Assessment and Optimisation of the Pancreas Allograft

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“Research is to see what everybody else has seen, and to think what nobody else has thought”

Albert Szent-Gyorgyi

16 September 1893 - 22 October 1986

Budapest, Hungary

DEDICATIONS

To my wife Ianire Irastorza and parents, Pramukh and Jayna Dholakia, thank you for putting up with my absence from home, grumpy, sleep deprived and hungry moods. You are always there to push me forward when I fall behind and pick me up when I am down, you are the source of my strength. I love you all very much, feeling very blessed to have you in my life.

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FORWARD

This thesis is a culmination of work performed during my DPhil at the University of Oxford 2015 to 2018, with the objective of the thesis being to improve whole organ pancreas transplantation, through optimisation and improved assessment of the allograft as well as reducing associated early morbidity following transplantation.

The structure of this thesis is influenced by the supervision, guidance and knowledge I have gained across the various projects I have performed. The introduction in the first chapter sets the overall theme for the thesis, while the second chapter draws on national data sets that have undergone new analysis to define the focus of the subsequent chapters. In chapters three and four, I have investigated potential markers for assessment and optimisation, as well as considered endpoints for future interventions of transplant pancreatitis. Chapter five shows more experimental results, finsihing with chapter six demonstrating the future avenues of research drawing on all the work, tying the threads together to offer a study design as the next step, as well as further ideas for future research and ending with some thesis conclusions.

Work done in collaboration

Some of the work described in this thesis has been performed in collaboration with others. I would like to acknowledge the following individuals for their help:

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ABSTRACT

Background

In this thesis, I have sought to improve the understanding of how pancreases are assessed with a particular focus on organ steatosis and allograft pancreatitis. Whole organ pancreas transplantation is a transformative treatment option, but despite the benefits since its inception, little evolution has been made tackling the change in donor habitus. With continued high morbidity and falling rates of referral, pancreas transplantation remains a niche treatment rather than a mainstay for patients with diabetes.

Aim

The key aim of the thesis is to assess current trends in pancreas organ utilisation nationally, and see how these compare with other international programs. I have started off by identifying areas of optimisation and considering factors that may be relevant, and I have then applied particular focus on assessment in regards to pancreas steatosis and associated allograft pancreatitis, thereafter focusing on how better to assess and optimise patients with this morbidity.

Results

- Lack of pancreases is not the primary issue when considering utility. Assessment of fatty pancreases in the context of organ steatosis is a key factor in the decision-making process with a very high percentage of donor organs assessed as unfit for purpose.
- Pancreas steatosis is common, heterogeneous and focal, with the use of MRI being quantitative and potentially clinically useful.

- Allograft pancreatitis is a common morbidity with an incidence of 44%. Day 5 CRP appears to be a suitable surrogate marker, with alternative methods of assessment also possible with further work (FACBC and glutathione).
- Inflammatory patterns suggest allograft pancreatitis may be a distinct process to native pancreatitis with classical monocytes and macrophages playing an important role.

Conclusion

Current donor utilisation and methods of assessment are imprecise, in particular, the causal associations between pancreas steatosis and the adverse outcome remains unproven. The need to minimise postoperative morbidity has clear merit with early organ dysfunction, due to allograft pancreatitis being shown to be associated with long-term graft loss. There is a clear clinical need to identify and test strategies that abrogate early graft inflammation in order to improve outcome.

ABBREVIATIONS

ACSL	acyl-CoA synthetase long-chain
ALT	Alanine Transaminase
AP	Acute Pancreatitis
Ap	Adipocyte protein
APC	Antigen Presenting Cell
AR	Acute Rejection
AST	Aspartate Transaminase
ATP	Adenosine Triphosphate
BMI	Body Mass Index
CASR	Calcium-Sensing Receptor
CCK	Cholecystokinin
CCL	C-C motif
CD	Cluster of Differentiation
CDC	Centre for Disease Control and prevention
CEL	Carboxyester lipase
CFTR	Cystic fibrosis transmembrane conductance regulator
CI	Confidence Interval
CIT	Cold Ischaemia Time
CMV	Cytomegalovirus
Cnos	Constitutive Nitric Oxide Synthase
CRAC	Calcium release-activated channel
CRP	C-reactive protein
CT	Computed Tomography
CTRC	Chymotrypsinogen C
CTRG	Clinical Trials and Research Governance
CTSI	Computed Tomography Severity Index
CXC	C-X-C motif
DAMP	Damage associated molecular patterns
DBD	Donor after Brain Death
DCD	Donor after Cardiac Death

dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DsDNA	Double Stranded Deoxyribonucleic acid
DSG	15-Deoxyspergualin
DSUR	Development Safety Update Report
ECAM	Epithelial Cell Adhesion Molecule
EIT	Electrical Impedance Tomography
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	endothelial Nitric Oxide Synthase
FABP	Fatty Acid Binding Protein
FACBC	Anti 1-amino-3-(18)-Flurocyclobutane-1-carboxylic acid
FOXp3	Forkhead box P3
GCP	Good Clinical Practice
H_2O_2	Hydrogen Peroxide
HLA	Human Leucocyte Antigen
ICAM	Intercellular Adhesion Molecule
ICH	International Conference on Harmonisation
IFN-γ	Interferon Gamma
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
IPTR	Internation pancreas transplant registry
IQR	Interquartile Range
IRI	Ischaemia Reperfusion Injury
IRS	Insulin Receptor Substrate
IVC	Inferior Vena Cava
Iκβ	Inhibitor of kappa-light-chain-enhancer of activated B cells
JAK	Janus Associated Kinase
MAPK	Mitogen-Activated Protein Kinase
MCP	Monocyte Chemoattractant Protein
MHC	Major Histocompatibility Complex

MHRA	Medicines and Healthcare Products Regulatory Agency
MIF	Macrophage Migration Inhibitory Factor
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAFLD	Non Alcoholic Fatty Liver Disease
NAFPD	Non Alcoholic Fatty Pancreas Disease
NEFA	Non Esterified Fatty Acid
NET	Neutrophil Extracellular Trap
NF-κβ	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NHSBT	National Health Service Blood and Transplant
NIH	National Institute of Health
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NRES	National Research Ethics Service
OPTN	Organ Procurement and Transplant Network
PAF	Platelet Activating Factor
PAK	Pancreas after Kidney
PARP	Poly ADP Ribose Polymerase
PCR	Polymerase Chain Reaction
PDRI	Pancreas Donor Risk Index
PDTC	Pyrrolidine Dithiocarbamate
PEG	Polyethene Glycol
PET	Positron Emission Tomography
PLRP	Pancreatic Lipase-like Protein
PPARγ	Peroxisome Proliferator-Activated Receptor gamma

PRSS1	Trypsin 1
PSC	Pancreatic Stellate Cell
PTA	Pancreas transplant alone
PTL	Pancreatic Triglyceride Lipase
R&D	NHS Trust R&D Department
REC	Research Ethics Committee
RNS	Reactive Nitrogen Species
ROCK	Rho-associated Protein Kinase
ROS	Reactive Oxygen Species
SABTO	Advisory committee on the Safety of Blood Tissue and Organs
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
SICAM	Soluble Intercellular Adhesion Molecule
SIRS	Systemic Inflammatory Response Syndrome
SPINK1	Serine Protease Inhibitor Kazal type 1
SPK	Simultaneous Pancreas Kidney Transplant
SPLK	Simultaneous deceased donor Pancreas and Live donor Kidney
SRTR	Scientific Registry of Transplant Recipients
STAT	Signal Transducer and Activator of Transcription
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TAP	Trypsinogen Activation Peptides
TH	T Helper
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
UFA	Unsaturated Fatty Acid
UK	United Kingdom
US	United States
USA	United States of America

Publications from thesis

1. Pancreas Transplantation: Past, Present, Future. *Dholakia S, Mittal S, Quiroga I, Gilbert J, Sharples E. Ploeg RJ, Friend PJ. Am J Med. 2016 Jul; 129(7):667-73. PMID 26965300.*
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4. Significance of steatosis in pancreatic transplantation. *Dholakia S, Sharples EJ, Ploeg RJ, Friend PJ. Transplant Rev (Orlando) 2017 21(4):225-231. PMID 28855081.*
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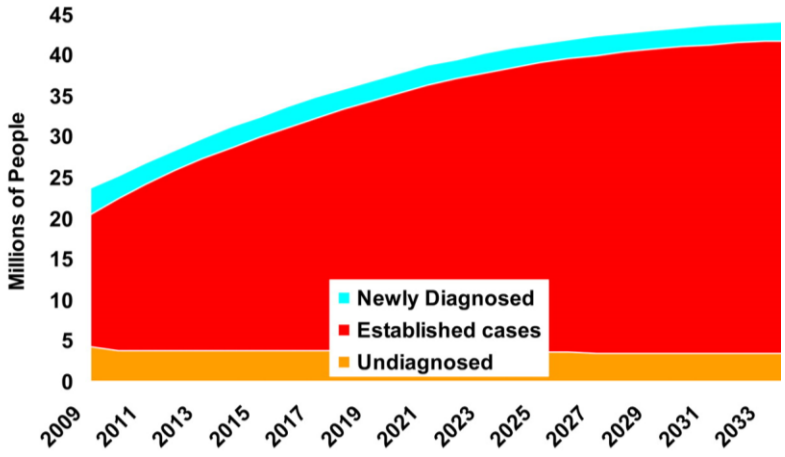
CHAPTER 1:

General introduction

1.1 Overview of diabetes

The National Institutes of Health (NIH) found that from 2002 to 2012, the rate of new diagnosed cases of type 1 diabetes mellitus (T1DM) increased by about 1.8% each year, while new diagnosed cases of type 2 diabetes (T2DM) increased even more quickly, at 4.8%, with T2DM regarded as the pandemic of the modern era [1]. Globally, the overall number of people with diabetes rose from 108 million in 1980 to 422 million in 2014 [2] (see Figure 1.1.1).

Figure 1.1.1: Projected distribution of newly diagnosed, established cases and undiagnosed cases of diabetes from 2009 to 2034. Image source: [3]

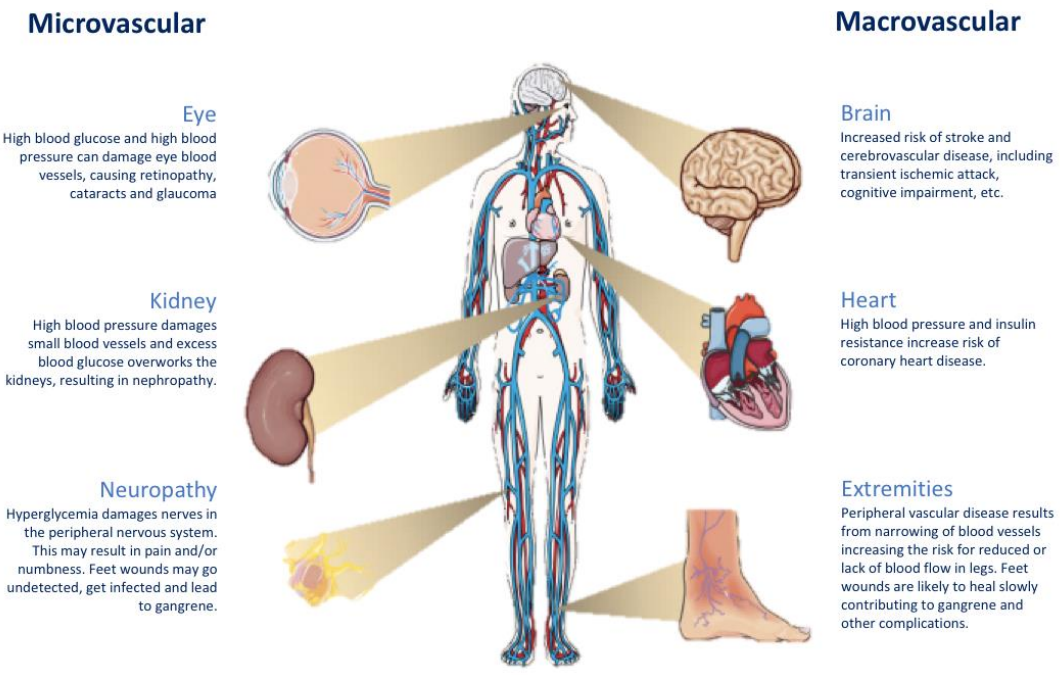


These figures mirror the current UK picture, with 1.4 million people with diabetes today expected to rise to 5 million sufferers by 2025. 10% of these patients have T1DM, of which 29,000 are children [2]. The UK rate of T1DM is double that of France and Italy and is the fifth highest worldwide, according to an international league table compiled by Diabetes UK [4].

T1DM is an autoimmune disease where the insulin-producing beta cells in the pancreas are destroyed and as a result, patients require insulin. T2DM is characterised by insulin resistance where the beta cells are overworked, and eventually can become damaged. Acute diabetic complications include diabetic ketoacidosis and non-ketotic hyperosmotic coma, affecting T1DM and T2DM respectively. Serious chronic complications include microvascular and macrovascular disease such as heart disease, stroke, renal failure, blindness, neuropathy and peripheral vascular disease [5].

Diabetic complications can be classified as primary, those that relate to problems of glucose control (typically hypoglycaemia unawareness or diabetic ketoacidosis), and secondary which include renal failure, cardiovascular disease, retinal disease, peripheral and autonomic neuropathy [2] (see Figure 1.1.2).

Figure 1.1.2: Major microvascular and macrovascular complications associated with diabetes. Image redrawn using adapted parts from Servier Medical Art.



The standard treatment for many people with diabetes includes therapeutic interventions to lower blood sugar levels through either oral or injected medication. Injectable exogenous insulin has been available for patients with diabetes since 1922, and the majority of patients remain reasonably (although not perfectly) controlled on this basis. Despite this, a proportion of T1DM sufferers still develop life-threatening complications, even when following the most careful insulin regimes and often benefit from continuous subcutaneous insulin infusion (CSII) [6].

The first insulin pump was developed by Dr. Arnold Kadish in 1963, utilising rapid acting insulin, but required patients to calculate the insulin boluses needed for food coverage and high glucose readings. Technological capabilities have advanced dramatically since then, with current pumps able to deliver basal insulin in increments of as little as 0.01 units per hour, and using automatic bolus insulin calculators [7].

The Diabetes Control and Complications Trial (DCCT) demonstrated the importance of intensive insulin therapy to delay the onset and slow the progression of diabetic microvascular complications such as retinopathy, nephropathy, and neuropathy [8].

Steineck et al. evaluated over 18,000 T1DM patients on CSII versus multiple daily injections where the authors found that CSII was associated with a 45% reduction in fatal coronary heart disease, a 42% reduction in fatal cardiovascular disease, and a 27% reduction in all-cause mortality [9].

Current advances in self-monitoring of blood glucose include meters that are able to communicate the readings directly and wirelessly to the pump via infrared or bluetooth

technology, thus eliminating manual glucose value entry into the pump. With implantable subcutaneous skin sensors such as the 14 day Abbott Freestyle Libre or the newly FDA approved Eversense system which can be worn up to 90 days, the ability to continually measure glucose levels is possible [6] [10].

The Medtronic Minimed and the Animas Vibe brands are examples of more commonly prescribed insulin pumps. With touch screen technology being incorporated in the Tandem Diabetes Care's T-Slim insulin pump and tubeless and fully disposable pumps also in existence (Omni Pod), pumps are now a desirable option for many patients [10].

The ability to fully mimic physiological demands using integrated and augmented glucose biosensors to providing real-time, data-driven glycaemic control and early detection of hypoglycaemia, creates the prospect of a functional, closed-loop "artificial pancreas" in the near future.

However, for a proportion of patients who chose not to pursue CSII or continue to have uncontrolled diabetes despite its use, solid organ pancreas or pancreatic islet transplantation is an option. The majority of these patients have T1DM, although some T2DM suffers have also benefitted from transplantation.

1.2 Overview of pancreas transplantation

Over 50,000 pancreas transplants have been recorded worldwide, predominantly for the treatment of T1DM [11]. Prolonging and improving the quality and duration of patients' lives makes pancreas transplantation a justifiable NHS funded treatment for severely

disadvantaged patients who have the most severe complications of diabetes. Currently, there are several types of beta cell replacement of which the main types include [12]:

1. **Simultaneous pancreas-kidney transplant (SPK)**: This is indicated in patients with chronic renal failure and diabetes. Organs are transplanted from the same deceased donor. UK organ allocation rules stipulate that the patient must have an estimated glomerular filtration rate of less than 20 ml/min/1.73m^2.
2. **Pancreas transplant alone (PTA):** Primarily for T1DM with frequent and life-threatening episodes of hypoglycemia unawareness. PTA patients reliably achieve euglycaemia and have a well-preserved renal function. This procedure is an alternative to pancreatic islet transplantation.
3. **Pancreas-after-kidney transplant (PAK):** Deceased donor pancreas transplant is performed after a previous living or deceased donor kidney transplant. Pancreatic-after-kidney transplant is indicated for patients with T1DM and a functioning renal transplant.
4. **Simultaneous deceased donor pancreas and live donor kidney (SPLK):** Only a few cases have been documented, and this procedure is now rarely performed. In theory, these patients benefit from a lower rate of delayed graft function than SPK recipients and significantly reduced waiting times for the kidney, resulting in improved outcomes compared to patients waiting for a SPK. However, surveillance for rejection is more complicated with tissue from more than one donor.
5. **Islet transplantation (isolated)**: Islet cells from donor pancreases are isolated from single or multiple (sequential) organs, and then injected into the liver of the recipient where islet cells engraft. Primarily indicated for T1DM with frequent and life-threatening episodes of hypoglycaemic unawareness, this procedure is less invasive and associated with much lower morbidity than solid organ transplantation. These patients

are required to have well-preserved renal function and may also be considered for transplantation of an isolated pancreas, either as an alternative or if islet transplantation is unsuccessful.

6. **Simultaneous islet and kidney transplantation**: There is a subset of patients with diabetes with chronic renal failure who have been considered for simultaneous kidney and pancreas (SPK) transplantation but categorised unsuitable due to co-morbidity or technical barriers. The alternative options for this cohort of patients are either kidney transplantation alone or islet after a kidney transplant.
7. **Islet after kidney transplantation:** This procedure may be considered for patients with type 1 diabetes who have undergone successful kidney transplantation and would benefit from beta cell replacement, but are unfit for whole organ transplantation.

1.3 History of whole organ transplantation

The first successful human pancreas transplant was carried out on December 12th, 1966 at the University of Minnesota by the team of Doctors Kelly, Lillehei, Merkel, Idezuki and Goetz. A pancreas with donor duodenum was transplanted together with a kidney into a 28-year-old woman who became insulin independent. However, she died shortly after due to pulmonary embolism [13].

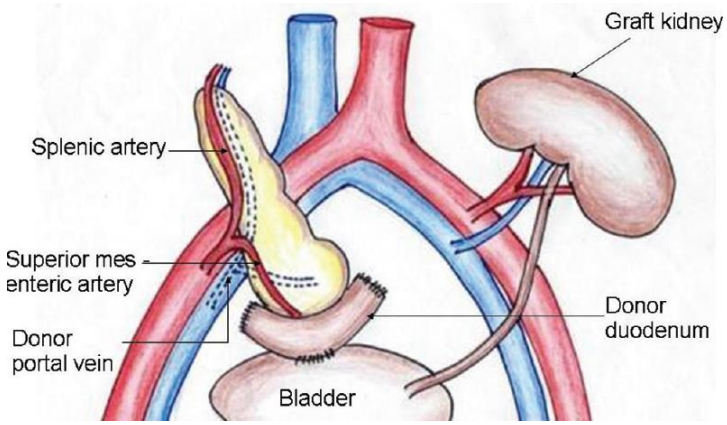
Deemed technically feasible, a series of whole organ pancreas transplantation began but due to the lack of potent immunosuppressive drugs, rejection and infections were common. Numerous techniques for diverting or dealing with the pancreas secretions were described, the variety of which attests to the fact of none of them being satisfactory. Broadly, the pancreas was transplanted as a 'segmental' graft (body and tail re-vascularised via the donor splenic vessels) or a whole graft (re-vascularised via the coeliac and superior mesenteric

arteries and the portal vein). The exocrine drainage was managed by obliteration of the pancreatic duct (in segmental grafts, using latex), by enteric drainage (by anastomosis of the donor duodenum or a 'button' of the duodenum to the patient's jejunum), or by bladder drainage. In the early days, management of the exocrine drainage earned the reputation of being the 'Achilles heel' of pancreas transplantation.

Between the late 1970s and late 1980s, three major events happened which boosted the development of pancreas transplantation: 1) The introduction of cyclosporine A 2) The renewed interest in urinary drainage of exocrine secretions 3) An emerging consensus that the whole pancreatico-duodenal block should be transplanted [14].

Exocrine pancreas drainage using duodeno-cystostomy allowed urinary amylase to be measured as a way of monitoring the pancreas graft (see Figure 1.3.1).

Figure 1.3.1: A diagrammatic overview of bladder-drained SPK transplant, where donor duodenal conduit is anastomosed to the recipient's bladder. Image source: [15]

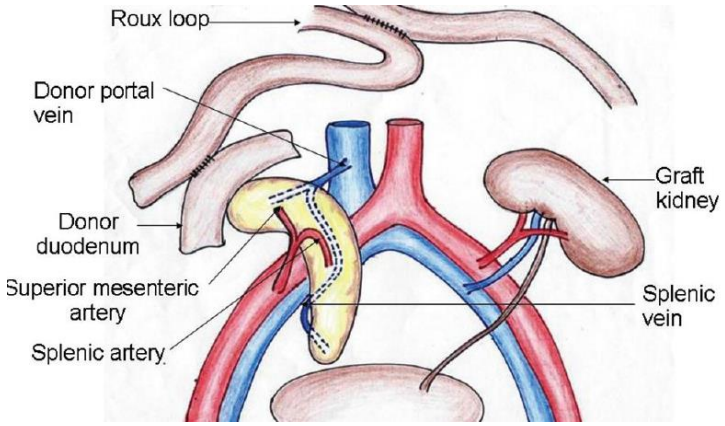


However, the loss of sodium bicarbonate in the urine resulted in severe metabolic acidosis and, not infrequently, in extracellular volume depletion requiring frequent hospitalisation.

Other complications associated with bladder drainage included chemical cystitis, urethritis, bladder leak, reflux pancreatitis, recurrent infections, bladder cancer, bladder stones, urethral strictures, urethral irritation, epididymitis, prostatitis, and prostatic abscess [16] [17].

During the 1990s improvements in immunosuppression, particularly the advent of tacrolimus and mycophenolate, greatly reduced the risk of rejection and rendered more feasible the prospect of enteric drainage, effectively removing the complications of urinary drainage listed above. However, it removed the ability to follow graft function through measurements of urinary amylase. Here, the head of the pancreas is joined to the small intestine using a donor duodenal conduit (see Figure 1.3.2).

