatic ductal organoid stem cells and growth medium can also be purchased commercially, which will act as a fall-back plan if significant problems are encountered.

Milestone 2 (7-day culture) has been achieved using acinar cell monocultures and specific growth factor-enhanced media. We hope to improve on this system by increasing functional longevity of acinar cells, establishing directional polarisation and prolonging a secretory phenotype, while reducing apoptosis/necrosis and ductal metaplasia.

Milestone 3 builds on the achievable goals of milestone 1 and 2 and establishes a phenotypic and functional profile of cultured acinar cells over one week of maintenance culture for the first time, using well established, frequently used (in our unit) and validated assays.

Details of Funds Requested:

Equipment:

Mimetas OrganoFlow Perfusion Rocker £995
GreinerBio 24-well magnetic 3D culture assembler
£1050

Consumables & reagents:

Cell culture plastic, perfusion culture plates (x6) £2500

Culture media, supplements, growth factors (EGF, FGF) £1800
Pancreatitis toxins (ACh, CCK, TLC-S, FOAEE) £600
Fluorescent dyes (propidium iodide, Fluo-4AM) £350
Assays (annexin V, elastase, trypsin/chymotrypsin, amylase) £1000
Antibodies (amylase; GFAP; Cytokeratin) £950

Animals:

CD1 mice (30x, purchase, housing and shipment)
£500.35

Total: £9745.35

Case for Funding:

Animal use and species:

Investigation of human acute pancreatitis currently necessitates animal models as it is impractical to safely sample human pancreatic tissue for use in high-volume assays. The mammalian pancreas is structurally and functionally different to that of other vertebrates, making murine models of experimental acute pancreatitis the most widely utilised. We have chosen CD1 mice as they are an outbred strain and have a higher body weight (and pancreatic mass) than other commonly used strains of mouse.

This project aims to replace whole animal experiments, often where only one substance/pathway is investigated per animal, with effective in vitro assays where the cell yield from a single animal leads to reproducible data investigating multiple therapies/pathways at any one time, thus reducing the need for whole animal experiments in early investigative work. This will form the basis for future grant applications to the 3Rs and the MRC for further development of the murine system as well as a parallel system of primary human acinar cell culture.

Equipment and consumables:

A common problem with culture of pancreatic acinar cells is that there is ongoing release and autoactivation of digestive enzymes in culture systems. This can be partially overcome by the addition of protease inhibitors, but one of the

hypotheses of this project is that acinar cell culture benefits from flow conditions through the washout of digestive enzymes from the cellular milieu. There are a number of perfusion/organ-on-a-chip systems currently available, however we have opted for the Mimetas OrganoPlate system for this set of experiments. This works by gravity-assisted, low velocity flow that has been demonstrated to be able to polarise vascular endothelial cells. This project will utilise the currently available 3-lane plate, which allows culture of cells in the middle lane, addition of toxins/drugs in the top lane and simultaneous sampling of media from the bottom lane to investigate cellular responses such as zymogen release. This system allows for 40 simultaneous experimental conditions on one plate, meeting our stated goal of developing a high-throughput system able to investigate multiple experimental conditions (including multiple models of pancreatitis) simultaneously.

Experience from work with other tissues, including liver, has demonstrated that parenchymal cell culture is improved both in physiological and pathological conditions by the addition of non-parenchymal support cells. This can be gradual, by sequential seeding of cells in sheets of collagen matrix, or rapidly induced using external forces. Magnetic culture works by coating cells in inert iron oxide nano-particles and then bringing them together into a spheroid by culturing them on top of a solid state magnet. This way, 3D spheroids are formed within 24 hours, leading to the generation of tight junctions and other 3D structures (such as bile canaliculi in liver) not seen in regular 2D co-culture. The rapid formation of 3D structures makes this an ideal method to investigate with exocrine pancreatic cells, which are known to deteriorate in structure and function much more rapidly than other cell types.