Figure 1.3.2: A diagrammatic overview of a SPK with an enterically drained pancreas, where donor duodenal conduit is anastomosed to recipient small bowel. Image source: [15]



Whole pancreas transplantation with enteric diversion has become the gold standard for SPK propelling expansion of pancreas transplantation. The other technical issue which continues to be debated and remains unresolved, includes the site of venous drainage. Although drainage into the portal circulation of the recipient is more physiological than into the systemic circulation, the former is more technically complex and has been adopted by a

small proportion of surgeons. There remains little evidence of a substantial or functional benefit, with many practitioners still opting to drain pancreas venous effluent for SPK, PTA and PAK, systemically into the IVC or common iliac vein [18].

1.4 Benefits of pancreas transplantation

Pancreas transplantation restores glucagon secretion, returns hepatic glucose production to normal, and improves lipid profiles and insulin-mediated protein kinetics [12]. As mentioned previously, some debate remains around the use of venous drainage via the portal vein as opposed to the IVC. What is more certain is that patients who undergo whole organ transplant seem to have clinical improvements in diabetic nephropathy [19] [20], neuropathy [21] [22], gastroparesis [23] [24], retinopathy [25] [26] (microvascular and macrovascular disease), cardiac function [27] [28] and sexual function [29]. There is also some evidence that shows how in patients with established diabetic nephropathy, there is an element of reversal in glomerular and cortical lesions, with one study showing almost complete normalisation of glomerular structure 10 years' post pancreas transplant [20]. The restored physiological control offered by pancreas transplantation supports the idea that restored glycaemic control, as CSII pumps aim to do, is beneficial [30]. However, the randomised control trial between insulin pumps and pancreas transplantation has not yet been done and so, it remains unknown which may offer better control.

1.5 Pancreas transplant survival

The International Pancreas Transplant Registry (IPTR) uses insulin independence as the definitive criterion for graft survival. Graft survival rates for SPK now reach more than 91% at 1st year after transplant, with PAK transplantation reaching 86% graft survival and PTA 85% at 1 year [31]. The longest surviving grafts recorded in this registry are 26 years, 24

years and 23 years for SPK, PAK and PTA respectively [8]. Table 1.5.1 shows the IPTR 5-year and 15-year graft survival rates following pancreas transplantation based on data from 1995-2015.

Table 1.5.1: IPTR 5-year and 15-year graft survival rates following pancreas transplantation based on data from 1995-2015 [11].

IPTR Graft Survival	SPK	PTA	PAK
5yr	71%	53%	57%
15yr	40%	18%	11%

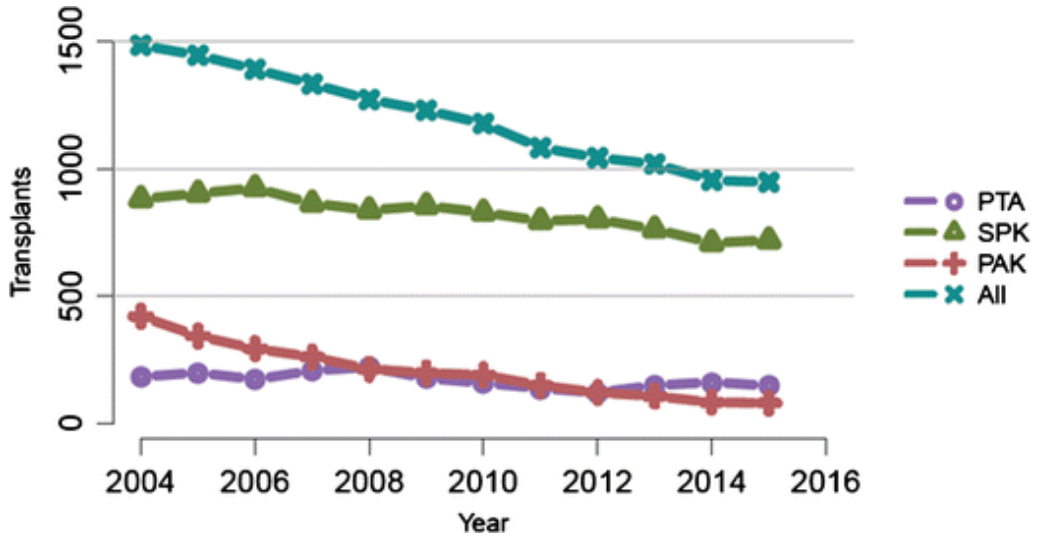
Pre-emptive SPK transplant recipients have better graft and patient survival outcomes compared to those already established on dialysis, but figures are likely to be affected by lead-time bias, and so further studies are warranted. The main advantage of SPK transplantation compared with PAK, apart from it being a cost-effective option [32], is the overall success rate of graft survival as acute rejection (AR) is assumed to be easier to detect. However, organ rejection is not always concordant and AR rates remain similar for SPK and PAK, with Dong et al. reporting the cumulative incidence for AR being 14.7%, 19.7%, 26.6% and 29.1% at 1, 2, 5 and 10 years, based on a retrospective sample of 56 SPK, 69 PTA and 102 PAK transplants [33].

1.6 Global pancreas transplant trends

Stratta et al. have identified that in 2014, 954 pancreas transplants (709 SPKs, 125 PTAs, and 120 PAKs) were performed in the United States (US) [34] [35]. During the same year, 194 pancreas transplants (175 SPKs 19 PTAs) were reported to the Eurotransplant Registry [36]. From 2004 to 2011, the annual number of SPK transplants in the US declined by 10%, whereas the corresponding annual decreases in PAK and PTA were 55% and 34%

respectively. Overall, the number of pancreas transplants have decreased by approximately 20% between 2005 and 2014 [37] (see Figure 1.6.1). In the UK, NHS blood and transplant (NHSBT) data from 2016, also shows the number of patients on the active pancreas list has decreased by 8% to 199, while the active islet transplant list has decreased by 22% to 28 patients in the same time period [38].

Figure 1.6.1: Declining US pancreas transplant volumes 2004-2015. Adapted from OPTN/UNOS data and ATC 2015 abstract 'Why are pancreas transplant volumes declining?' Carrico R, Fridell J, Odorico J et al. and image source [34].



The reasons for this decline are complex but clearly, the number of suitable patients referred to a service is a key consideration. Suggested reasons include: (i) improved non-transplant management of complex diabetes including the use of insulin pumps and continuous glucose monitoring devices (ii) a substantial increase in the proportion of suboptimal donor organs and (iii) insufficient referrals to transplant units [37].

1.7 Current issues with pancreas transplantation

A key requirement to promote the growth of pancreas transplantation is overcoming the current morbidity and mortality associated with the procedure. Allograft pancreatitis, graft thrombosis, and longer stay in the hospital due to complications requiring further intervention, all contribute to poorer outcomes. In addition to the medical implication, additional morbidity also has measurable financial implications. For these reasons, clinicians have been selective in the use of organs which are deemed optimal (although the criteria used are largely unproven and partly subjective). In the context of the short waiting lists for pancreas transplantation when assessing potential risk versus benefit, current practice has resulted in a conservative approach to donor criteria [39].

There is no doubt that this risk-adverse strategy has affected acceptance and transplant rates, resulting in lower organ utilisation and also higher organ discard rates in many transplant centres. In the UK, many retrieved organs are documented to have been discarded based on their fatty appearance (described as and inferring steatosis), damage during organ procurement or issues in the donor history [40]. These reasons are mirrored across the globe with both the Eurotransplant 2015 annual report showing an overall rate of pancreas organ discard of 78% (945 offers, 544 accepted, 259 transplanted) [36] and the US Organ procurement and transplant network/scientific registry of transplant recipients (OPTN/SRTR) 2015 report publishing a rate of discard of high BMI donor organs of approximately 50% [41].

The significance of steatosis in pancreas transplantation remains poorly understood, firstly because the terms ‘steatosis’ or ‘fatty’ have been used interchangeably to describe both macroscopic appearance of pancreases, as well as parenchymal fat deposits within pancreases. The reasons for this interchange are due to the concept that macroscopic

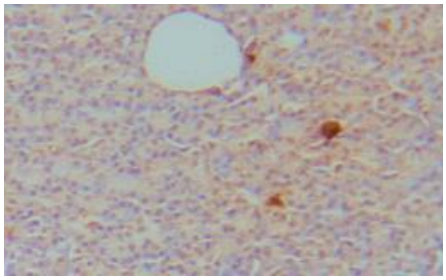
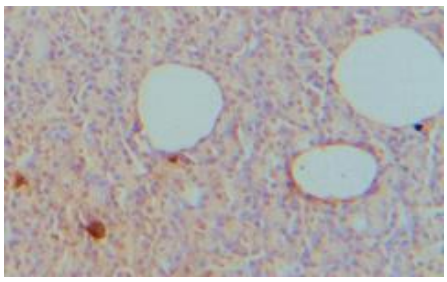
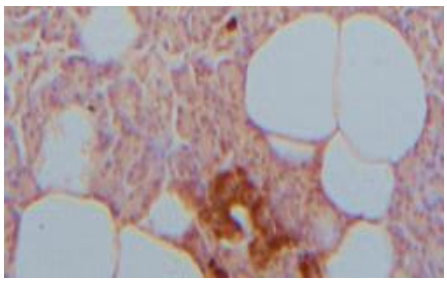
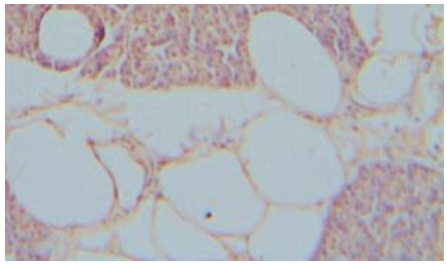
appearance is thought to relate to the intra-parenchymal appearance [42]. However, in the context of pancreas transplantation, the term steatosis should strictly be used in reference only to the ectopic deposition of adipose tissue within the pancreas parenchyma, while descriptive terms such as fatty may be in reference to the macroscopic appearance.

Currently, fatty pancreases are less often used for solid organ transplantation, due to mixed evidence that suggests that patients transplanted with fatty organs have poorer outcomes due to increased pancreas steatosis. This appears to corroborate evidence from animal models which suggested that when steatotic organs are placed under conditions of oxidative stress (common in transplant), fat-derived cytokines may be released and promote a local inflammatory process resulting in organ dysfunction [43].

1.8 Pancreatic steatosis

Pancreatic steatosis was first described by Ogilvie in 1933, where the degree of pancreatic adiposity was found to be higher in obese patients compared to non-obese subjects [44]. In 1978, Olsen evaluated the presence of pancreatic steatosis in 394 autopsies, grading pancreatic fat into four categories. Grade 1 showed sections with few scattered fat cells in the exocrine parenchyma while grade 4, had partial or total replacement of exocrine lobules with fatty tissue (see Table 1.8.1). Ogilvie demonstrated a correlation between increased age and being overweight with increased fat content in pancreatic tissue [45].

Table 1.8.1: Histological appearance of tissue describing the Olsen pancreas steatosis grading. The tissue has been stained with haematoxylin, insulin, and perilipin. Pancreas parenchymal tissue is pink, islets are identified as brown stained tissue containing insulin (>10cells), and adipocytes appear as white globular structures with golden perilipin stained rim.

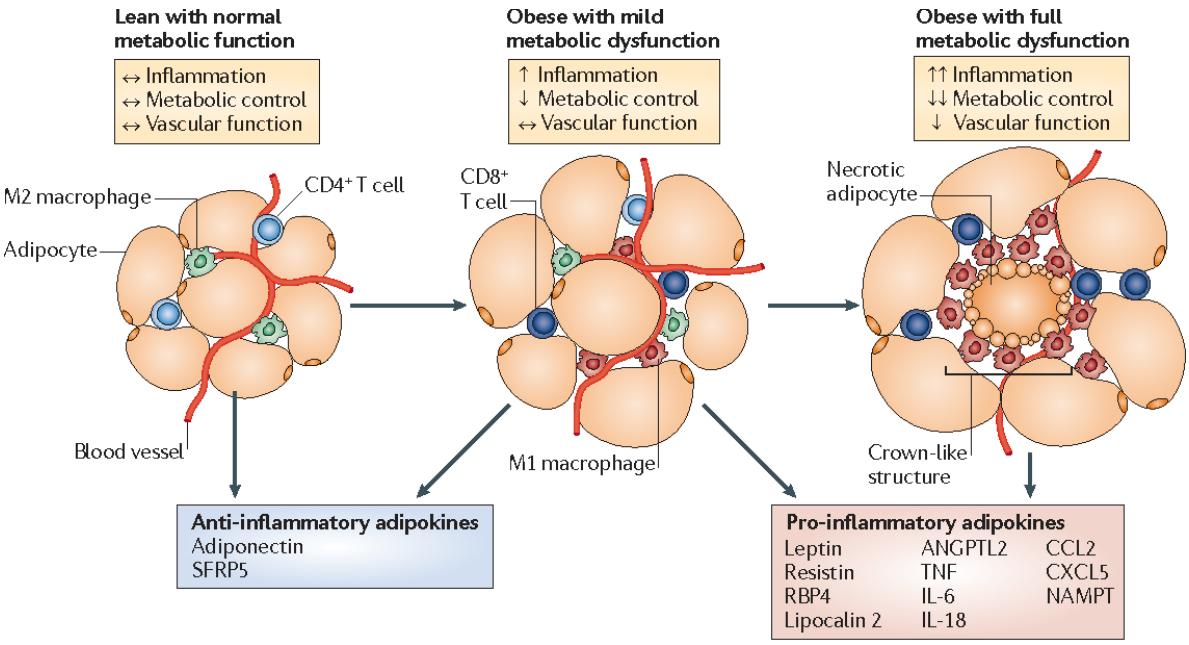
Grade	Description	Histological Appearance
1	Section with few scattered fat cells in the exocrine parenchyma	
2	Section with <50% scattered fat cells	
3	Section >50% scattered fat cells	
4	Partial or total replacement of exocrine lobules with fatty tissue	

In 1984, Stamm et al. identified significant correlations between T2DM and pancreas steatosis in patients with more than 25% fat in the pancreas, which led to the consensus that having an element of steatosis in the pancreas was deemed to be pathological [46]. This was further supported in 2010 where tissue from 80 postmortem patients identified a correlation between non-alcoholic fatty liver disease (NAFLD) and non-alcoholic fatty pancreas disease (NAFPD). The study suggested that pancreas interlobular fat infiltration and total pancreatic fat were increased in patients with NAFLD and so, total pancreatic fat may be a significant predictor of NAFLD. Investigators further hypothesised, that the presence of intralobular pancreatic fat may be related to non-alcoholic steatohepatitis [47] [48]. This suggestion highlights the importance of not only the amount of fat involved in steatosis but also its location, as well as a potential link to surrounding organs.

1.9 Pancreas steatosis and inflammatory mechanisms

Adipocytes perform an important function in storing excess lipid as cellular fuel but also have an endocrine function, releasing adipokines and cytokines in response to specific stimuli. These cytokines can promote a local inflammatory process resulting in cellular dysfunction and cell death. Lipolysis occurring within adipocytes build stores of non-esterified fatty acids (NEFA), which may be released into the tissue if adipocytes are damaged [49]. In the case of the pancreas, NEFA released into exocrine parenchyma, not removed by the circulation, is thought to promote cytotoxicity [50]. Thus, disruption of pancreatic adipocytes are believed to contribute to pancreatitis and other functional disorders affecting the pancreas (see Figure 1.9.1).

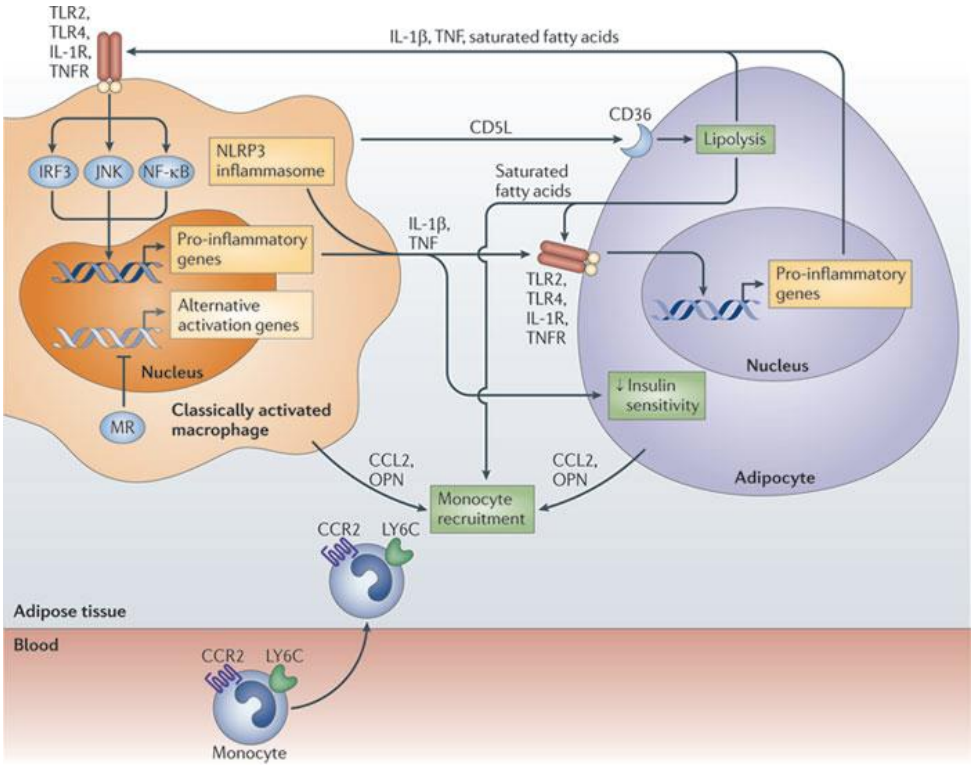
Figure 1.9.1: Phenotypic modulation of adipose tissue showing how obesity impacts inflammation in adipose tissue. Image source: [51]



Weisberg et al. demonstrated that adipose tissue macrophages are responsible for almost all adipose tissue TNF-α expression and significant amounts of iNOS and IL-6 expression. Adipose tissue macrophages are known to accumulate through chemokine (C-C motif) receptor 2 and the ligand for this receptor, monocyte chemo-attractant protein-1 (MCP-1), which is considered to be pivotal for the development of pancreatitis [52]. Additionally, Shirakawa et al., demonstrated that CD4+ T cell activation was increased in the visceral adipose tissue of obese mice. These cells also expressed PD-1 and CD153, which are characteristic of cellular senescence. Once activated, Th1 and Th2 cells orchestrated inflammation by secreting large amounts of cytokines. Th1 cells produced interferon gamma (IFN-γ), interleukin-2 (IL-2), and tumour necrosis factor (TNF) alpha/beta, triggering cell-mediated immunity and phagocyte-dependent inflammation. Th2 cells, in contrast,

produced IL-4, IL-5, IL-6, IL-9, and IL-13 to regulate antibody responses [53] (see Figure 1.9.2).

Figure 1.9.2: Interaction between macrophages, adipocytes and associated inflammatory cytokines in the stimulation of inflammation. Image source: [52]



1.10 Aetiology of pancreatic steatosis

The precise aetiology of pancreatic steatosis remains elusive but current estimates based on NAFLD patterns and small series biopsy studies, suggest up to 10% of the general population may have ≥5% of fat infiltration within the pancreas [43]. Aging, obesity and impaired glycaemia are known causative factors that are associated with pancreatic steatosis, with patients with T2DM or even impaired glucose tolerance, having greater pancreatic fat infiltration [54] [55]. Numerous other conditions associated with inflammation (e.g. myelodysplastic syndrome and haemochromatosis) are also associated with pancreas steatosis [56]. Pancreatic fat replacement has also been shown to be

associated with a number of congenital syndromes, including cystic fibrosis [57], Shwachman–Diamond syndrome [58], Johanson–Blizzard syndrome [59], and heterozygous carboxyl-ester lipase (CEL) mutations [60]. These syndromes share pancreatic exocrine insufficiency as a feature, but the exact cause of the steatosis remains unknown. In addition, viral infections such as HIV [61], reovirus and hepatitis B and C can also cause pancreatic steatosis where damaged tissue is replaced by fat as a response to the inflammation [62]. Malnutrition also increases pancreatic steatosis as seen in patients with Kwashiorkor [63].

Drugs are another major cause of pancreatic steatosis, with steroid use well documented to promote steatosis [64]. Other drugs such as rosiglitazone [65] and chemotherapy agent gemcitabine are also documented causes [66]. Al-Haddad et al. reported that alcohol intake of greater than 14 grams/week was associated with significantly more pancreatic hyper-echogenicity than those with lower intake [67]. This result was controversial as one unit of alcohol (UK) is defined as 10 millilitres/8 grams of pure alcohol, and so 14 grams = 1.75units. Subsequent studies have shown up to 20g of alcohol intake a day is safe [68], suggesting the evidence for an acceptable alcohol level as a risk factor for pancreas steatosis is yet to be determined.

1.11 Obesity and pancreas steatosis

Data collected between 1980 and 2013 by Ng et al., reported that more people are overweight than at any other time in the past three decades. The UK has the third-highest rate of excess weight in Western Europe, with 67% of men and 57% of women overweight or obese, according to a BMI classification (25-30 = overweight and ≥30 = obese) [69]. Obesity is now a global epidemic, with the CDC 2015 report showing the prevalence of

obesity in the USA to be 36.5% during 2011-2014 rising to 40.2% in adults between 40-59 years old [70].

Based on the historic links between obesity, pancreas steatosis and BMI being a measure of obesity, many transplant units use BMI as a surrogate marker for pancreatic steatosis and to assess donor suitability. Yet many people with pancreatic steatosis are asymptomatic, having normal BMI, endocrine and exocrine function and so, are considered as potential pancreas donors.

Furthermore, studies investigating BMI as a surrogate for pancreas steatosis have reported conflicting results, perpetuating uncertainty and contributing to inconsistency in donor organs acceptance. Consequently, potentially suitable organs may be unnecessarily declined, disadvantaging patients on waiting lists.

Humar et al. examined the impact of donor obesity on surgical complications and graft function after pancreas transplants for three groups based on donor body mass index (BMI): <25 kg/m2 (n=434), 25-30 (n=196), and >30 (n=81). Donor characteristics were similar between the three groups except for donor cause of death. In the BMI >30 group, recipient surgical complications were significantly more common. Furthermore, technical surgical failure rates were higher and short-term graft survival was inferior.

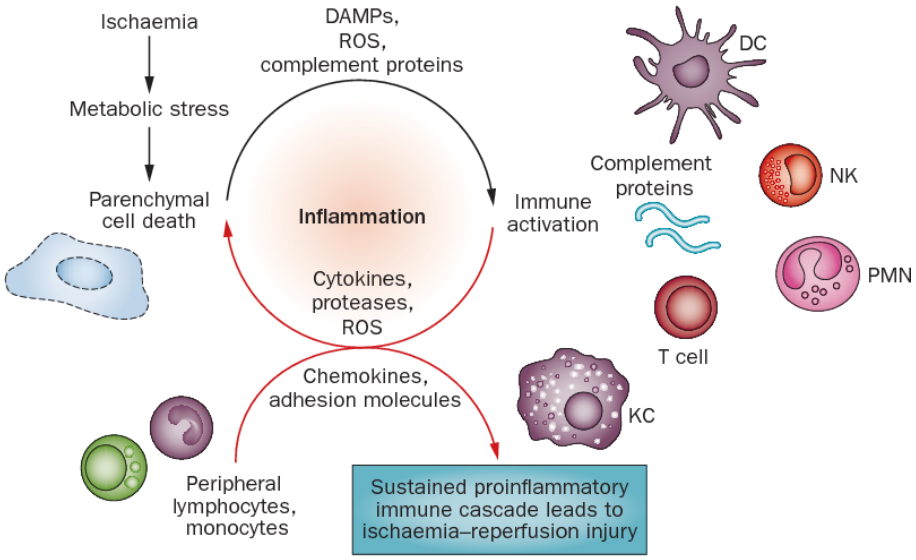
The incidence of technical (i.e. non-immunological) failure was 9.7% in the BMI <25 group, 16.3% in the BMI 25-30 group, and 21.0% in the BMI >30 group ($p = 0.04$). However, multiple confounding factors, including variations in operator experience and overall comorbidities of the recipient, should be considered when interpreting these results, and with no

regression analysis performed, there are limits into the insights that can be drawn. Interestingly, when examining technically successful transplants, no significant difference was identified between the three groups with regard to graft survival at 1 and 3 years' post-transplant [71]. Conversely, Krieger et al. reported in 2003 a series of 620 SPK transplants where no difference in survival or morbidity using obese donors was observed [72]. This has been further supported by Fridell et al. who compared the outcomes of 308 pancreas transplant recipients with donor BMI <25 kg/m^2 compared with >30kg/m^2. Of the 308 donors, 84 (27%) were overweight and 43 (14%) were obese. These donors were older and had higher rates of hypertension, yet there was no significant difference in graft loss, morbidity or one-year survival when compared to normal BMI donors [73].

1.12 Allograft pancreatitis

One major concern regarding pancreatic steatosis in the context of transplantation is the risk of reperfusion pancreatitis, triggered by the unavoidable ischaemia-reperfusion (IR) insult. Pancreas tissue injury and/or death occurs as a result of the initial ischaemic insult and is determined primarily by the magnitude and duration of the interruption in the blood supply followed by subsequent damage induced by reperfusion. During prolonged ischemia, ATP levels and intracellular pH decrease as a result of anaerobic metabolism with lactate accumulating. As a consequence, ATPase-dependent ion transport mechanisms become dysfunctional, contributing to increased intracellular and mitochondrial calcium levels (calcium overload), promoting cell swelling, rupture, and cell death by both necrotic and apoptotic mechanisms [74] (see Figure 1.12.1).

Figure 1.12.1: Stages of pancreas ischaemia reperfusion injury. Image source: [75]



As oxygen levels are restored upon reperfusion, a surge in the generation of reactive oxygen species (ROS) occurs and pro-inflammatory leucocytes, infiltrate ischaemic pancreas tissue to exacerbate the injury. The pathologic events induced by IR orchestrate the opening of the mitochondrial permeability transition pore, which appears to represent a common end-effector of the pathologic events initiated by IR [74] [75].

Xu et al. have demonstrated that obesity is a state of low-grade chronic inflammation that triggers the release of lipids, aberrant adipokines, and pro-inflammatory cytokines from adipose tissue [77]. Human pancreas sections obtained during autopsies of patients with acute native pancreatitis reveal significant adipocyte necrosis that were rich in calcium, suggesting NEFA release into the parenchyma, causing saponification exacerbated pancreatic injury. This supports the concept of adipokine up-regulation in obesity and links to evidence showing obesity is associated with more severe pancreatitis [78]. In native pancreatitis, peri-pancreatic necrotic collections have shown elevated concentrations of adipokines, including TNFα, IL-6, leptin, adiponectin, visfatin, resistin, apelin, retinol binding

protein (Rbp4), proteases like trypsin, unsaturated fatty acids (UFAs) such as oleic and linoleic acid, as well as cytokines including IL-1β, CXCL-8 and CCL-2 [79].

Pancreatic acinar cells produce four lipases; pancreatic triglyceride lipase (PTL), carboxy ester lipase (CEL) and pancreatic lipase-like protein 1 and 2 (PLRP1 and PLRP2). These are released from the apical pole into the ductal lumen, and normally are not in contact with adipocytes (located on the basal surface). Damage to acinar cells during transplantation may promote leakage of lipolytic enzymes stimulating adipocyte release of inflammatory mediators [80]. Linoleic acid - but not palmitic acid - has been shown to inhibit mitochondrial complex V, causing a drop in intracellular ATP, which induces cell necrosis. Additionally, higher levels were found to increase mRNA levels of CXCL1 and CXCL2, which play a major role in neutrophil chemotaxis [79] [80].

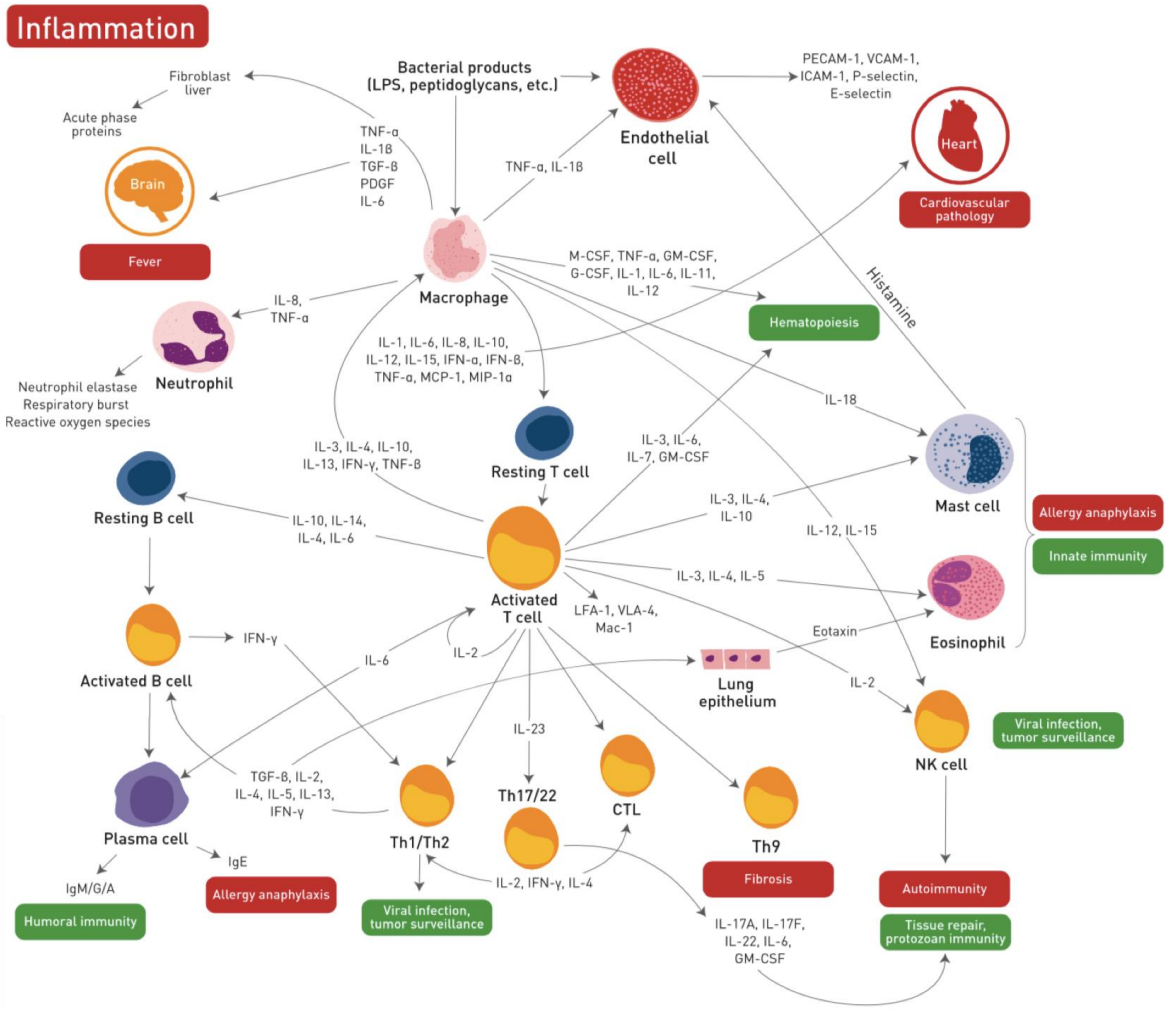
1.13 The critical balance between pro- and anti-inflammatory mediators

Many of the ideas surrounding allograft pancreatitis have been adopted from native pancreatitis, as what appears evident is that acute inflammation, regardless of the trigger, leads to a common effector pathway. One concept of this inflammatory pathway is one of cause and effect, like a line of dominos where a stimulus cascades promoting downstream cytokine activation leading to inflammation. Therefore, using pancreases from obese donors (thought to have increased pancreatic steatosis) may increase pancreatitis incidence and severity.

However, the amplification of the response (i.e. cytokine release is both cause and the effect) may mean, the size of the stimulus itself is less important than the amplification and the effect of pancreatitis is driven by the recipient's response to inflammation.

Pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are responsible for early responses and known to amplify inflammatory reactions, whereas anti-inflammatory cytokines, including IL-4, IL-10, and IL-13 limit the inflammatory responses. The interaction of pro- and anti-inflammatory cytokine/chemokine networks makes it crucial to examine these mediators in functional groups rather than on an individual basis [81].

Figure 1.13.1: Overview of main human inflammatory pathways highlighting its complexity with common disease triggers and effects. Redrawn from Cellular and Molecular Immunology; Elsevier 2017; Abbas AK, Litchman AH, Pillai S. 4th Edition.



1.14 Aims of the thesis

Following the introductory chapter, the first objective of the thesis in Chapter two will be to assess the UK perspective of pancreas allocation and utilisation. Additionally, I will attempt to assess how UK practice compares to other programs around the globe and whether the same issues highlighted in this introduction are present, allowing for further optimisation.

Chapter three will focus on the assessment of the allograft and pancreas steatosis. By evaluating current practice and comparing this to a number of other methods, a consideration to whether current methods of assessment are optimal or whether there is potential to improve practice will be examined. Understanding the issues of what a fatty pancreas versus a steatotic pancreas means, I will attempt to build a hypothesis regarding the clinical significance of pancreatic adipose tissue.

Chapter four aims to address the issue of transplant allograft pancreatitis, which is one of the main issues stunting the growth of pancreas transplantation. The associated morbidity, of which steatosis-related allograft pancreatitis is a major component, will be investigated with the incidence and then the aetiology of allograft pancreatitis assessed in order to better understand this morbidity. Currently, there is no reliable clinical grading system to assess allograft pancreatitis. Investigating reliable methods to measure the incidence and severity of transplant pancreatitis will be a major objective of chapter four and five, where chapter five will aim to consider the use of alternative imaging to quantify pancreatitis severity. The exploration of the associated inflammation patterns will also be examined, with chapter six bringing together the key findings of the thesis, considering the implications and where these might lead with respect to clinical implementation and future research.

CHAPTER 2:

Utilisation and trends of whole organ pancreas transplantation in the UK: 2005 to 2017

2.1 Global trends in pancreas utilisation

When looking across the globe to understand what type of donors are considered for pancreas transplantation, it becomes very evident that there is significant variation in utilisation and donor trends between countries. IPTR and United Network for Organ Sharing (UNOS) provide detailed information about US activity and some data on how it compares against non-US counterparts. In addition to UK-based NHSBT, there are also large datasets accessible from the Eurotransplant consortium, and Australia and New Zealand Islet and Pancreas Transplant Registry (ANZIPTR).

IPTR data shows 74% of all SPK donors are under 30 years in the US, with 79% under 30 years for PTA donors and 78% for PAK. Only approximately 15% of all US deceased donors in 2013 donated a pancreas for transplantation, which given the trends of increasing age, obesity and incidence of diabetes is not surprising [82].

Eurotransplant 2008 data, identified 2233 pancreas donors which translated into 880 pancreas transplant offers, resulting in 257 pancreas transplants where the median donor age was 33. However, in 2017 a similar number of donor offers resulted in only 142 whole organ pancreas transplants reflecting a fall of 45% [83].

The targeted use of young donors with a normal BMI is also seen in Australia and New Zealand. Peng et al. analysed data from ANZIPTR between 1984 to 2014 and showed 628 pancreas donors resulted in 582 pancreas transplants, where median donor BMI was 24 (range 21.9-24.0) and median donor age 27.5 years (range 23.9-28.5) [84].

2.2 Aim

Since inception of the UK program, 2981 whole organ pancreas transplants have been performed, with a yearly average of approximately 200 transplants since 2005. With the mean age of UK organ donors increasing from 46.7 years in 1990 to 59.3 years in 2013 [85]. The aim of this chapter is to examine UK practice via the NHSBT whole organ pancreas transplant dataset [86].

2.3 Methods

Examining trends and patterns using national data records (2005-2017) has allowed a unique analysis, complementary to the annual reports produced by NHSBT. In addition, differences in practice across the eight UK pancreas transplant centres were assessed using an e-questionnaire and results from the NHSBT organ discard audit - performed on behalf of NHSBT by the Oxford Transplant Centre in collaboration with Edinburgh.

UK registry data from 2005 to 2017, in unpublished data tables provided by NHSBT, were organised in Microsoft Excel format. This data was sorted and analysed using Pearl programming language and then tested for normality. GraphPad Prism version 7.0 was used for statistical analysis. Data was found to be non-parametric, with Spearman Rank, Mann Whitney U, Kruskal Wallis and Chi-Squared tests being employed where significant values were deemed $p<0.05$. The work was done in collaboration with Dr. Claire Counter from NHSBT statistics.

The NHSBT prospective national study of pancreas damage and discard audit was devised and approved through the Research, Innovation and Novel Technologies Advisory Group (RINTAG) at NHSBT. Pancreases accepted for implant by centres but later discarded

following retrieval, were transported to an audit centre (Oxford or Edinburgh) for a second opinion, prior to being allocated for national research projects. Each centre was on call for 7 days at a time, operating a week on/week off rota coordinated by NHSBT.

Pancreases were evaluated by two consultant pancreas transplant surgeons and scored against predetermined criteria designed by RINTAG (see Appendix 1) to assess whether the organ could have been used or not, after which they were photographed and videoed. 53 pancreases were examined (27 at Edinburgh and 26 at Oxford).

2.4 Waiting list overview

National UK data shows the total number of pancreases transplanted annually increased from 181 transplants in 2005 to 246 in 2013 (an increase of 36%). The start of this increase coincided with the designation and funding of solid organ pancreas transplantation in 2004 as a highly-specialised NHS-funded service. In 2005/6 transplantation activity matched the number of patients on the waiting list (roughly 200). In 2007 however, the waiting list increased from 245 (an increase of 22%) to 293 by 2008 and peaked in 2009 at 335. However, the expansion was short-lived with the number of patients on the waiting list declining from 2013 onward. Only 185 whole pancreas transplants (SPK, PTA and PAK) were performed in 2015/16, and 179 in 2016/17.

The median time wait for a pancreas transplant has fallen from 682 days (IQR 246-841) in 2011 to 426 days (IQR 211-594) in 2016 (see Table 2.4.1).

Table 2.4.1: Overview of the falling UK number of patients and the median waiting time in days with IQR (Q1 and Q3) for whole organ pancreas transplant. Data supplied by NHSBT.

Median waiting time (days)	2011	2012	2013	2014	2015	2016
Number of patients	200	209	220	199	198	163
Median	682	479	417	362	394	426
Q1	246	226	247	236	243	211
Q3	841	704	578	529	524	594

One of the pivotal reasons explaining the reduced waiting time on the list was the UK pancreas allocation scheme. Introduced in December 2010, it aimed to develop the fair allocation of donor pancreases for both whole organ and islet transplantation, allowing recipients an equal opportunity of getting a pancreas and reducing variability between centres.

As demographics between UK and US waiting lists were deemed broadly similar, data was compared identifying similar factors affecting wait times and outcomes following transplantation. Each factor was given a weighting and the person on the waiting list with the highest total points would be offered the transplant first.

Factors included cold ischemic time, sensitization to HLA, dialysis status, waiting time, donor recipient age matching, donor BMI, and donor-recipient HLA mismatch. Travel time by road from the donor hospital to the recipient's transplant centre or islet isolation centre was used as a surrogate for cold ischemic time such that the closest three transplant centres or closest islet isolation centre had priority. For donation after cardiac death (DCD) donors, the travel time weighting was higher than DBD donors to minimize cold ischemia further. Pancreases

were allocated to blood group identical recipients; with the exception of those who were blood group AB, who also had access to donors of blood group A.

Priority was given to highly sensitized patients (cRF 75%) over non-sensitized patients, with an exponential weighting to increasingly raise their chances of a transplant with increasing sensitization. Increased weighting was also given for patients on dialysis, and for those who had waited longer. Islet recipients awaiting a second graft were prioritized further to try to ensure a second graft within a median of 6 months.

Age was weighted to minimise the difference between donor and recipient and BMI was given a weighting such that donor pancreases with a lower BMI would be preferentially allocated to whole organ transplantation, and those with a higher BMI would be allocated to islet transplantation (since low BMI donors tend to give poorer islet yields) [87]. Implementation of the scheme led to a reduction in the number of long waiting patients, with only 31% waiting more than a year compared to 54% at the introduction of the scheme [88].

In the following sections the term ‘used’ refers to pancreases that have been offered for donation, retrieved and transplanted, while ‘unused’ refers to pancreases that have been offered for donation, retrieved but not transplanted. Data referring to organs that were within criteria, offered and not accepted without retrieval, have been discussed in section 2.10.

2.5 Pancreas offers

The destination and outcome of every organ offered or retrieved for transplantation in the UK is coded by NHSBT. Data fields are pre-determined by NHSBT and have been compared over a 13-year period. Understanding the current pattern of pancreas offers will help delineate whether the issue of failed expansion is due to insufficient referrals for transplantation or insufficient pancreas offers that have contributed to smaller waiting lists.

Figure 2.5.1: The total number of pancreas offers and number of offers declined from 2005 to 2017.

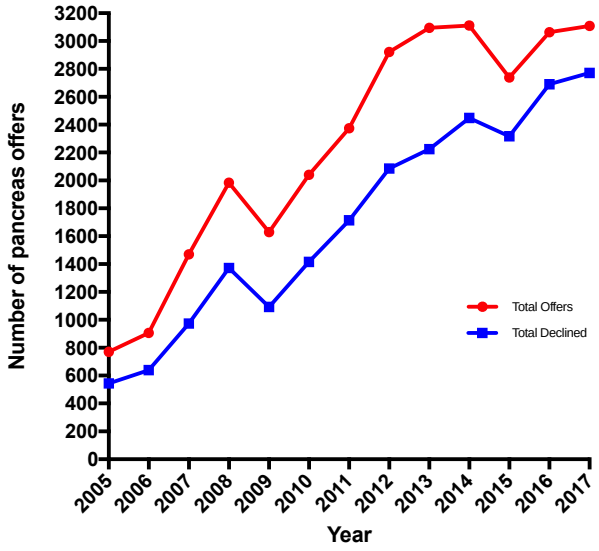
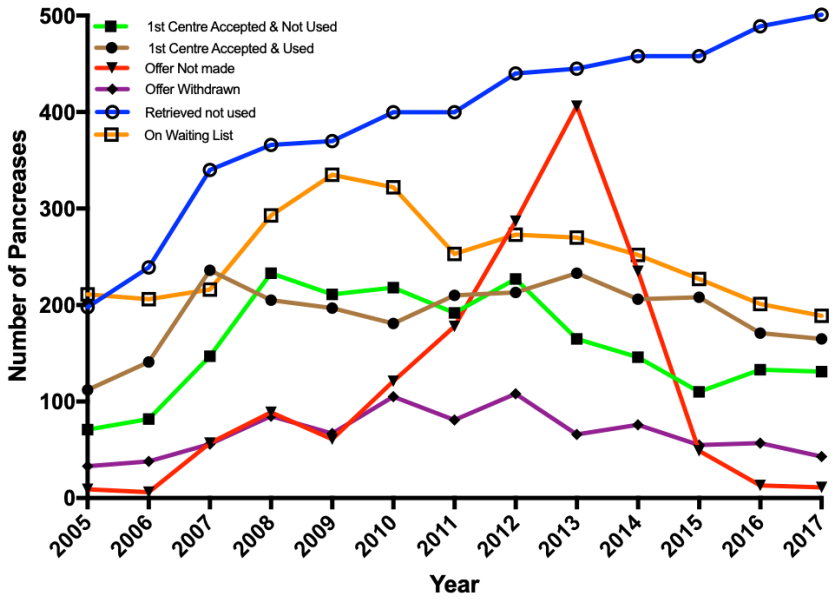


Figure 2.5.1 demonstrates a 4-fold increase in pancreas offers from 770 in 2005 peaking at 3111 in 2014. Yet the 2016/17 NHSBT annual report states that a total of 1413 donors translated into 3710 transplants (of all organ types). Having over 3000 pancreas offers in 2016, reflects that pancreases are being offered multiple times and coded as separate offers. To substantiate this assertion, further data examination identified the actual retrieved number of pancreases ranged from 300-700 over the 13-year period. Pancreases were offered alone or combined with another organ (usually the kidney), and then re-offered in various formats which included fast track and research offers before being

discarded. Occasionally, the organ was re-offered with new information such as outstanding virology/microbiology or assessment of frozen sections. With offers managed by NHSBT duty office and discussed with transplant implanting teams, the system of assessing offers remains laborious, particularly of organs that are so often declined.

Figure 2.5.2 below identifies pancreases retrieved that were later unused have increased steadily over the 13-year period. In 2005, 198 pancreases were retrieved but not used increasing to 501 in 2017 (a 2.5-fold increase). One reason contributing to this increase was the 2015 change in requirement, requiring assessment of all organs on the back table and so, compulsory removal of the pancreas before declaring it non-transplantable.

Figure 2.5.2: NHSBT pancreas offer outcomes from 2005 to 2017.