The choice of culture media, growth factors, pancreatitis toxins and functional assays are based on published and established methodologies of pancreatic acinar cell culture and experimental acute pancreatitis.

References:

1. Petrov, M.S. and D. Yadav, Global epidemiology and holistic prevention of pancreatitis. Nat Rev

	<p>Gastroenterol Hepatol, 2019. 16(3): p. 175-184.</p> <p>2. Fleming Martinez, A.K. and P. Storz, Mimicking and Manipulating Pancreatic Acinar-to-Ductal Metaplasia in 3-dimensional Cell Culture. J Vis Exp, 2019(144).</p> <p>3. Lerch, M.M. and F.S. Gorelick, Models of acute and chronic pancreatitis. Gastroenterology, 2013. 144(6): p. 1180-93.</p> <p>4. Breslin, S. and L. O'Driscoll, Three-dimensional cell culture: the missing link in drug discovery. Drug Discov Today, 2013. 18(5-6): p. 240-9.</p> <p>5. Blauer, M., et al., A novel explant outgrowth culture model for mouse pancreatic acinar cells with long-term maintenance of secretory phenotype. Eur J Cell Biol, 2011. 90(12): p. 1052-60.</p>
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Timetable

Name	Isolation, purification and maintenance of murine (CD1 Swiss) pancreatic acinar cell monocultures (stellate cells, ductal cells, Igr5+ stem cells).
Date	April - May 2020

Name	Culture of pancreatic ductal organoids (reproduction of published protocols and optimisation of conditions).
Date	May - August 2020

Name	Optimisation of co-culture (stellate/ductal and acinar cell) - sandwich culture, magnetic co-culture and perfusion culture.
Date	August - November 2020

Name	Comparison of responses to physiological (CCK, ACh) and pathological (CCK, TLC-S, fatty acid ethyl ester) stimuli in each of first 7 days of culture versus freshly isolated pancreatic acinar cells.
Date	November 2020 - March 2021

Funding

Name	Mimetas OrganoFlow Perfusion Rocker
Amount	995.0

Name	GreinerBio 24-well magnetic 3D culture assembler kit
Amount	1050.0

Name	Cell culture plastic, incl. perfusion culture plates (x6)
Amount	2500.0

Name	Culture media, supplements and growth factors (EGF, FGF)
Amount	1800.0

Name	Pancreatitis toxins (ACh, CCK, TLC-S, FAOEE)
Amount	600.0

Name	Fluorescent dyes (propidium iodide, Fluo-4AM)
Amount	350.0

Name	Assays (annexin V, elastase, trypsin/chymotrypsin, amylase)
Amount	1000.0

Name	Antibodies (amylase, GFAP, cytokeratin)
Amount	950.0

Name	CD1 mice (30x; purchase, housing and shipment)
Amount	500.35

Details of ethical approval

The project has been designed in order to reduce the need for animal experiments in acute pancreatitis, replace animal tissues with primary human cells and refine exocrine pancreatic cell culture methods for both species.

It has been reviewed and approved by the Animal Welfare and Ethical Review Body of the University of Liverpool (AWERB Ref: AWC0166).

The acquisition of primary human cells from pancreas resection patients has previously been approved (LREC Ref: 03/12/242A) and is currently in the process of being renewed.

Institutional approval information

This proposal has undergone a formal Institutional review process and has received the support of the finance department as well as the head of department.

Mr Keith Huckle was the Research Support Officer at the University of Liverpool checking the budget for the application, and found that "... the figures provided are accurate and reflect the true cost of the proposed work." (RSO Ref: 166130).

Prof Paul Ghaneh (Director of the Centre for Pancreatic and Hepatobiliary Disease) has lent this project her support and approved the project on behalf of Prof Andrew Pettitt as head of the Department of Molecular and Clinical Cancer Medicine (Ref: 166131).

Declaration

Confirm Declaration: Yes

Head of Department

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