The remaining variables, including organs received by the 1st centre (used/unused) and offers withdrawn remained relatively static, while a number of patients on the waiting list trended downward from its peak in 2009 (n=335) to below 200 in 2017. This figure also shows the number of offers not made, increased from 9 in 2005 to 406 in 2013, but then

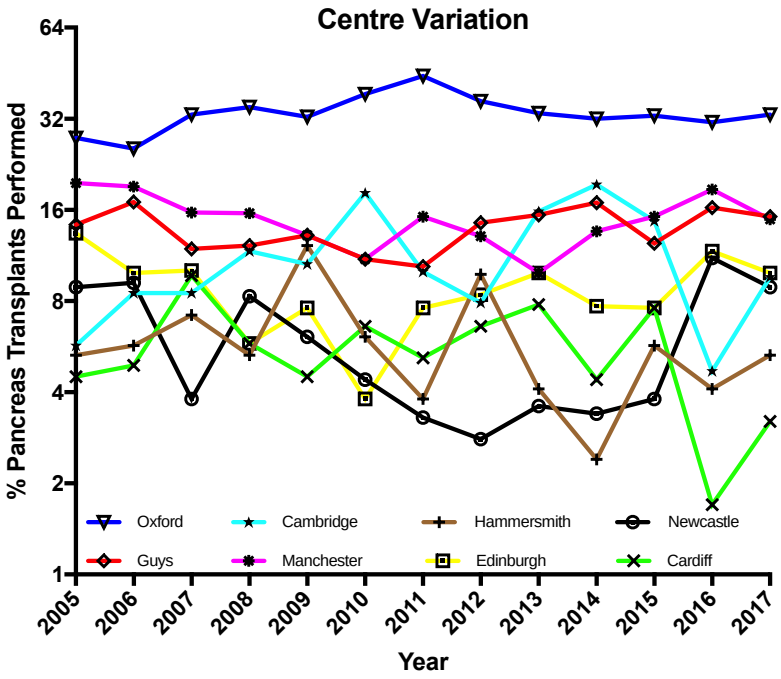
rapidly fell to 13 in 2016. One reason for this may have been a change in national donor eligibility criteria in 2012.

In 2005/6, 23.5% of all offers were converted into actual transplants, while in 2016/17 this had fallen to 6.2%. One of the reasons for this decline may be the reduction in referral rate and fewer patients on the waiting list, combined with the excessive number of organ offers. Data suggests current utilisation can be improved by reducing the significant number of offers that never translate into transplant and only increases the burden and cost. Additional analysis of data highlighted that once a centre accepted an offer, the rate of conversion into proceeding to transplant was variable.

2.6 Proportions of transplants performed

The variation in the number of pancreas transplants performed between the eight UK centres can be seen in Figure 2.6.1.

Figure 2.6.1: Volume of whole organ pancreases transplanted across the eight UK centres.



The results show how the level of activity varies widely with yearly variations between centres. Roughly 33% of all pancreas transplants are performed at a single centre (Oxford), while other high volume centres such as Guys and Manchester, roughly transplant 15% each of the total number each year. Low yielding centres such as Cardiff and Newcastle are only contributing around 5% of the transplants per annum. The reasons for this variation may include (i) a reduced number of patients registered on the local waiting list at the lower volumes centres or (ii) a more conservative approach to organ offers. What is worthy of note is that throughout the 13-year period there is a lot of variation and overlap amongst all centres year-on-year in regard to the number of transplants performed, suggesting the overall patterns are more complex.

Complementing Figure 2.6.1, Table 2.6.2 shows the overall median for the 13-year period for each centre, with both IQR and the overall maximum and minimum levels recorded, highlighting the overlap between centres.

Table 2.6.2: Summary of centre activity between 2005-2017.

Centre	Median & IQR (%)	Min - Max Range (%)
Oxford	33 (31.6-35.8)	25.5-44.3
Manchester	15.2 (13.15-17.2)	9.9-19.6
Guys	14.3 (12.05-15.8)	10.4-17
Cambridge	10 (8.2-15.2)	4.7-19.4
Edinburgh	8.4 (7.6-10)	3.8-13.4
Hammersmith	5.3 (4.1-6.6)	2.4-12.2
Cardiff	5.2 (4.4-7.1)	1.7-9.7
Newcastle	4.4 (3.5-8.9)	2.8-11.1

Kopp et al., identified that patient and graft survival following pancreas transplantation was superior in higher volume centres within the Eurotransplant region (low volume <5

transplants each year in the 5 preceding years, medium volume 5-13/year, or high volume ≥13/year). The authors identified that higher volume centres had better results, even though they transplanted organs with the highest pancreas donor risk index (PDRI), suggesting a volume effect [89].

Using the median values from Table 2.6.2 to differentiate low and high volume UK centres (≤7 and ≥20), the UK results demonstrated that centre volume correlated with a higher rate of acceptance of offers (p = 0.01) and a higher conversion rate to transplant (p = 0.03). However, the centres in the middle of the activity range (7-20) were less easy to differentiate; some lower volume centres maintaining good graft survival outcomes, while others less so. The results have been summarised in Table 2.6.3.

Table 2.6.3: Shows the differences between high, medium and low volume centres, comparing the rate of acceptance as the 1st offered centre and the conversion to transplant once an offer has been accepted. Graft Survival data (all pancreas transplant SPK/PTA/PAK) are taken from the NHSBT annual report 2016/2017.

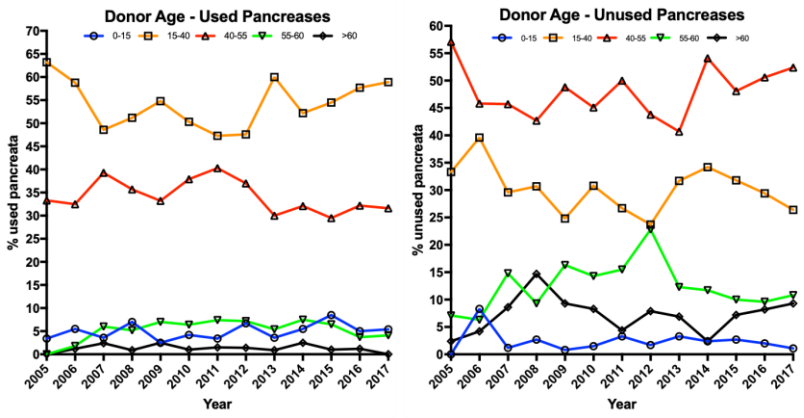
Centre	**Rate of acceptance as 1st Centre**	**Conversion rate to transplant once accepted**	**NHSBT 5yr Graft Survival data**
High volume			
Oxford	84%	61%	81%
Medium volume			
Cambridge	89%	70%	88%
Manchester	77%	53%	72%
Guys	69%	49%	81%
Edinburgh	69%	43%	83%
Low volume			
Cardiff	22.2%	11%	73%
Newcastle	36.36%	18%	59%
Hammersmith	52.5%	28%	62%

Centres with lower volumes may have fewer patients waiting or possibly, more complicated patients on their waiting list. This may result in the need to wait or pick only the best organs resulting in a reduced rate of acceptance and conversion to transplant. Additionally, low volume centres may have less experience leading to a more conservative approach. The inconsistency of conversion rates between centres (one component of organ utilisation) is a marker of the challenge, and reflects the absence of a reliable means to determine the real risk posed by each organ. This is a problem that remains to be solved.

2.7 Donor age

NHSBT donor criteria consider pancreas donors from 1-65 years old. Results show the trends for donor age have remained relatively consistent, with 50-65% of used transplanted pancreases coming from donors between 15-40 years each year. However, 25-35% of discarded unused pancreases also come from this same age group. Donors between 40-55 years are the second most common age group to provide pancreases that are used, contributing roughly a third of all the pancreases that are actually transplanted. They also are the largest contributors to unused organs, with 45-55% pancreases coming from this age range (see Figure 2.7.1).

Figure 2.7.1: NHSBT data (2005 to 2017) showing the variation in donor age in years for whole organ pancreases that were used and unused.



The trends in Figure 2.7.1 have been summarised using median and IQR values over the 13-year period in Table 2.7.2.

Table 2.7.2: Used and unused pancreas proportions of all donor age groups, from 2005 to 2017 expressed as median and IQR.

Donor Age (years)	% Pancreases retrieved & transplanted (median & IQR)	% Pancreases retrieved & not transplanted (median & IQR)
0-15	5 (3.5-6.1)	2 (1.1-8.3)
15-40	54.5 (49.4 – 58.8)	30.7 (26.5-32.5)
40-55	33.2 (31.8 - 37.4)	48.1 (44.4-51.5)
55-60	6 (3.9-7.1)	11.7 (9.4-15.1)
>60	1.2 (0.9-1.9)	7.9 (4.3-9.9)

At the extremes of the age range <15 years and >55 years, the numbers of pancreases retrieved, used and unused are small. The reasons for this are either a small donor pool with good utilisation, or a large donor pool with poor utilisation. Table 2.7.2 shows that donors above 55 years only contribute 6-7% of pancreases that are retrieved and transplanted, while 11.7% of pancreases from donors above 55 are retrieved and not used.

It is important to note that the above data relate only to organs that were initially accepted for transplantation. Organ utilisation includes also those organs within nationally agreed criteria that were offered and not accepted by any transplant centre. Organ utilisation is therefore, considerably worse than the above numbers may immediately suggest.

At the other end of the age spectrum, the results demonstrate donors from 0-15 years contribute on average less than 5% of pancreases used for transplantation. However, the issue here is more complex and not only due to poor utilisation but also due to a small sample size. There is mixed evidence to suggest that use of paediatric organs can lead to adverse outcomes due to the small vessels increasing the risk of thrombosis [90]. However,

there is also evidence to show good outcomes with the use of paediatric organs [91]. Variation in practice has led to poor utilisation in this age group.

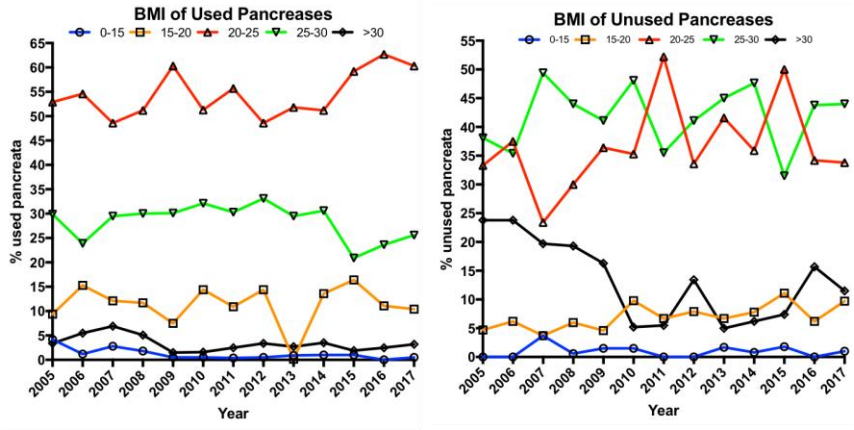
For pancreases retrieved from donors <15 years, 81% (median value from 2005-2017) were transplanted, but with less than 2% of all donors coming from this population, the overall contribution to the transplant pool is small. Further stratification of the group identified 93% of donors were aged 10-15years, with 100% being used when the lower end of the range was decreased to 5 years, suggesting donors under 5 years are rare and less frequent, but possibly less likely to be used.

Individual pancreas transplant units provide NHSBT with donor upper age limit of age. With an apparent abundance of organs for a small national waiting list, a discussion to whether it is necessary to continue to include any donors above 55 years or under five years should be considered, based on the assumption that these extreme groups are fundamentally less suitable.

2.8 Donor BMI

The used and unused numbers of pancreases at each BMI interval were calculated, with the median and IQR plotted from 2005-2017. Results identified that over 50% of used pancreases came from donors with a normal BMI (20-25), with a further 30% from overweight donors (BMI>25-30). The volume of donors at the extremes of BMI (at either end of the spectrum) did not contribute significantly. Donors with BMI >30 had a median contribution of 3.2% (range 1.5-6.9%), reflecting the nationally agreed BMI limit for solid organ donors (32 kg/m^2). Paediatric donors with BMI <20 contributed roughly 10% to the overall number used (see Figure 2.8.1).

Figure 2.8.1: Data from 2005 to 2017 showing the variation in donor BMI for whole organ pancreases that were used and unused.



Considering organs that were retrieved but not transplanted, the difference between normal BMI (20-25) and the overweight category (25-30) was less pronounced. The median unused rate of donors with BMI 20-25 was 35.3% and 43.8% for BMI 25-30. The high proportion of discarded organs within the normal BMI range suggests that either pancreases were discarded for other reasons than fattiness/steatosis or that BMI is an insensitive marker of pancreatic fattiness and presumed steatosis. Analysis from 2016/17 data identified 53% of discarded pancreases with donor BMI 20-25 and 68% with BMI 25-30 being discarded on the basis of visual macroscopic fattiness which was imprecisely described as steatosis (see Figure 2.8.1). With conflicting evidence described in chapter one around the use of BMI and outcome, another hypothesis to consider is whether the macroscopic appearance of the pancreas allograft alone is a predictor of poor transplant outcome.

Interestingly, unused donors with BMI >30 fell from 2005 to 2017 (23.8% to 10.5%), suggesting that offers with high BMI less likely to be considered as well as being influenced by the pancreas allocation system introduced in 2010, where higher BMI donors are now preferentially allocated to islet isolation. Moreover, donors with BMI >32 are not offered

for solid organ recipients, which may also contribute to the low rate [88]. The trends in the graphs above can be summarised with the median and IQR values over the 13-year period being calculated and presented in Table 2.8.2.

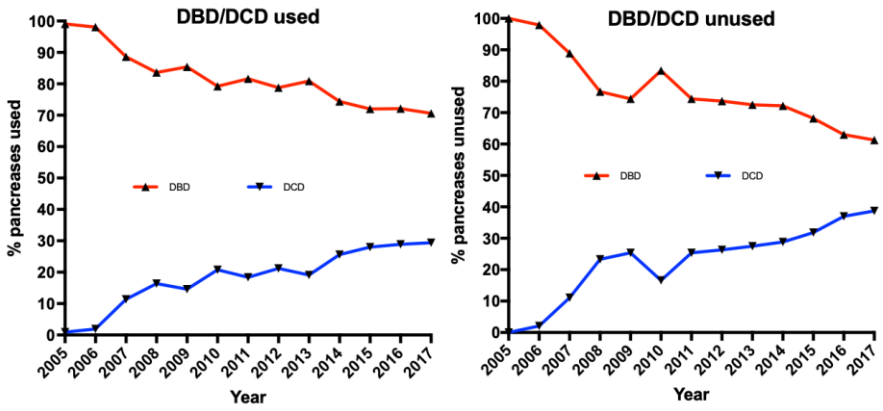
Table 2.8.2: Used and unused pancreas proportions of all donor BMI groups, from 2005 to 2017 expressed as median and IQR.

Donor BMI	% Used Pancreases (median & IQR)	% Unused Pancreases (median & IQR)
0-15	0.9 (0.5-1.5)	0.8 (0-1.6)
15-20	11.7 (9.9-14.4)	6.7 (5.3)
20-25	52.9 (51.2 – 59.5)	35.3 (33.4-39.5)
25-30	29.9 (24.7-30.5)	43.8 (36.8-46.8)
>30	3.2 (2.2-4.3)	13.4 (5.8-19.5)

2.9 Brain death

Since 2005 the proportion of UK transplanted pancreases provided by DBD donors has fallen from 99% to 71% in 2017. In that same time period, the trend of increased DCD donor use has progressively increased from 1% to 29% (see Figure 2.9.1). This pattern fits with national data showing DCD donation has increased substantially over the last decade, from 288 donors in 2008-2009 to 619 in 2017-2018, representing 39% of all deceased organ donors in 2017.

Figure 2.9.1: Variation in the usage of donor pancreases from DBD and DCD donors 2005-2017.



The same pattern (fall in DBD donors and increase in DCD donors) can be seen for unused pancreases. Published data suggest that DCD pancreas transplantation has similar graft and patient survival rate compared to DBD donation, as shown in Table 2.9.2. However, conflicting results regarding the risk of early complications, with allograft thrombosis and pancreatitis being reported more frequently in organs from DCD donors, may be one of the reasons why unused DCD pancreases are increasing.

Table 2.9.2: UK SPK graft and patient survival of DBD and DCD pancreas transplants at 1 year and 5-year median values with IQR (2012-2015).

	1yr Graft Survival	5yr Graft Survival	1yr Patient Survival	5yr Patient Survival
DCD	89 (80-95)	87(77-93)	99(91-100)	88% (83-91)
DBD	88 (85-91)	77 (72-81)	96 (94-98)	87% (83-90)

UK SPK data suggests that one-year and five-year allograft and patient survival is higher in DCD donated organs than DBD donors (see Table 2.9.2 above). Although statistically not significant, one explanation for this may be the use of stricter criteria both for accepting DCD organs (i.e. the avoidance of other risk factors) and the medical fitness of potential

recipients (lower risk recipients selected for higher risk organs). The second of these explanations is less likely in the context of the national organ allocation system which allocates organs to individual patients, and effectively prevents organs from being locally allocated (other than for 'fast-track' organ offers).

2.10 First centre offer and outcome

There are many reasons that offered pancreases are declined at the point of offering. Although NHSBT collects the primary reason for the decline, the reality is that often a number of risk factors in combination usually drives the overall decision, and generally only one of these is captured. Data recorded by NHSBT between 2010 and 2016 was analysed and the results identified that the majority of discarded pancreases are declined by all centres at the point of offer and so, are never retrieved. For those that are retrieved, if the first centre offered a pancreas declines for donor-related reasons, then data suggests subsequent centres often also decline, unless the issue was for recipient or logistic reasons, where cold ischaemia time was the greatest controlling factor.

Figure 2.10.1 summarises the reasons why an organ was declined, based on the current NHSBT labelling. A logarithmic scale has been used to enable both very rare and frequent reasons to be displayed on the same graph. As there is a lot of data in this graph, with many of the reasons having very little effect, the overall findings have been summarised in Figure 2.10.2, where factors that have less than 1% effect were omitted.

Figure 2.10.1: Percentage of pancreas offers declined by the 1st centre offered and then are not used – documented NHSBT reasons.

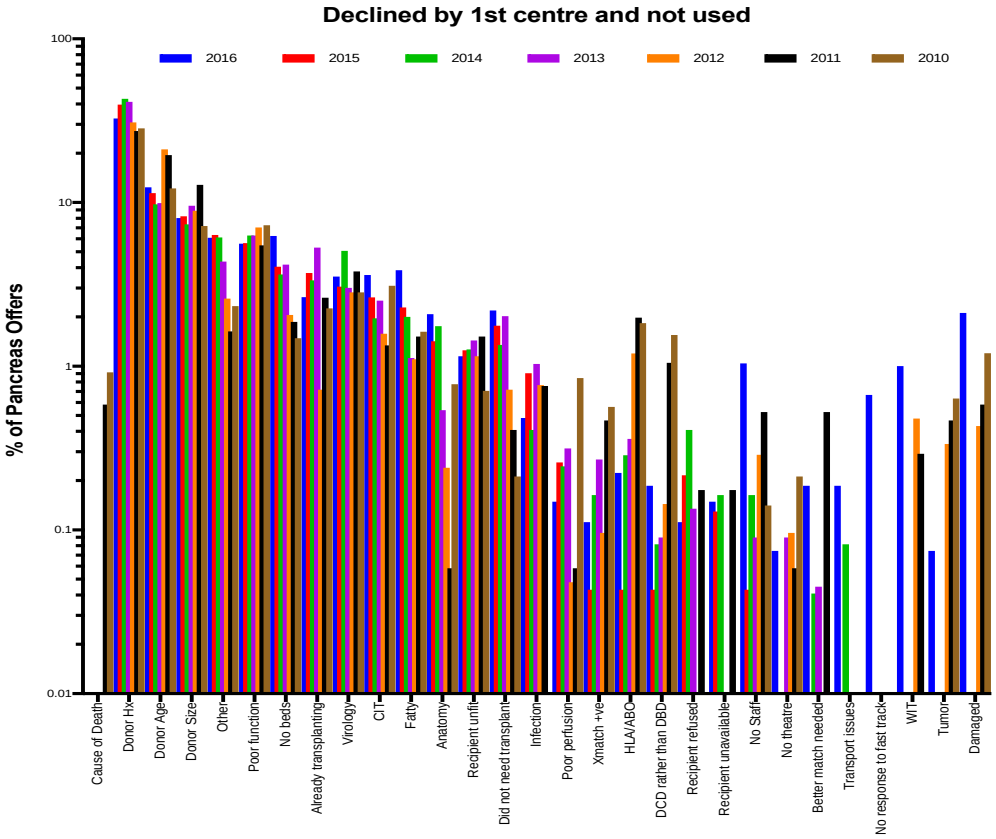
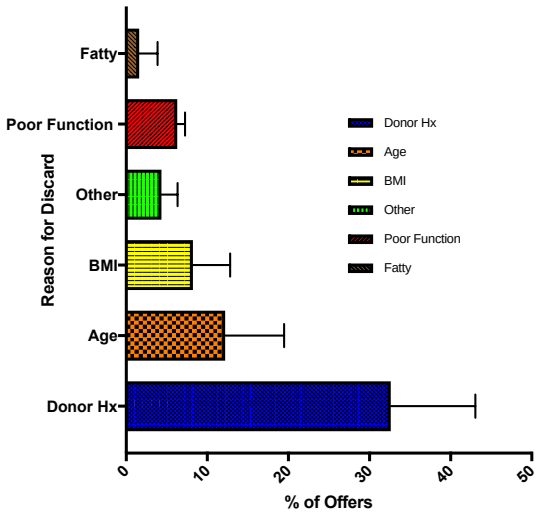


Figure 2.10.2: Summary of the data from Figure 2.10.1. Reasons that offered pancreases were declined by the 1st centre (on the basis of history or at the time of retrieval) and subsequently not transplanted. Data from 2010-2016.



Between 2010-2016 a median of 32.6% (IQR 27.4-43.1) of pancreas offers were discarded on the basis of donor history, which is the most frequent indicator of rejecting a pancreas offer. However, the specific features (single or combination) of the history that caused the decline are lacking, limiting conclusions.

Donor age was the primary reason for a median discard rate of 12.2% (9.7-19.5), BMI 8.24% (7.2-12.8), other 4.36% (1.6-6.3) and poor function 6.3% (5.5-7.3). Organs deemed fatty based on visualised appearance at the point of offer accounted for only 1.6% (1.1-3.8) of rejected offers, and should be considered with BMI – as this is the surrogate marker used for fatty pancreases.

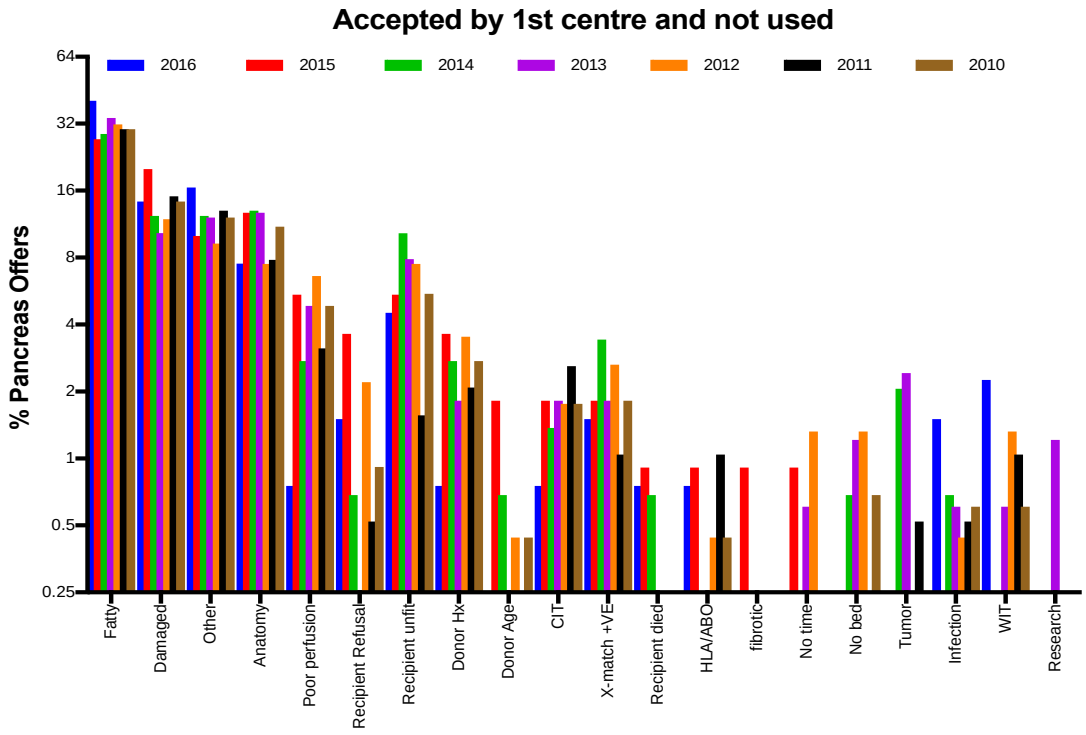
Examining the NHSBT code 'other', free text data entered by the retrieval team as additional information or by the duty office staff who were recording the outcome of the offer was captured when this code was selected, however it was poorly documented and often left blank. 'Other' documented reasons included issues during retrieval prolonging warm ischaemia time or damage to the organ during retrieval, tumor discovery at the time of retrieval or other new information.

2.11 Discard at the point of retrieval or inspection at implanting centre

Standard UK organ retrieval practice mobilises retrieval teams once organs have been placed. Following organ offering, the retrieval is the second point at which organs may be declined when additional information is conveyed to implanting centres, typically regarding steatosis, fibrosis or poor perfusion. Pancreases are retrieved and transported to the accepting centre, which is the third point at which organs may be declined. Percentage of

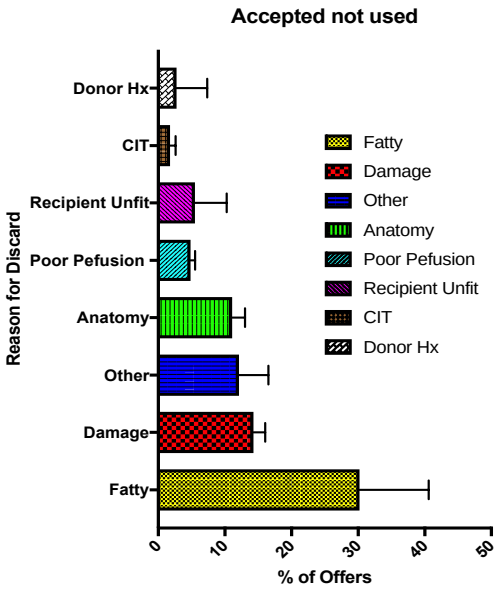
pancreases initially accepted by the 1st centre but not transplanted using the NHSBT codes, were examined (see Figure 2.11.1).

Figure 2.11.1: NHSBT data from 2010-2016 identifying the most frequently reported reasons for discarding pancreases once they have been accepted by the first centre and retrieved.



Between 2010-2016, the most frequent discard reason with a median of 30.20% (IQR 27.3-40.6) was 'Fattiness', also documented as steatosis. 'Damage' was the next most common reason with a median of 14.3% (10.3-16.1), 'Anatomy' 11.1% (7.48-13.01), 'Other' 12.1% (9.2-16.5), 'Poor perfusion' 4.8% (0.7-5.5), 'Unfit recipient' 5.5%(1.6-10.3), 'CIT' 1.8% (0.7-2.6) and interestingly, 'Donor history' again 2.77% (0.4-7.4%). Presumably donor history came into the decision at this point only in the context of other risk factors revealed at the time of retrieval/inspection. The remaining reasons had contributions below 1% and therefore, have been omitted from the following summary graph (see Figure 2.11.2).

Figure 2.11.2: Summary of the most commonly reported reasons for discarding a pancreas after it had been accepted by the implanting centre. NHSBT data, 2010-2016.



'Fattiness' and 'Organ injury' remain the most common reasons for centres not to use pancreases, with 'Other' and 'Anatomy' codes as the next most frequent reasons. As with the coding summary above, there is a free text option to allow capture the 'Other' fields. However, these values are not recorded by NHSBT, and if documented (often left blank) by the implanting unit are not routinely transcribed into NHSBT data sets.

To address this, an additional analysis of post-transplant return sheets from 500 pancreases was examined from historical Oxford cases, where organs had been accepted but then discarded. Out of the 500 pancreases, 29.4% (n=147) pancreases had been coded with 'Other' (n=88) or 'Anatomy' (n=59). The term 'Other' was found almost exclusively with reference to the donor iliac Y graft being absent or unusable or if the duodenal staple line had opened causing contamination. Organs refused on the basis of 'poor perfusion' were reported to either have blood still present in the splenic vein or were incompletely flushed

during organ retrieval, possibly increasing the risk of thrombosis. However again, our ability to draw conclusions is limited due to the lack of data being captured.

An NHSBT initiated and supported audit was performed jointly over two centres (Edinburgh and Oxford) where all nationally discarded pancreases were sent for a second assessment (visual and tactile as per current clinical practice). This assessment was done, in every case, by two pancreas transplant surgeons over a period of six months (October 2015 to March 2016). Cornateanu et al. reported that 35.8% of discarded pancreases were deemed transplantable by second opinion assessment [92] [93].

This analysis identified the degree of fattiness and size of capsular injury as being the most frequent reasons for discard. It was clear that the assessment of these discard factors was heterogeneous between different centres and between surgeons in the same centres. This result, combined with the data presented here, highlights the need for a stronger consensus (and preferably more objective criteria) of what constitutes an acceptable degree of fattiness and injury.

2.12 E Questionnaire

An 11-question (14-part) survey was designed electronically (surveymonkey.com) and aimed to determine donor criteria that influence surgeons' decision to accept a pancreas for transplantation. The primary objective was to better understand what components of the donor history were important to clinician decision making, uncovering why donor history was selected as the reason to decline a pancreas. The secondary objective of the questionnaire was to evaluate whether the questions effectively captured the topic under

investigation, flagging any confusion questions. As this questionnaire was newly designed for this pilot study, validation was not addressed.

The questionnaire was given to two surgeons at each of the eight UK pancreas transplant centres. They were asked a number of questions regarding clinical practice in the context of solid organ pancreas transplantation. Based on the initial responses, the question would open up for further detail if appropriate or allow for free text option.

2.12.1 – Copy of E Questionnaire

1. **Do you have formal written unit criteria of which pancreases you accept? (Please select one)**

   Yes/No/Other – free text

2. **Donor cause of death (COD) – What categories of COD would you not consider? (Select all that apply)**

   Intracranial haemorrhage/hanging/infection – cause unknown/infection cause known (please state – free text)/ drug overdose/hypoxic injury/out of hospital cardiac arrest/other (please state- free text)

3. **Downtime – How long is an acceptable downtime period? (Please state in hours and minutes) (HH:MM)**

   Other – Free text

4. **Cold Ischaemia Time (CIT) – What is the longest CIT you would accept and still proceed with transplantation? (Please state in hours and minutes) (HH:MM)**

   Other – Free text

5. **Insulin Use – Would you consider a donor on insulin?**

   Yes/No

If 'Yes' what is the acceptable cut off before you would decline? (Please state units of insulin per hour)

Other – Free text

6. **Alcohol Use – would you consider a donor with alcohol use?**

   Yes/No

   If 'Yes' what is the acceptable cut off limit for alcohol use before you would decline?

   Please state number of units a week/no limit/ Other – Free text

7. **Drug Use – would you consider a donor with drug use?**

   Yes/No

   If 'Yes' which of the following drugs would you accept? (Select all that apply)

   Stimulants/Depressants/Hallucinogens/Dissociatives/Opioids/Inhalants/ Cannabis/Oral Use/ Inhaled use/Intravenous use/Other – Free text

   If 'No' which of the following drug use would you decline? (Select all that apply)

   Stimulants/Depressants/Hallucinogens/Dissociatives/Opioids/Inhalants/ Cannabis/Oral Use/ Inhaled use/Intravenous use/ Other – Free text

8. **Enzymes – Which enzymes would you want to know the values for and what is the upper limit of what you would accept? Please select from the following (Select all that apply)**

   Amylase - Upper limit/Lipase - Upper limit/ALT - Upper limit/AST - Upper limit/Gamma GT - Upper limit

9. **Age – what are the lower and upper limits of what you would accept? (Please highlight your range)**

   1/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/19/20/21/22/23/24/25/26/27/28/29/30/31/32/33/34/35/36/37/38/39/40/41/42/43/44/45/46/47/48/49/50/51/52/53/54/55/56/57/58/59/60/61/62/63/64/65/Other – Free text

10. **BMI – What are the lower and upper limits of what you would accept? (Please highlight your range)**

    1/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/19/20/21/22/23/24/25/26/27/28/29/30/31/32/33/34/35/Other – Free text

11. **What is your attitude toward donor risk factors? (Select all that apply)**

    Cumulative effect of multiple factors/ Single factor sufficient to decline / Right organ for right patient/ Other – Free text

END – thank you for your participation

All 16 responses were returned highlighting the ease of completion and good compliance from users. Responses were enriched by the comments under the 'Free text' option, however the significant amount of information captured through this options suggests that the questions did not cover all areas fully and so further work is needed to make the questionnaire more robust. Based on this pilot, a future larger cohort may be selected to validate the questionnaire and assess the reliability of responses.

The results of the questionnaire were anonymised and outlined below. Results show only two centres had formal written criteria while the rest of the centres used a case-by-case assessment based on informal unit guidelines. The results identified substantial heterogeneity, not only between centres but also between the two surgeons from the same centre.

2.12.2 Cause of death (COD)

The majority of donor COD are related to intracranial haemorrhage and cardiovascular disease. However, discrepancies around trauma, hanging and infective causes with presumed pathology in the absence of identified pathogens were the main variations seen

in responses. Some surgeons were very conservative, discounting organs that deviated from guidance on the cause of death and not accepting any unconfirmed cause of death (even if all screening virology and cultures were negative and all other factors appeared favourable).

Donors with HIV and/or hepatitis make up a very small proportion of the UK donor pool. All centres agreed that they would decline donors with HIV and most with hepatitis, but one centre accepted organs from hepatitis C antibody positive donors if hepatitis C had been eradicated following treatment and if all other donor factors regarding the offer were acceptable.

2.12.3 Downtime

Long downtimes, including hanging with the unknown timeframe and other out-of-hospital events such as trauma, are thought to constitute additional warm ischemia. Longer periods of warm ischaemia are thought to be associated with more severe ischaemia reperfusion, potentially increasing reperfusion pancreatitis.

Variation in the acceptable maximum downtime ranged from 30 minutes to one hour (unsurprisingly, the same as the acceptable functional warm ischemia time in DCD retrieval). Unanimously, all responses agreed a time of up to one hour would be acceptable in this context. A better definition of acceptable time limits would be helpful, possibly by considering donor systolic blood pressure as the marker of the start of hypoxia, and liver enzyme levels as the marker of outcome.

2.12.4 Cold Ischaemia Time (CIT)

Prolonged pancreas CIT is associated with inferior graft survival rates. All responses agreed that the acceptable CIT should be under 12 hours however, only one centre has this as a fixed written criterion. With nationally documented transplants (CIT≤18 hours), CIT seems not limited to transport time alone, and may be contributed to by implanting centre complications or logistic issues delaying implantation.

2.12.5 Insulin use

Donors requiring insulin was another variable affecting whether centres would accept organs or not. There is some evidence to suggest that low-level donor insulin use is not detrimental but the upper limit of what is acceptable is less clear. Two respondents refused all offers if donors were on insulin while the rest had varying levels of acceptable insulin requirement. The reasons of why insulin was started was not captured by the questionnaire and it is an important point which needs to be included in the future revisions. The acceptable level of insulin on which a donor may safely be dependent prior to organ retrieval without affecting outcomes remains to be determined and warrants further study.

2.12.6 Alcohol use

When asked about the importance of extensive alcohol use, many regarded this as a criterion to decline an offer with the median peak level of acceptability as 25 units/week. Four responders felt that in the absence of proven pancreatitis, high alcohol use on its own was not a reason to decline an organ. The exact definition of what was regarded as ‘extensive’ use was difficult to define especially as a recording of alcohol units is not always clear in NHSBT electronic donor record.

Motallebzadeh et al. used the UK Transplant Registry to determine whether donor alcohol intake influenced pancreas survival in the simultaneous pancreas-kidney (SPK) transplants performed between 2006 and 2012 (n = 770). Recipients were stratified by donor alcohol intake: group I (n = 122) - high recent alcohol intake (>21 or >14 units of alcohol/week in males or females, respectively) or previous alcohol abuse, and group II (n = 648) - low/unknown current intake and no previous alcohol abuse. The authors found no difference in unadjusted graft survival in donor history of alcohol abuse or donors with high current intake (>50 units/week), suggesting that high donor alcohol intake, by itself, should not exclude consideration of pancreas transplantation [94].

2.12.7 Drug use

One centre had a fixed policy regarding donor drug use, not accepting donors who had used any substances except cannabis. The remaining 14 responses agreed that drug use was an important consideration in the donor history but most reported that a decision was made on a case-by-case basis. The use of injectable drugs was not a blanket reason to exclude donors as long as virology was negative, while 3 respondents stated that they would discount offers from intravenous drug users.

2.12.8 Enzymes

As raised transaminases (ALT and/or AST) may be a marker of a severe hypoxic injury, all centres agreed that any evidence of acute liver injury (suggested by raised liver enzymes) or any enzymatic sign of acute pancreatitis were reasons to decline an organ offer. However, the acceptable level of enzyme rise was variable, demonstrating considerable heterogeneity. A raised amylase was another point of contention in the survey, with the acceptable threshold ranging from 100-250U/L. Some centres and surgeons declined offers

on amylase elevation while others did not regard it absolute contraindication, particularly if the cause of death is hanging, (salivary gland trauma may account for the phenomenon).

2.12.9 Age

While one centre had a fixed criterion of donor age less than 55 years, another centre accepted donors up to 65 years, and the remaining centres would consider offers up to 60 years. However, all responses showed as age increased, the tolerance for other risk factors decreased. The exact accumulation of what risk factors were acceptable was difficult to ascertain but it is apparent from the registry data, that very few organs from older donors are used.

2.12.10 BMI

Only one centre had written criteria with an upper limit of BMI of 28, with the remaining centres using a case-by-case assessment, in general considering BMIs up to 30. However, five responses suggested that BMIs up to 32 might be accepted. With the overall contribution of transplanted pancreases from donors with BMI>30 only 3%, it was notable that 11 responses took into account the measurement of girth to assess the distribution of fat, with <100cm being the upper limit.

2.13 Discussion

The results of this chapter have identified that the falling number of pancreas transplants is primarily due to a reduced number of patients on the waiting list and not a lack of organs and so, the challenge remains to ensure patients receive the best donor organ possible amongst all the offers they may potentially receive.

The drive to grow pancreas transplantation needs further consideration and larger emphasis on the referral of appropriate patients to transplant units. With a conversion rate from offer to transplant as high as 23.5% in 2005, falling to 6.2% in 2016, there appears to be little evidence of an overall shortage of donor organs. It may be that the criteria for eligibility for pancreas transplantation should be reviewed, especially in light of increasing transplantation to a wider number of people with diabetes.

Utilisation data suggests that current evidence and practice do not align in what is deemed acceptable. The results highlight a disproportionate workload associated with the high and increasing level of discard. This needs to be addressed as, at the very least, it generates a significant amount of waste.

The heterogeneity in clinical practice when considering factors such as donor history, fattiness or damage, raises a number of possible questions: Should there be more rigorous guidelines nationally to ensure more consistent practice? Should this extend to referral practice as well as donor policies? Should the relative over-availability of donor organs mean that higher-risk organs should be declined routinely, or is the evidence insufficient to support this?

Introducing standardisation in order to improve pancreas utilisation and avoid the continued increase of unused organs that have been retrieved, may be achievable in a number of ways. One may be to have a national protocol with standardised criteria rather than variations between centres to improve utility. Another would be to develop strategies to increase patient referrals to transplant units and fully utilise the surplus of organs as this will increase experience in lower volume centres.

Eliminating the use of donors at the extremes of age and BMI would consolidate the number of acceptable donors, without impacting on the number of transplanted organs. With low current utilisation of donors >55years and those BMI >30, the rationale to include these demographics in the donor organ pool should be investigated further.

It is also necessary to study all the available evidence regarding the risk of donors of these different characteristics, so that risk can be minimised for current patients. Accepting that age or BMI alone may be insufficient to discard an otherwise perfect pancreas, continued use of donors at the extremes may be an opportunity to explore how best optimise extended criteria pancreases.

Donor history at the point of the offer has been shown to be the single most influential factor in determining whether an organ is accepted or not. However, the term itself encompasses many different components and so, understanding which combination and to which threshold is acceptable needs further investigation.

With 32.6% of pancreas discards being attributable to donor history, a variable currently coded as a single factor, the survey highlights the fact that donor history is highly heterogeneous and complex, causing considerable variation in both intra-centre and inter-centre practice. This raises the question of whether the current NHSBT coding system is adequate, and suggests that revising it will improve the granularity of the detail needed for analysis.

Results from the E Questionnaire demonstrated that the cumulative effect of multiple factors was critical in decision making. However, this is not captured in the current way offer information is recorded by NHSBT. As a result, it is impossible to understand what aspects

of the donor history and what combination of factors are important leading to pancreas decline. Revised data collection is essential to allow capture of missed information. The E Questionnaire also identified its own design did not fully capture key information, as evidenced by the fact that relevant additional information was added as free text, elaborating on these questions prior to validation will be necessary.

When considering the risk of raised enzymes, in particular the effect of amylase, Hesse et al. presented a donor series measuring amylase in (n=47) and plasma glucose in (n=94). 20 donors had elevated amylase (110-994 IU/L) and 51 had hyperglycaemia (200-980 mg/dl). The post-transplant pancreas graft function led authors to conclude that hyperamylasaemia and hyperglycaemia were not contraindications for donation unless associated with overt pancreatic trauma, pancreatitis, or a history of diabetes [95].

The Advisory Committee on the Safety of Blood, Tissues and Organs (SaBTO) offers guidance on the microbiological safety of human organs, tissues and cells used in transplantation, in order to minimise risk. The guidelines highlight that material from cases of meningoencephalitis for which no infection is identified should not be used for donation [96]. Nonetheless, data from NHSBT suggests that over a 10-year period, 159 transplants (all organ types) were undertaken from 52 donors that had meningoencephalitis with no firm aetiology [97]. The justification for transplanting such high-risk organs is based on the balance of risks in patients for whom delaying for a lower-risk organ would incur an even higher risk. Although the decision to use such organs ultimately lies with the implanting transplant surgeon, in the context of pancreas transplantation where there is no organ shortage the use of pancreases without confirmed cause of death diagnosis raises complex questions of balance of risk [98].

Results from this chapter have demonstrated damage accounts for 14.3% of discarded pancreases. The NHSBT discard audit highlighted the most frequent reason for this was a capsular tear followed by damage of and around the portal vein [83]. Continued attention to the training of retrieval teams to combat lack of experience or errors made due to aberrant anatomy are clearly important in order to reduce the rates of organ damage. Many retrieval surgeons may have not been exposed to pancreas transplantation during training, and may be less aware of the extremely vulnerable nature of the organ. Consensus is needed between pancreas transplant surgeons as to what level of injury (grade/severity) is acceptable. This may be achieved through the annual national pancreas forum where all pancreas centres meet to discuss issues of common interest and concern. It is possible that the apparent lack of consensus is a reflection of differential experience between high and low volume centres, and it also most probably reflects the lack of objective evidence.

Considerations to overcome iliac conduit problems include a possible vessel bank to allow the use of alternative conduits, as well as training retrieval teams to identify other possible grafts including carotid or brachiocephalic trunk during procurement.

The significance of fatty pancreases and presumed pancreatic steatosis remains an important area to clarify since it is a major source of underutilisation with 30.2% (IQR 27.3-40.6) of pancreases offered discarded on this basis. This is critical as these organs have been already accepted in light of knowing the donor BMI and girth, suggesting that the sensitivity and specificity of these markers is not robust. Improving the assessment of pancreas steatosis will be a focus of the forthcoming chapter.

CHAPTER 3:

Quantification of pancreatic steatosis in whole organ pancreas transplantation

3.1 Overview

The aim of this chapter is to develop an objective method to assess pancreases in terms of fattiness, in order to help resolve the diagnostic uncertainty surrounding the use of organs that are discarded as fatty and/or steatotic. Based on the evidence in the previous chapters, macroscopic fattiness presumes parenchymal steatosis and is the most frequent reason not to use pancreases once retrieved. As mentioned in chapter 1 the synonymous use of these terms is incorrect and contributes to the issues seen in utilisation, as steatosis is deemed a subset of being fatty.

However, chapter two identified the majority of discarded donor pancreases deemed fatty and steatotic came from donors with normal BMIs suggesting that either BMI is not a suitable surrogate or that the assessment of steatosis is neither objective nor appropriate.

Chapter three focuses on the definition of pancreas steatosis in the context of pancreas transplantation, aiming to identify what an acceptable threshold may be. The assumption that perceived pancreatic steatosis is associated with poor outcomes and will also be explored in this chapter.

I have investigated several methods to quantify pancreatic steatosis and identify its distribution within the pancreas, to ascertain the true level of ectopic fat deposition from pancreas donors. These assessments were performed in parallel, using the same donor organ set, allowing comparison of methods. Below is a short summary of each method to justify the rationale for inclusion into this assessment of pancreas steatosis.

3.1.1 Visual assessment

Current clinical practice evaluates parenchymal steatosis through visual assessment by transplant surgeons, and uses overlapping terms 'steatosis' and 'fattiness'. Findings are recorded using the qualitative terms "very", "severe", "moderate" or "mildly" to describe and attempt to classify organs in terms of their usability referring to the macroscopic fatty appearance. As shown in chapter two, there is significant variation on what constitutes mild or severe fattiness/steatosis, as well as ambiguity regarding what is acceptable. Understanding the problems of visual assessment and the variation of human interpretation are critical in the correct quantification.

3.1.2 Conventional histology

Microscopic analysis of stained tissue sections provides a wealth of data at the cellular level and is the current gold standard to assess pancreatic steatosis. Understanding the differences in the type of biopsies (and so the amount of tissue examinable) is important, as fine needle, tru-cut, punch, and full cross-sections all provide differing amounts of information. Processing methods (fresh, formalin fixed, frozen), location (head, body, tail) and a number of (single versus multiple) biopsies, give context to the conflicting results in current literature where variations in methods have been used. Biopsy interpretation is traditionally carried out by histopathologists, but the use of digital pathology and machine learning to analyse biopsies in combination with traditional histopathologic assessment may more accurately assess donor pancreases.

3.1.3 Digital image analysis

ImageJ was developed by Wayne Rasband and is a Java image processing and analysis program based on NIH Image for the Macintosh. ImageJ is free and runs on any computer

that is a Java 1.5 or later virtual machine, making it widely accessible. It evaluates digitally captured images using indices of positivity for photographs, immunohistochemistry and fluorescence images. Analysis principles work with all pixels being assigned the value of black, which then have additive colour. As the amount of reflected light is increased from the original capture of the digital image, the value of the pixel will be adjusted allowing differentiation between colours and also shades of the same colour. Primary colours are used as a reference, which for a 24-bit colour image, white pixels would be made up of red, yellow and blue values of = R255, Y255, B255 and black = R0, Y0, B0. All other colours are displayed by varying these pixel values. Each colour shade (hue) has a value, the saturation is equivalent to intensity and brightness [99].

3.1.4 Immunohistochemistry

The techniques of staining ectopic steatosis have been well validated, with a number of stains available including Perilipin for formalin fixed sections and Oil-Red-O for frozen sections. In addition to assessing steatosis, formalin fixed slides were stained for insulin to identify islets, with the aim to assess whether beta cell mass is also affected by steatosis. Current evidence is conflicting with Szczepaniak et al. identifying significant correlation between pancreas steatosis and beta cell dysfunction with potential reduced beta cell mass [100], while Murakami et al. identified that pancreatic fat deposits had little effect [101].

3.1.5 Adiponectin

Adiponectin is a potential biomarker for steatosis. This is a hormone of adipocyte origin that is involved in the homeostatic control of circulating glucose and lipid levels. It has been shown to be an indirect regulator of glucose metabolism, increasing insulin sensitivity, improving glucose tolerance and also inhibiting inflammation. Unlike most adipokines,

which positively correlate with obesity, high adiponectin levels show a strong inverse relationship with measures of body fat. Flechtner-Mors et al. examined 1349 subjects (age 18-65 years) who underwent an ultrasound examination of the liver as well as having adiponectin measured. This showed an inverse relationship between adiponectin and hepatic steatosis, but the utility of adiponectin in the assessment of pancreatic steatosis is yet to be determined [102].

3.1.6 MRI

The best imaging technique quantifying fat in the pancreas is magnetic resonance imaging (MRI). However, which MRI sequence is optimal remains unclear and as a result, variation between studies makes comparison difficult. In 1984, Dixon published the first paper on a simple spectroscopic imaging technique for water and fat separation. This utilises the frequency shift between the proton resonances in fat and water to generate in-phase and opposed-phase images where the difference between the net magnetisation vectors of fat and water are at a maximum (in-phase, 0° between vectors) or a minimum (opposed-phase, 180° between vectors). A linear combination of these images in post-processing yields a fat fraction parametric map as well as images of only fat or water. This method is quick, simple and available on all MRI systems, which makes it more usable than more complex sequences that are only available on certain machines or specialist centres [103].

3.1.7 Splenic DNA and PNPLA3

Correlations between non-alcoholic fatty pancreas disease (NAFPD) and non-alcoholic fatty liver disease (NAFLD) are increasingly recognised, with studies showing similarities between these two pathologies. Andrade et al. evaluated 576 ultrasonography exams, showing a

positive correlation between the two [104]. withPatel et al. demonstrating the same association using MRI in 43 patients [105].

The human patatin-like phospholipase-3 (PNPLA3) gene is localised on human chromosome 22. The PNPLA3 protein (adiponutrin) is expressed in both adipocytes and hepatocytes. PNPLA3 exhibits lipase activity against triglycerides and acylglycerol transacetylase activity, with its expression highly responsive in energy mobilisation and the storage of lipid droplets. The PNPLA3 gene is one of the potential candidate genes currently related to NAFLD susceptibility [106].

In 1998, Romeo et al. noted that a single nucleotide polymorphism in residue 148 (I 148 M, rs738409), was a strong genetic determinant of NAFLD. However, it is unclear whether this polymorphism is associated with simple steatosis only or also associated with NAFLD. Investigating whether an association between the rs738409 polymorphism and histological parameters of pancreas steatosis exists may be of benefit in the assessment of donors [107].

3.1.8 Electrical Impedance Tomography (EIT)

EIT is a non-invasive method of medical imaging in which the electrical conductivity, permittivity and impedance of a material is inferred from surface electrode measurements and used to form a tomographic image. Electrical impedance is the measure of the opposition that a circuit presents to a current when a voltage is applied. Hence, using very small alternating electrical currents applied between pairs of electrodes, allows the resulting voltage to be measured. Knowledge of the current and how it changes, allows interpretations of the measurement and build the image through repeat measurements of

the electrical potential field at various points on the boundary of the substance being measured. Capacitance is the ratio of the change in an electric charge in a system, where the change corresponds to the change in its electric potential. Hence this will also change when a substance is being measured using EIT.

Gonzalo et al. have shown electrical bio-impedance to indirectly measure steatosis, using a porcine model where they found a significant correlation between EIT and biopsy-proven hepatic steatosis [108]. EIT may also provide a useful method to quantify pancreatic steatosis at the point of retrieval, and allow implanting centres to make more informed decisions on potential pancreas offers.

3.2 Material – source of pancreases

3.2.1 Animal material

East London Abattoir, Romford Halal Meats Ltd, supplied 6 sheep pancreases which were explanted from sheep of varying age and weight, aimed to correlate to various measures of steatosis defined as low, medium and high based on work performed by Helman et al. in 1995 (lamb less than eight weeks (10kg) = low steatosis, two-year-old sheep (40kg) = medium steatosis and five-year-old sheep (120kg) = high steatosis) [109].

3.2.2 Human material

Access to human pancreases was granted by registering the research study with NHSBT, where all pancreases with research consent were made available once discarded from clinical use. 66 organs were accepted into the study. (REC approval: 16/WA/0182, IRAS: 206897).

Whole discarded pancreases were retrieved by the NHSBT National Organ Retrieval Service (NORS) with the intention of clinical transplantation between Sept 2015 and Oct 2017. Organs were transported on ice, triple bagged in University of Wisconsin (UW) solution. Donor data was taken from the accompanying Electronic Offering System (EOS) document. Donor blood that accompanied the organ was also taken for assessment in an ethylenediaminetetraacetic acid (EDTA) tube, from which plasma was later separated. All samples had research consent and the study had national research ethics approval. (REC approval: 16/WA/0182, IRAS: 206897).

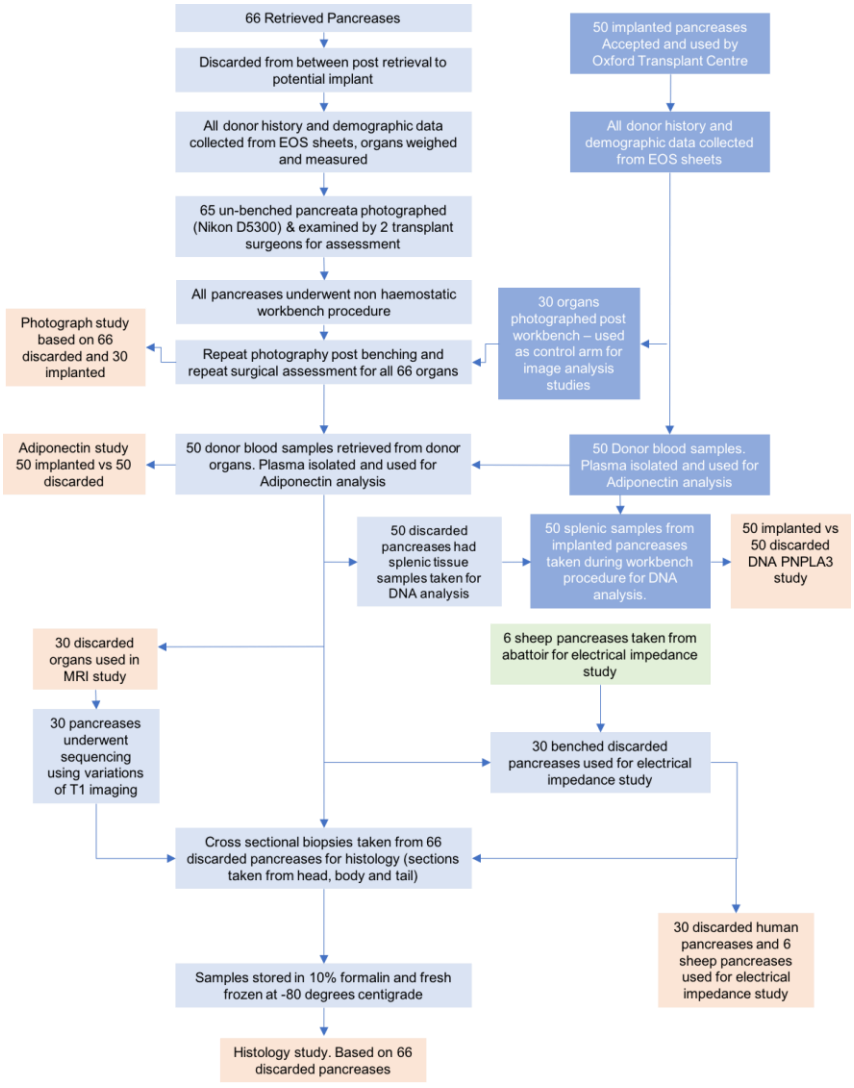
A further 50 pancreases which were accepted for implantation by the Oxford transplant centre were examined as a control group. Donor EDTA blood and photographs of implanted pancreases were taken during workbench procedure. No biopsy material was taken from pancreases that were implanted. Organ demographics are shown in Table 3.2.2.1.

Table 3.2.2.1: Demographics of human donor pancreases (implanted and discarded) expressed as median and IQR where applicable.

	Discarded
Total Number of Organs	66
Age	48 (38.7-54)
Male: Female	43:23
BMI	24.1 (22.4-27.4)
Girth	89.5(66-98.2)
DBD: DCD	48:18
Ethnicity (Caucasian: Other)	64:2
CIT (Median & IQ Range)	10.1 (9.1-12.1)
Organ Weight	207g (117-298)
Organ height (H), length (L) and thickness (T)	H = 3.3cm (2.1-5.2), L = 22.1cm (13.4-28.9), T = 2.7cm (1.2-3.4)
Discard Reason: damage, steatosis, anatomy, recipient issue	44%, 39%, 11%, 6%

	Implanted
Total Number of Organs	50
Age	32 (23-49)
Male: Female	22:28
BMI	22.6 (21.1-25.2)
Girth	83.5(76.5-90.2)
DBD: DCD	42:8
Ethnicity (Caucasian: Other)	49:1
CIT	10 (8.5-11.3)
Organ Weight	213g (115-307)
Organ height (H), length (L) and thickness (T)	H = 3.4cm (2.2-5.1), L = 20.5cm (10.4-25.6), T = 2.5cm (1.3-3.3)

3.3 Experimental flow diagram - Outlines the number and type of pancreas tissue used for each experiment

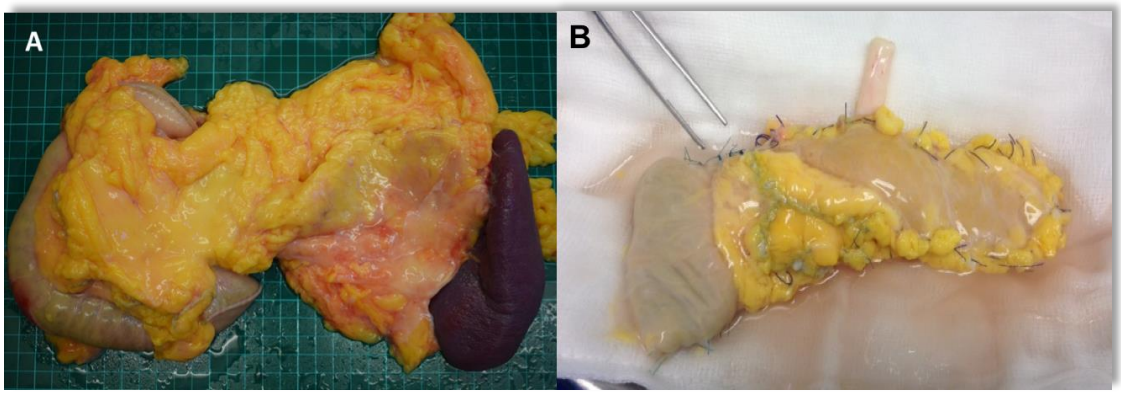


3.4 Methods

3.4.1 Workbench procedure

All pancreases underwent standard workbench preparation ('benching'), where mesentery and omental fat were removed from the pancreas, the spleen disconnected and the duodenum shortened (closed at both ends). Figure 3.4.1.1 below shows the pancreas before and after benching. When considering the embryological fusion of the pancreas buds, the uncinate process and true head of the pancreas were combined and labelled as the head region adjacent to the duodenum. Following benching, organs were weighed with length and width measured.

Figure 3.4.1.1: Image A: Un-benched pancreas as retrieved, showing spleen attached to the tail of the pancreas and the omentum/mesentery covering the organ. B: Pancreas following completed workbench procedure, showing a shortened duodenal cuff at the head of the organ, multiple ligations, and staple lines, with the arterial Y graft attached to the superior mesenteric artery (SMA) and splenic artery openings.



3.4.2 Current practice assessment

Consultant transplant surgeons were asked to estimate the amount of steatosis based on current practices of visual and tactile assessment. Assessments were performed twice ,before and after workbench preparation, and reassessed using a second consultant surgeon who was not aware of the first assessment results. Steatosis was estimated to the closest percentage (see Figure 3.4.2.1).

Figure 3.4.2.1: Consultant surgeon assessment of discarded pancreases at various stages of the workbench procedure.



3.4.3 Digital image procurement

Plan view digital photographs were taken with SLR camera (Nikon D5300) at a resolution of 24 megapixels. The camera rig was constructed in-house using two Vivitar 62-inch camera tripods and CameraTrax automated camera slider. As pancreases are normally transported on ice and kept cool (0-5°C) until reperfusion, a cooling mat was constructed for the pancreases to be laid out for photography while minimising further warm ischaemic injury. A polyester sponge and polyvinyl chloride (PVC) composite with inner liquid cooling gel (Marukan by Malayas) was kept in the freezer and placed under an overlying mat which was

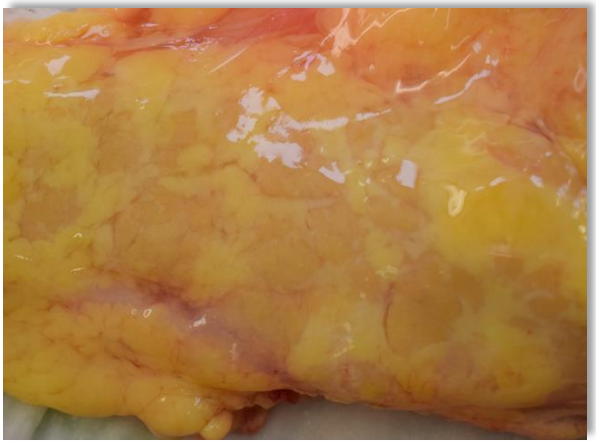
cooled. The overlying mat was a green plastic cutting mat that could be sterilised. The temperature was measured using an attached digital thermometer (see Figure 3.4.3.1).

Figure 3.4.3.1: Camera set up used to create standardised and reproducible photographs of discarded pancreases.



Organs were photographed both pre-and post-workbench preparation at a fixed distance of 50cm. Lighting conditions were standardised and each organ had eight photographs capturing anterior, posterior, superior and inferior views. High resolution images were saved as PNG files (see Figure 3.4.3.2).

Figure 3.4.3.2: Plan view photograph of a benched organ with the marbling effect of steatosis mixed with the pancreas parenchyma clearly visible.



3.4.4 Image analysis

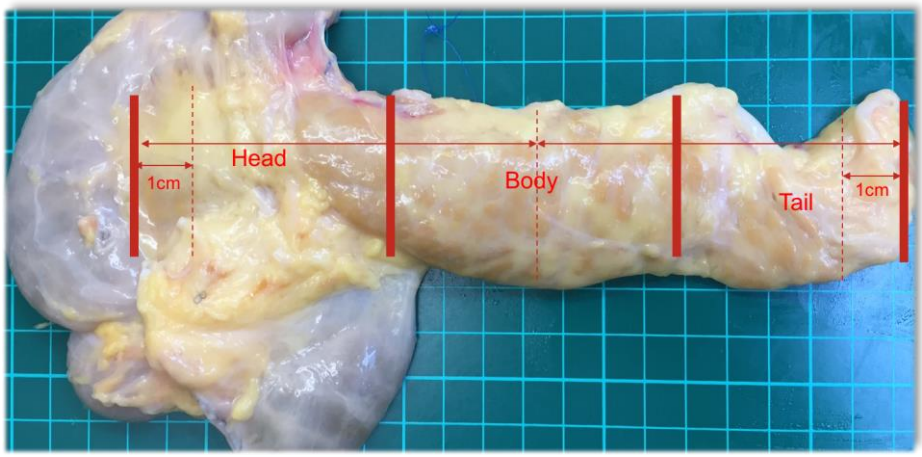
ImageJ was used for digital photo colour analysis. Plan view macroscopic photographs and scanned tissue sections were uploaded and analysed in image J. By identifying Hue Saturation Brightness/Value (HSB/V) and Red Green Blue (RGB) values, using a pre-set threshold value and colour histogram, the true pancreas tissue could be separated from surrounding fat. Organ edges were outlined using an automated process allowing reproducibility, delivering a fat percentage for each image. This method was used for macroscopic photographs and scanned slides.

3.4.5 Biopsy procurement

Following the workbench procedure, two full pancreas cross sections of 2.5mm in thickness were taken from fixed distances: one centimetre from the tip of the tail, one centimetre from the head at the boundary of the duodenum, and at the midpoint of the organ. Samples

were named as Head, Body and Tail, one stored in formalin being later fixed in paraffin and the other fresh frozen at -80°C (see Figure 3.4.5.1).

Figure 3.4.5.1: Diagrammatic overview of where pancreas biopsies were taken.



3.4.6 Wax processing of tissues for histology

Tissues were placed in plastic cassettes and processed using a Leica Histokinette on an overnight programme from neutral buffered formalin (10%, 30mins) through graded alcohols (70%, 1hr; 90%, 1hr; 95%, 1hr; 100%, 1hr; 100%, 2hrs; 100% 2.5hrs) to xylene (3 buckets; 1hr, 1hr, 1.5hrs) to molten paraffin wax (2 buckets; 4hrs, 5hrs).

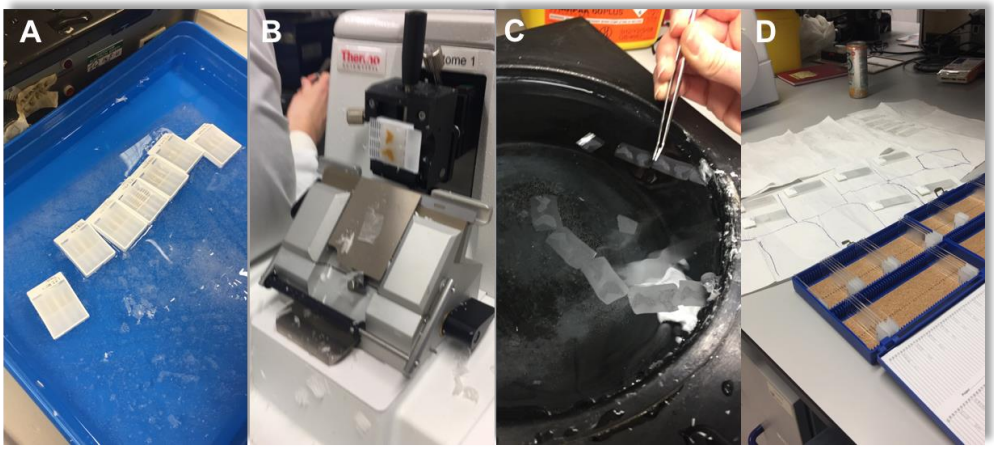
3.4.7 Embedding tissues in paraffin

Embedding was performed on a Leica embedding centre maintained by the NDS QUOD biobank. Molten wax was poured into plastic moulds containing wax processed pancreas tissue and samples were orientated to allow a full cross-section on cutting. The wax hardens around the sample in the plastic cassettes and makes up the finished block, allowing blocks to be easily cut.

3.4.8 Cutting paraffin sections and mounting slides

Sections 5µm thick were cut using a microtome and then floated onto the water surface at 40°C. Once creases were smoothed out, sections were lifted out of the water, using the paraffin to adhere to the glass slides. Slides were then allowed to dry out for 12hrs (see Figure 3.4.8.1).

Figure 3.4.8.1: Four panel photo showing processing of slides. Panel A shows the cooling of wax blocks, allowing them to harden in order to be cut. Panel B shows the microtome to cut sections. Panel C shows the floatation of cut sections to allow glass slides to adhere to them. Panel D shows the adhered tissue drying onto the glass slides ready for labelling and staining.



3.4.9 H&E staining of sections

Slides were heated in an oven at 60°C for 35mins. Sections were de-waxed and taken to tap water using the following method: Xylene (3mins x2), ethanol (100%, 3mins x2; 90%, 3mins x2; 70%, 3mins x2) and then running tap water (3mins x2). Firstly, the slides were stained with Harris haematoxylin for 2mins and then washed with running water for 3mins. The background was reduced by dipping twice in 1% acid/alcohol, a quick rinse in running tap water followed by 30seconds (s) in Scott's tap water substitute, and a final rinse in running

water. Slides were then stained with 1% eosin for 2mins and the excess stain was washed off with running tap water. Sections were then dehydrated with ethanol (70%, 30s; 90%, 1min; 100%, 3mins x2) and xylene (3mins x2). A glass coverslip was then mounted over the tissue from xylene with DPX mountant (Sigma Aldrich), ensuring no air bubbles were trapped between tissue and coverslip.

3.4.10 Frozen sections and lipid staining

Using frozen samples, full cross-sections of frozen tissue were cut at 10µm thickness. Frozen sections were then placed upon clean glass slides. Slides were fixed in 10% formalin and then washed in distilled water. Propylene glycol was used with two changes, 5 minutes each, then stained with Oil Red O for 7 minutes and then agitated. 85% Propylene glycol was used for 3 minutes. Slides were then rinsed in distilled water and then kept in Haematoxylin for 1 minute after which they were again washed in water. Bluing solution was used, then slides again were rinsed in distilled water. Slides were mounted with aqueous mounting media-glycerin jelly, with the slide image ready for scanning and image analysis.

3.4.11 Insulin staining

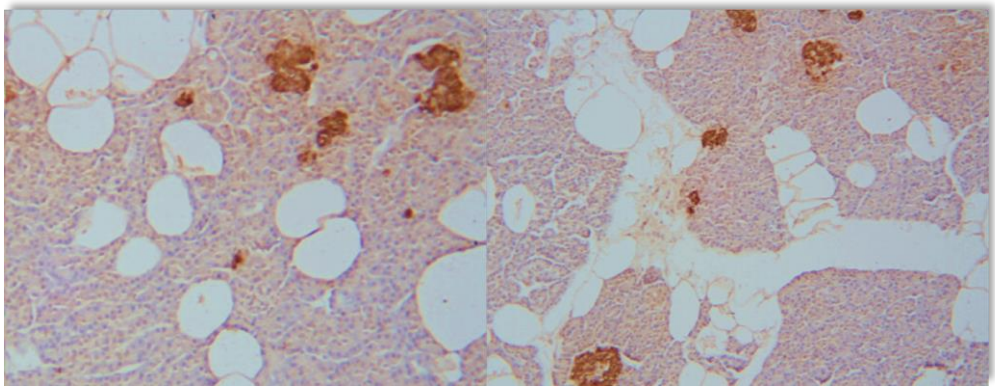
Previously published islet morphometrical analysis have shown both β-cell (insulin stained) and α-cell (glucagon stained) composition helps identify islets. Differences in size distribution, number and volume of human islets were measured and the variation in insulin content and volume was analysed. Published results identified that core β-cells of large islets contained less insulin and had cells with a lower insulin granule density than peripheral smaller β-cells. Using this data, the threshold of identifying 10 adjacent cells stained for insulin was deemed sufficient to identify an islet [110].

In this study, islets were identified using insulin staining from formalin fixed paraffin embedded sections, the brown stained insulin-positive cells, where 10 cells or more clustered together were deemed to be islets [111]. Paraffin-embedded sections were cut with a thickness of 6µm and fixed onto glass slides. Sections were then stained with a polyclonal guinea pig anti-porcine insulin primary antibody. The primary antibody was detected using Cy2-conjugated secondary antibody. Haematoxylin staining was then used to highlight the acinar tissue. Areas of positive labeling were determined in relation to the total area of acinar tissue examined (see Figure 3.4.12.1).

3.4.12 Histology image acquisition

Stained slides were scanned at ×40 using a single z-plane via an Aperio XT whole slide scanner (Leica Biosystems, USA). Scanned images were evaluated for quality and to ensure that they were in focus. Digital files in (.svs) format were stored on an image server for remote evaluation using the WebScope viewer from Aperio. Each .svs file was 817 ± 242 MB in size. Acquisition time for each slide averaged 1 min (see Figure 3.4.12.1).

Figure 3.4.12.1: Scanned stained tissue sections showing islets with insulin staining in brown, and normal pancreas parenchyma in pink (H&E staining). Circular white areas represent adipocytes while straight branches show the interlobular fat.



3.4.13 Histological analysis

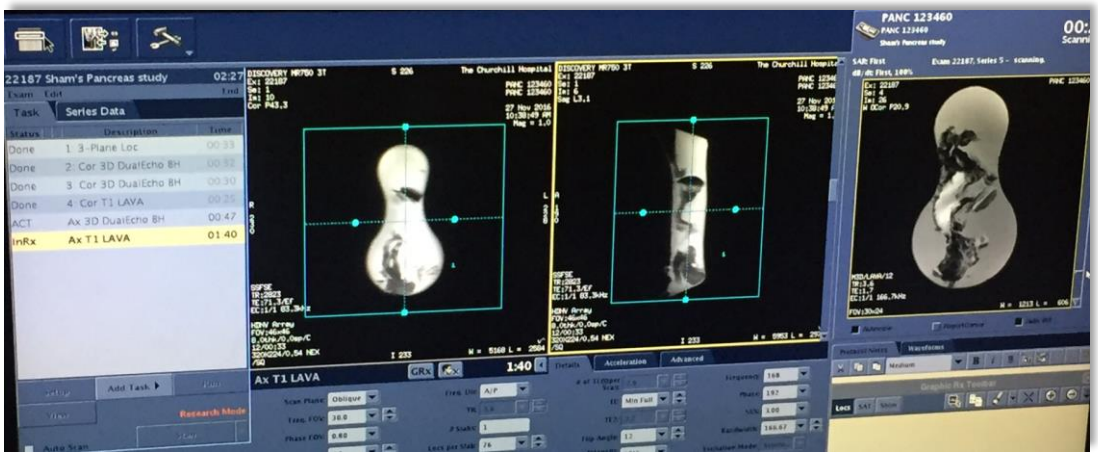
The percentage area of free fat and adipocytes occupying human pancreatic tissue was determined from images of histological sections as well as the number and size of islets in proportion to tissue. Sections were scored by two independent consultant histopathologists as well as digitally estimated by ImageJ. Fat and adipocytes were identified by labelling for Oil-red-O staining. The area of positive labelled tissue was determined in relation to the total area of acinar tissue examined. Interlobular adipose tissue was also included while large ductal structures were not. A mean section area of 891mm^2 was examined for each human specimen.

3.4.14 MRI acquisition

30 human pancreases underwent MRI assessment using a 3T clinical scanner (GE Healthcare, Waukesha, WI, USA). Organs were laid out to length, submerged in cold UW. Some had undergone workbench procedure but others had not, depending at the point at which they had been discarded. Temperature control was confirmed using a digital thermometer on a subset of organs.

A dual-echo 3D fast spoiled gradient echo pulse sequence ("LAVA flex", GE Healthcare) was used to acquire a stack of in-phase and opposed-phase axial images. The imaging parameters were: 3.6ms TR, 4.0ms TE, 12° flip angle, 256 × 256 × 144 matrix size, 300 × 300 × 288 mm^3 field of view, and 2mm slice thickness. In five pancreases, images were acquired with the same protocol except in a coronal plane. Standard online reconstruction was performed on the scanner to generate fat-only and water-only images (see Figure 3.4.14.1).

Figure 3.4.14.1: Screenshot of the MRI sequencing used to capture data from the discarded pancreases on 3T MRI machine.



3.4.15 MRI post-processing

A fat fraction (FF) map was computed pixel-wise from the axial images in Matlab according to:

$$\eta = \frac{F}{W + F}$$

Where η is the computed fat fraction, F and W are the magnitude signal intensities in the fat and water images [103]. This method ensures that the resulting fat fraction map is independent of the spatially-varying receive coil sensitivity [112]. The pancreas was then manually contoured on the water-only image with the pancreas volume and average fat fraction being calculated. The average fat fraction at the head, tail and in the body, was assessed by averaging over one image slice chosen to match the biopsy position. To remove a sampling bias, the average fat fraction across all pancreas voxels was computed to give an overall fat fraction for each pancreas.

3.4.16 Human high molecular weight & total adiponectin ELISA

Sample handling

Donor serum samples accompany all donated pancreases for further use by tissue typing teams if necessary. These samples were collected accompanying organs (both implanted and discarded) and underwent 2000xg centrifugation for 20 minutes at 2-8°C. 500µL of serum was aliquoted into cryotubes and stored at -20°C. Upon thawing samples were kept on ice until performing the assay, avoiding repeated freeze/thaw cycles, and were pre-treated with citrate buffer and SDS, which reduces multimeric adiponectin to dimers.

Serum Control Untreated

1. Combine 5µL of reconstituted serum control with 295µL of Adiponectin Assay Buffer in a tube and vortex to mix thoroughly.
2. In a separate tube, combine 12µL of the previously diluted serum control and 988µL of Adiponectin Assay Buffer and vortex to mix thoroughly.
3. The serum control is now effectively diluted 1:5000. Diluted serum control must be used within two hours of dilution when stored at room temperature.

Total Adiponectin Samples

1. Combine 5µL of the sample with 295µL of Adiponectin Assay Buffer in a tube and vortex to mix thoroughly.
2. In a separate tube, combine 12µL of the previously diluted sample and 988µL of Adiponectin Assay Buffer and vortex to mix thoroughly. The sample is now effectively diluted 1:5000. Samples must be used within two hours of dilution when stored at room temperature.

Full assay

1. The microplate was equilibrated to room temperature prior to opening the foil pouch.
2. 50 µL of each standard, control, and the diluted sample was pipetted into their respective wells.
   a) See table below of standards and their concentrations.
   b) Controls include adiponectin control (low & high) and adiponectin serum control.

Standard	Source	Assay Buffer	Standard Concentration (ng/mL)
A	Reconstituted Standard (see C of A*)	See C of A	5.000
B	300 µL (Std A)	300 µL	2.500
C	300 µL (Std B)	300 µL	1.250
D	300 µL (Std C)	300 µL	0.625
E	300 µL (Std D)	300 µL	0.313
F	300 µL (Std E)	300 µL	0.156
G	300 µL (Std F)	300 µL	0.078

3. 50 µL of working strength detector antibody solution was then pipetted into each well.

Number of plates	Amount of Adiponectin Detector Antibody	Amount of Detector Antibody Buffer
0.25 (3 strips)	15 µL	1.5 mL
0.5 (6 strips)	30 µL	3.0 mL
0.75 (9 strips)	45 µL	4.5 mL
1.0 (12 strips)	60 µL	6.0 mL

4. The microplate was covered with a plate sealer and incubated for two hours at room temperature, shaking at 700-900 rpm on a microplate shaker.
5. Well contents were decanted and the microplate washed six times with 350 µL of working strength wash buffer per well, using an automated microplate washer (NB: the wash buffer is provided in a concentrated form, dilute with dH_2o (see table below).

Number of plates	Amount of Concentrate (manual wash)	Amount of dH_2O (manual wash)
0.25 (3 strips)	12.5 mL	250 mL
0.50 (6 strips)	25.0 mL	500 mL
0.75 (9 strips)	37.5 mL	750 mL
1.0 (12 strips)	50.0 mL	1,000 mL

6. 100 µL of working strength streptavidin-horseradish peroxidase (SA-HRP) solution was pipetted into each well (see table below).

Number of plates	Amount of Concentrate	Amount of SA-HRP Buffer
0.25 (3 strips)	30 µL	3.0 mL
0.50 (6 strips)	60 µL	6.0 mL
0.75 (9 strips)	90 µL	9.0 mL
1.0 (12 strips)	120 µL	12.0 mL

7. The microplate was covered again with a plate sealer and incubated for 30 minutes at room temperature, shaking at 700-900 rpm on a microplate shaker.
8. Well contents were decanted and the microplate washed six times as in Step 5 above.
9. 100 µL of TMB Substrate was pipetted into each well.
10. The microplate was covered with a plate sealer and incubated for 15 minutes at room temperature, shaking 700-900rpm on a microplate shaker.
11. 100 µL of Stop Solution was pipetted into each well and the microplate gently shaken to mix the contents. Air bubbles were removed before proceeding to the next step.
12. The microplate was placed in the microplate reader at an absorbance of 450nm.

3.4.17 Spleen DNA extraction and analysis

Material: Small sections of donor splenic tissue were digested with proteinase K overnight (approximately 16hours) and the resulting lysate was processed using the Promega Maxwell RSC As1400 Blood DNA kit to extract the DNA.

PCR Method

1µl DNA at a concentration of 100ng/µl was added to the reagents below in a 1.5ml Eppendorf tube.

KAPA+$MgCl_2$ Mix*	10.2µl
PCR Primer mix♦	5µl
dH_2O	3.8µl

*125µl 50mM MgCl2 (Bioline) is added to 6.25ml KAPA2G Fast Ready Mix (KK5609, Kapa Biosystems) on arrival.

♦ The primer mix consists of forward (F) and reverse (R) primers at a concentration of 2µM each.

Primer sequences: F: TGTGTGAGCACACTTCAGAGG, R: GGGTTGTTAAGGGGATTATGC

Reagents were mixed and transferred to a PCR plate (total volume 20µl).

Once placed on the PCR machine, cycling conditions were set to the following parameters:

1) 95°C for 3mins
2) 95°C 10 seconds, 60°C 10 seconds, 72°C 1 second
3) Repeat step 2, 35 times
4) 72°C for 1 min

Sequencing Conditions

Once PCR programme had completed, 5µl of product was cleaned usingAmpure XP on an automated protocol using a Biomek NX^{p}:

1) Removed 5µl PCR product and placed in a new PCR plate
2) Added 9µl AMPure XP (1.8 µL AMPure XP per 1.0 µL of sample)
3) Bind DNA fragments to paramagnetic beads
4) Separation of beads + DNA fragments from contaminants
5) Washed beads + DNA fragments twice with 70% Ethanol to remove contaminants
6) Elute purified DNA fragments from beads
7) Transferred to new plate

Bidirectional sequencing

To 2.5µl purified DNA, the following reagents were added:

1) 0.2µl BigDye v3.1
2) 1.3µl 5x Buffer
3) 0.5µl Primer (F or R at a concentration of 2µM)
4) 0.5µl Water
5) 5.00µl Total volume of reaction

Solution placed onto PCR machine and cycling conditions were set to the following parameters:

1) 96°C for 5 min
2) 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 2.5 mins
3) Repeat step 2 for 30 cycles

Sequencing products were then purified using Agencourt Cleanseq on an automated protocol using a Biomek NX^{p}:

1) The CleanSEQ reagent was shaken to fully re-suspend the magnetic beads ensuring that no visible bead pellet remains in the bottle.
2) 10 μL of CleanSEQ reagent was added to each sample.
3) 85% ethanol was added to each sample according to the table below. The mix was then pipetted seven times, so the solution is homogenous (ethanol floats to the top of the sample, while the CleanSEQ sinks to the bottom).

Sequencing Reaction Volume (μL)	Volume of 85% Ethanol (μL)
5	31
10	42
15	52
20	62
25	73

4) Sample plate was placed onto a magnetic plate (Beckman Coulter Agencourt SPRIPlate 96R) for 5 minutes until the magnetic beads formed a ring on the side of the well.
5) The next steps were performed whilst the plate was still on the magnet:
    a. Supernatant was aspirated and discarded;
    b. 100 μL of 85% ethanol was added into each well and timed for 1minute;
    c. Repeat 'a' and 'b' twice.

6) Samples then were air dried for 10 minutes at room temperature.
7) 40 μL of elution buffer (reagent grade water or 0.1mM EDTA (pH 8.0)) was added into each well and incubated for 3 minutes at room temperature to elute until a solution is clear.
8) 35 μL of the clear sample was transferred into a new plate for loading on the detector.

Products were then run on an ABI3730 and results were analysed using Mutation Surveyor software.

3.4.18 Electrical impedance

The sensor was commercially produced by Zedsen Ltd. Its surface consisted of alternating transmitter (T) and receiver (R) electrodes across perpendicular directions - x and y (see Figure 3.4.18.1). The electrodes were printed on opposite sides of a membrane and did not touch, with the outermost electrodes across both directions being transmitters. Data was obtained by applying a voltage pulse on one of the transmitter electrodes and measuring the voltage on one of the receiver electrodes after it passed through the pancreas which was laid out on the sensor. The measured voltage depends on the complex permittivity of the materials between the two electrodes. A full scan involves scanning all possible pairs of parallel transmitters and receivers across the entire sensor surface and grouping them into layers (see Figure 3.4.18.2).

Figure 3.4.18.1: (a) Top view of the sensor. The white cross shows the origin of the coordinate system. (b) Zoomed in view of one of the membrane edges. The black and white lines are overlaid with x transmitters and receivers.

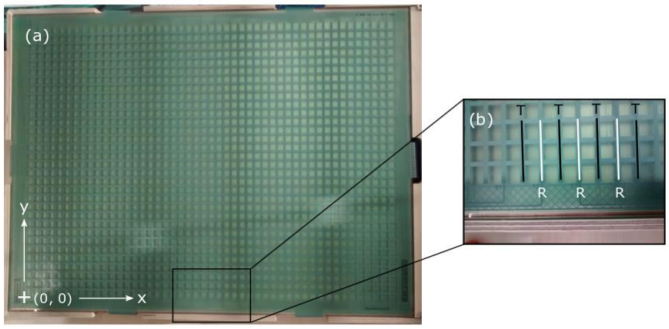
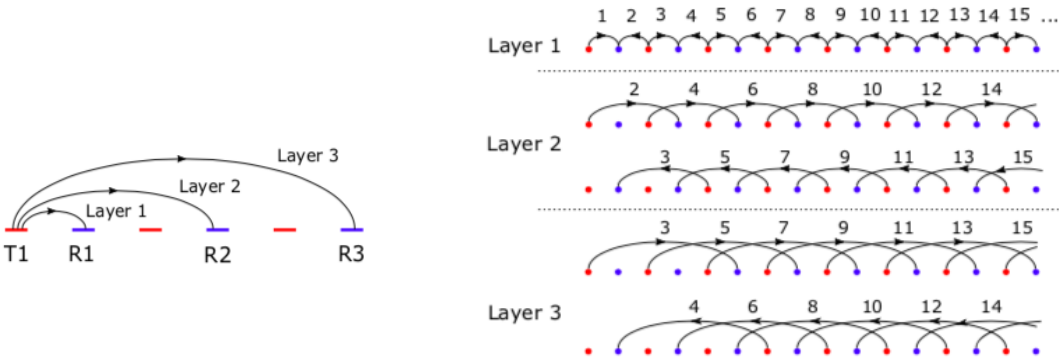
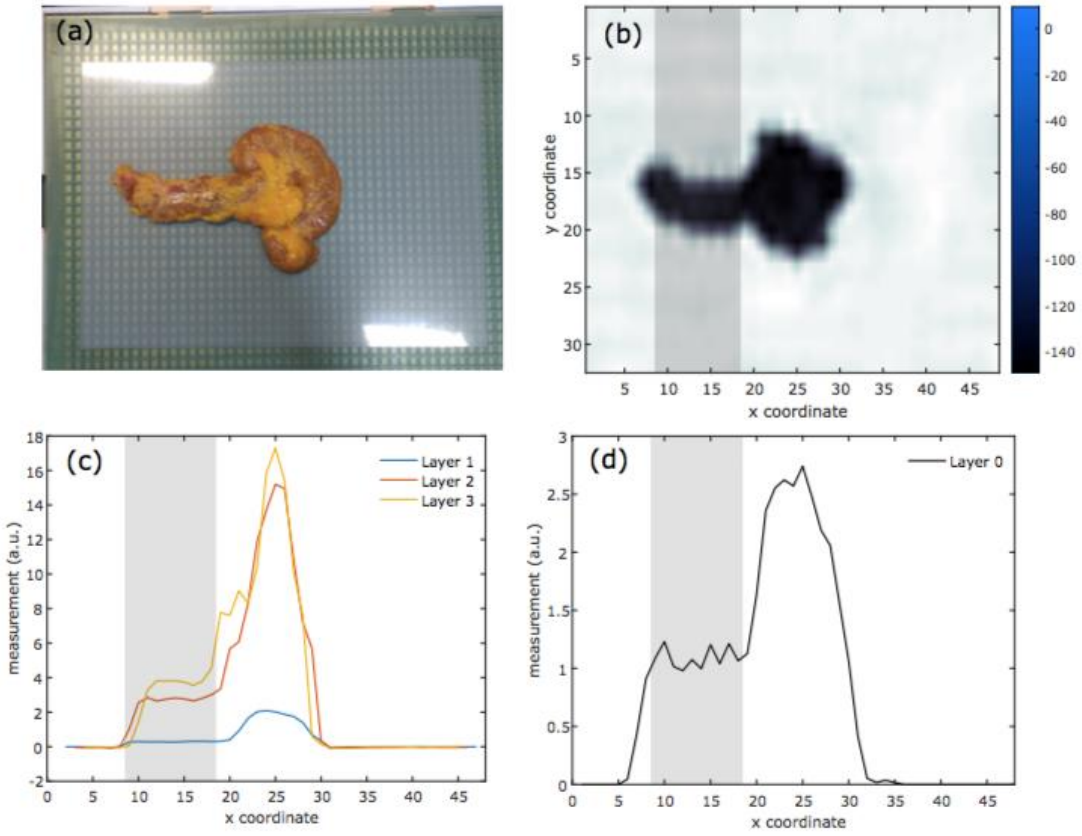


Figure 3.4.18.2: The measurements are categorised into layers according to the distance of the transmitter and receiver electrodes, in order to generate penetration depth. The 3rd layer (L3), for example, corresponds to all the measurements between a transmitter and its 3rd nearest receiver, on either side. Multiple readings are taken at each layer, using different variations of electrodes to create more data points.



Benched pancreases were placed on the sensor as shown in Figure 3.4.18.3, allowing the electrical outline of the pancreas to be visualised. Variations in electrical fields through the different layers were measured with permittivity levels recorded using prebuilt algorithms within the sensor. These results were then correlated against macroscopic and histological steatosis values.

Figure 3.4.18.3: a) Shows a human pancreas on the sensor. b) The electrical image outline of the organ can be seen using the electrical permittivity. c) Variations of frequency and layering create multiple data plots of the same organ. d) The differences are compared to the baseline which is inferred as layer 0.



3.4.19 Statistical analysis

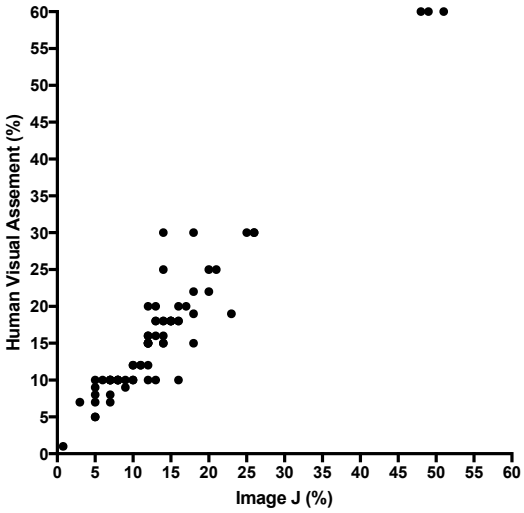
All analyses were conducted in GraphPad Prism V7.0. Data was deemed non-parametric following normality testing and so the statistics were used: median, interquartile range (IQR), Spearman Rank, Mann Whitney U and Wilcoxon signed rank tests. Comparisons among the three groups were performed by Kruskal-Wallis test. Where multiple assessments were made, the coefficient of variation was measured. Multivariate analysis was used to adjust for confounding factors such as age and body mass index (BMI). $P<0.05$ was deemed significant.

3.5 Results

3.5.1 Visual assessment versus digital photography

66 discarded pancreases were assessed by two consultant retrieval surgeons and then photographed and analysed through ImageJ. A positive and significant linear correlation between human estimation of macroscopic pancreatic steatosis and digital photograph analysis of benched pancreases was identified ($p = 0.04$) $R^2 = 0.72$. Human estimation was found to be 18.3% higher than computed analysis, with the tendency for human results to round to whole numbers while the digital software provided higher precision and was able to delineate subtle differences in pixels that may appear similar to visual assessment. The coefficient of variance between surgeons was 28% (see Figure 3.5.1.1).

Figure 3.5.1.1: Correlation between macroscopic visual assessments of pancreatic steatosis using photographs assessed by surgeons and computed analysis.

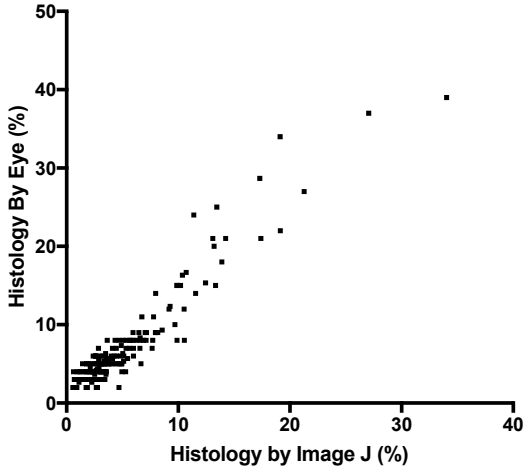


3.5.2 Interpretation of human histopathology versus digital pathology of stained slides

Stained pancreas tissue cross-section slides were scored by two consultant histopathologists for steatosis as well as islet proportions. The coefficient of variation between pathologists was low at 8.4%. Mean pathology results were compared against

digital image analysis of the scanned slide. A significant correlation was identified ($p<0.001$). $R^2 = 0.86$ (see Figure 3.5.2.1).

Figure 3.5.2.1: Correlation between visual assessments of steatosis by a histopathologist (mean result) and digital image analysis software using imageJ.



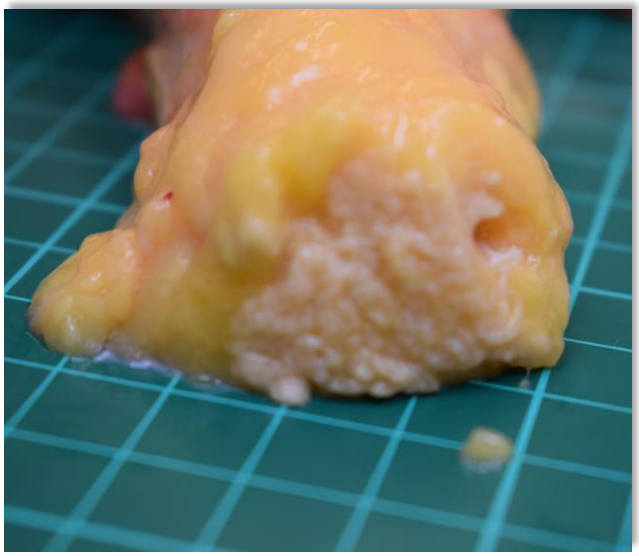
3.5.3 Islets and pancreas steatosis

Islet volumes were measured on insulin-stained cross sections using imageJ. Circumferential outlines of islets and adipocytes that had undergone appropriate staining were measured providing a proportion to the overall cross section for all 66 pancreases. Islet number and volume did not correlate with donor BMI or the steatosis volume present in sections ($p = 0.67$ and $p = 0.39$ respectively).

3.5.4 Macroscopic versus microscopic assessment

In all 66 discarded pancreases, neither macroscopic human assessment nor macroscopic digital photographs correlated with histology, which implies that the external appearance of the pancreas does not correlate with the histological assessment of steatosis ($p = 0.83$, $R^2 = 0.08$) (see Figure 3.5.4.1).

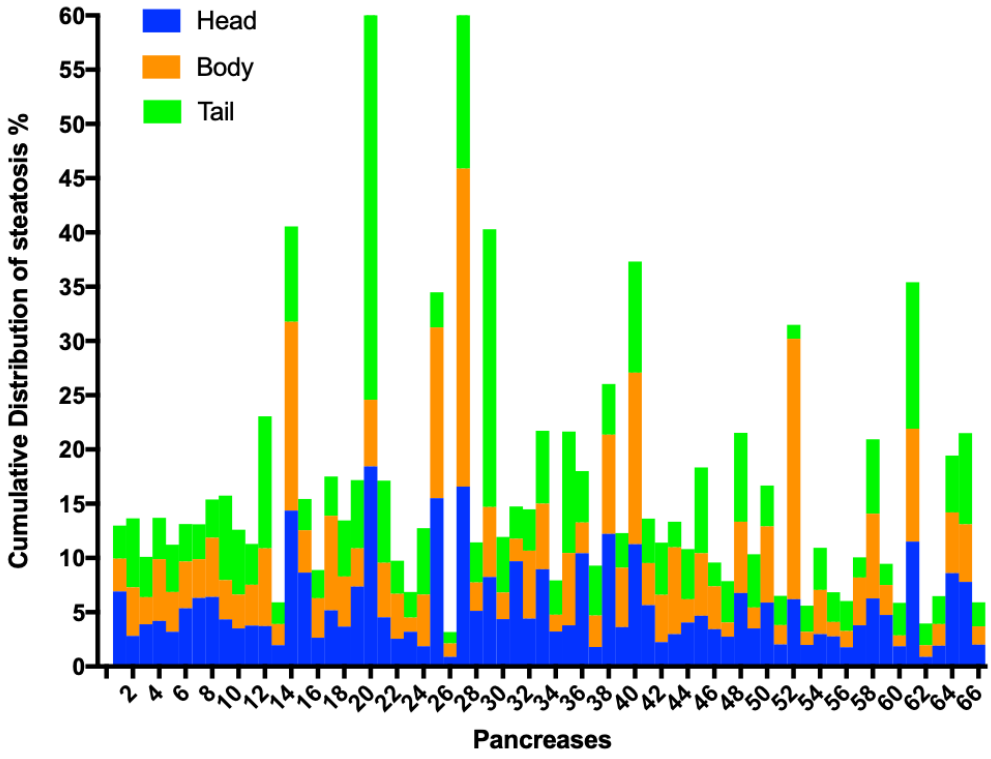
Figure 3.5.4.1: Cross section of the pancreas where the biopsy was taken. Adipose tissue encases the pancreas, with normal pancreas parenchyma seen centrally, explaining the discordance between macroscopic and histological assessment.



3.5.5 Histopathology assessments

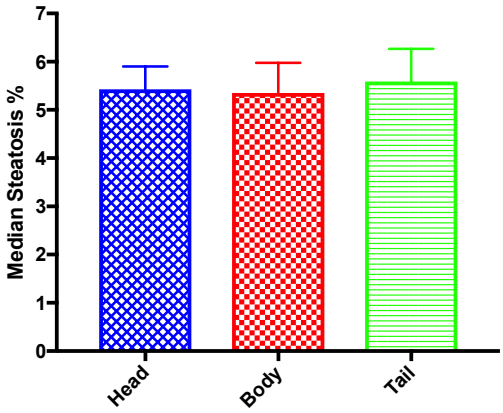
A large variation in steatosis distribution was seen, with fat deposition seen within interlobular septa as well as free adipocytes interspersed between pancreas parenchyma. No free lipid droplets within exocrine tissue were observed. This ectopic deposition was present in all 66 sections. Within a pancreas, the distribution of fat was extremely heterogeneous with areas having focal fat infiltration in either head, body or tail, while other areas remained relatively spared. There was no significant correlation between segments of the same organ: head vs tail $p = 0.79$ $R^2 = 0.02$, head vs body $p = 0.19$ $R^2 = 0.05$, tail vs body $p = 0.28$ $R^2 = 0.01$ (see Figure 3.5.5.1).

Figure 3.5.5.1: Stacked column histogram of histological assessment of steatosis in human pancreases demonstrating the intra-pancreatic variation between biopsies of the same pancreas and the variation in steatosis between different pancreases.



When comparing the overall median level and IQR of steatosis of all organs, the tail of pancreas was found to have the highest level of steatosis at 5.6% (2.9-6.3), followed by the head of the organ 5.4% (2.8-6.8) and body 5.3% (2.4 – 6.2) having the least. There was statistically no significant difference between them in this series (p = 0.31) (see Figure 3.5.5.2).

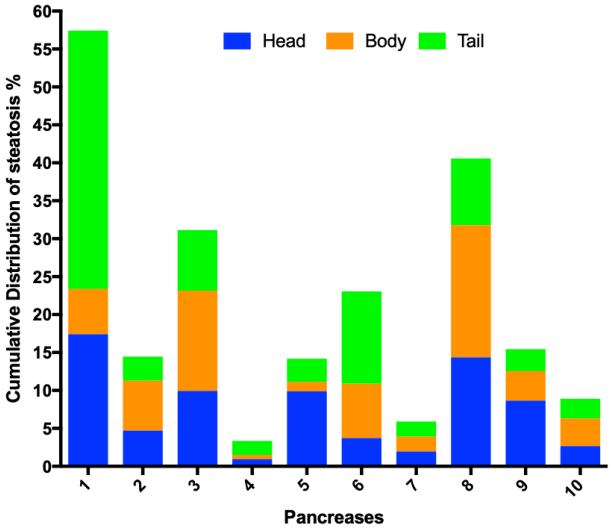
Figure 3.5.5.2: Comparison of median steatosis levels measured across the three biopsy sites (head, body and tail).



All permutations of biopsy results were used when assessing the relationship between histological steatosis and BMI, but no significant correlation between measures was found (head vs BMI p = 0.11, body vs BMI p = 0.29, tail vs BMI p = 0.54, head+body vs BMI p = 0.58, head+tail vs BMI p = 0.27, body+tail vs BMI p = 0.12 and head+body+tail vs BMI p = 0.43). What was noted was a high coefficient of variation between biopsies of 40%, showing heterogeneous distribution of biopsies taken from the same pancreas at different locations providing different results.

From the 66 organs, 10 pancreases were selected all with matched donor characteristics (female DBD donor, aged 40, BMI 22, with intracranial haemorrhage as the cause of death) to assess the variability in steatosis. The distribution and amount of steatosis within each organ and between organs was highly variable and found to be significantly different (p = 0.02) (see Figure 3.5.5.3).

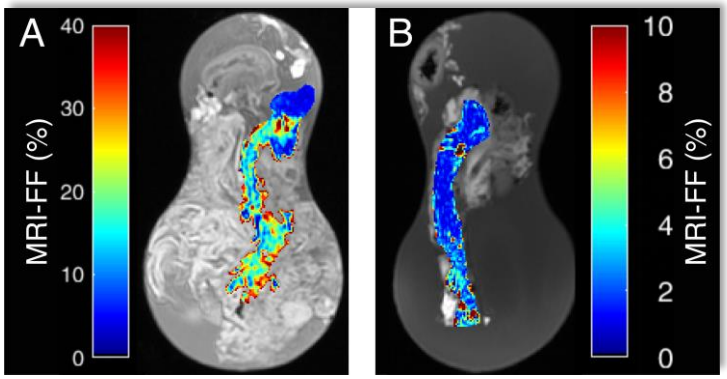
Figure 3.5.5.3: Stacked column histogram of 10 pancreas donors with matched demographics, showing variation in steatosis despite controlled donor factors.



3.5.6 MRI versus Histology

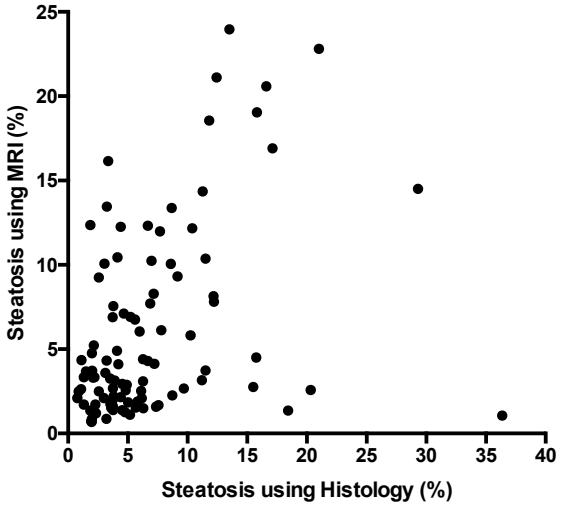
30 pancreases were used and the MRI-FF was found to have a positive correlation with histological steatosis in matched slices, with large differences clearly visible between fat and lean pancreases ($r = 0.669$, CI 0.458-0.770, $P<0.001$). Figure 3.5.6.1 shows the fat fraction (FF) maps of steatotic and non-steatotic pancreases.

Figure 3.5.6.1: MRI fat fraction maps of two contoured pancreases overlaid on water-only images. (A) A steatotic pancreas with median MRI-FF = 21.11% and large variation in MRI-FF along the entire length of the pancreas (0-40%), FF scale 0-40% (B) A non-steatotic pancreas with median MRI-FF = 3.34% still showing focal fat infiltration ranging from 0-10%, FF scale 0-10%.



A positive correlation was also seen between the average histologic steatosis grade for each pancreas taken from 3 biopsies and the average MRI-FF calculated across all pancreas voxels r = 0.739, CI 0.645-1.45, p<0.001 (see Figure 3.5.6.2).

Figure 3.5.6.2: Scatter plot showing a correlation between histological biopsy and MRI assessments of steatosis.

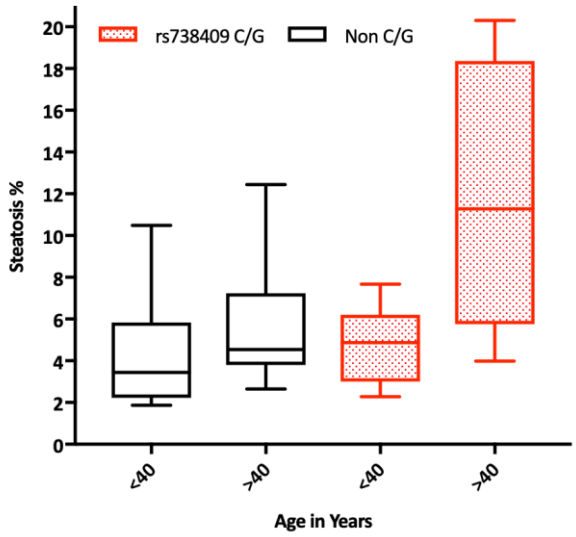


3.5.7 Spleen DNA

50 discarded and 50 implanted pancreases had concurrent donor spleen samples analysed, demographics of the tw o groups were matched. Discarded pancreases had median donor age of 42 (IQR 7 -59 years), while implanted pancreases had a median of 40 years(IQR 9 - 51 years). Mean and median levels of steatosis were calculated based on tissue biopsy results of all the discarded pancreases. 19 of 50 discarded organs had the rs738409 C/G polymorphism (38%), 11 had CC genotype (22%) and 20 patients had GG (40%). 17 of the 50 implanted organs had the C/G mutation (34%), with 30% having CC genotype and 36% having GG. There was no difference between the groups in regard to the incidence of rs738409 ($p = 0.71$).

In the non C/G group there was no difference between donors <40 years (median steatosis 3.44% IQR 2.2-5.8) compared with donors >40 years (median steatosis 4.5% IQR 3.8-5.7), $p = 0.1$. There were also no differences between C/G and non C/G groups in donors less than <40 years $p = 0.4$. Within the C/G polymorphism group (n=19) donors >40 years (n=11) had significantly more steatosis (median steatosis 11.3% IQR 5.7-18.4) than donors <40 years (median steatosis 4.8 IQR 3-6.2) $p = 0.02$ (see Figure 3.5.7.1).

Figure 3.5.7.1: Box and whisker plot of median histological steatosis with IQR, in donors <40 and >40 years old with and without the rs738409 polymorphism causing C/G genotype.



Using the assumption that C/G mutation carriers >40 years had increased steatosis present in donated pancreases, the outcome of 50 implanted organs was assessed by looking at one year graft survival data of patients with and without rs738409 (all over 40 years).

Of the 17 patients having the C/G mutation, 8 were over 40 years while 16 organs were implanted from donors with either CC or GG genotypes, giving a total sample size of 24 pancreases. No difference in graft survival was identified between groups ($p = 0.32$), suggesting that the presence of pancreatic steatosis is not associated with adverse outcome.

3.5.8 Adiponectin

Donor serum adiponectin was measured in 50 implanted and 50 discarded pancreases. There was a trend of higher adiponectin levels in donors of implanted organs but this did not reach statistical significance (maybe due to the sample size). The median adiponectin

level for discarded pancreases = 4.4μg/ml (IQR 3.9-6.6) and 6.1μg/ml (IQR 3.89-7.1) for implanted pancreases (p = 0.07) (see Figure 3.5.8.1).

Figure 3.5.8.1: Box and whisker plot showing median and IQR of adiponectin levels of implanted and discarded pancreases.

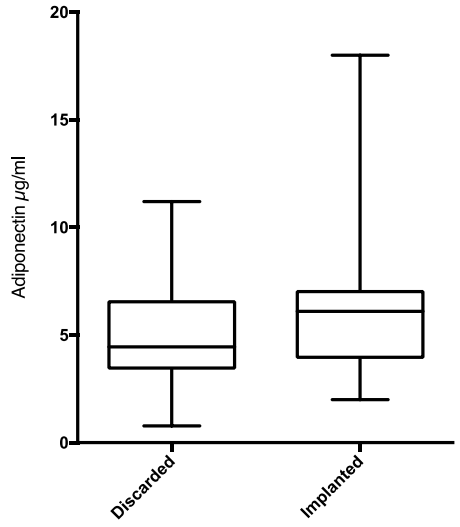
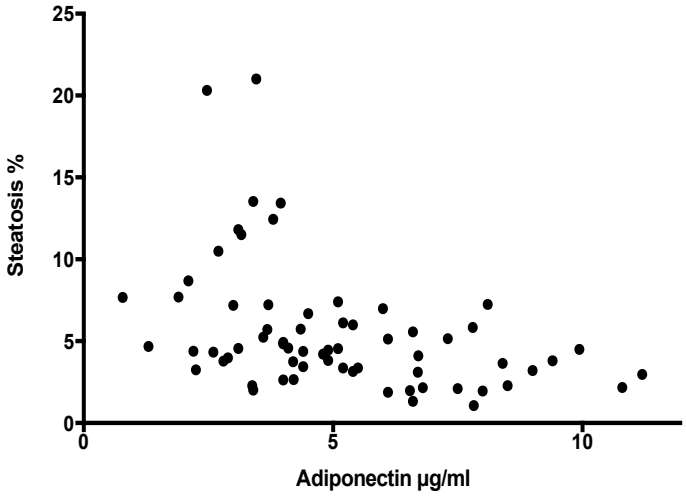


Figure 3.5.8.2 shows the inverse correlation of plasma adiponectin levels when compared with histological steatosis, based on the sample of 50 discarded organs, Spearman rank idenified an r value = -0.472 (IQR -0.645 to -0.253) and p <0.01.

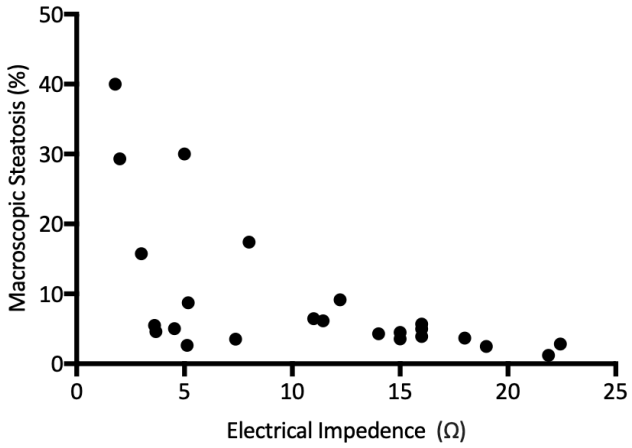
Figure 3.5.8.2: Correlation of median histological steatosis and donor serum adiponectin level (p <0.01).



3.5.9 Electrical impedance

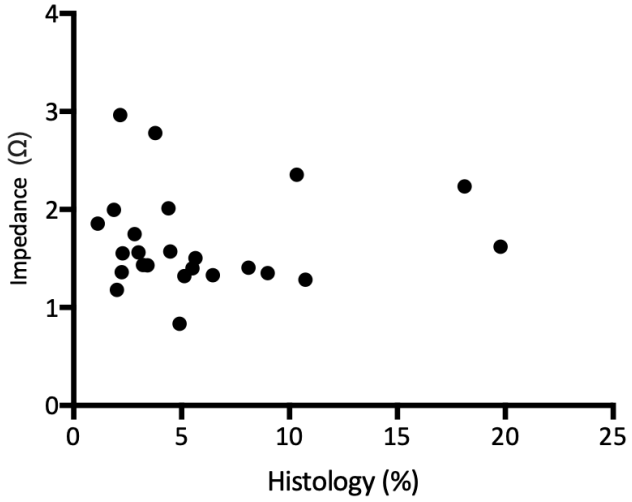
Correlation between surface fattiness (the macroscopic appearance) and impedance, provided a positive and significant inverse correlation r = -0.58 (-0.796 to -0.237) p = 0.02 (see Figure 3.5.9.1).

Figure 3.5.9.1: Correlation between macroscopic steatosis and electrical impedance.



However, no significant correlation was found between histological steatosis and electrical impedance r = 0.0338 (-0.37to 0.43) p = 0.875 (see Figure 3.5.9.2).

Figure 3.5.9.2: Correlation between histological steatosis and electrical impedance.



3.5.10 Discussion

In the context of transplantation, the term 'pancreatic steatosis' has clearly remained ill-defined, with surgeons using it to describe the macroscopic fatty appearance of a retrieved organ (inferring that internally, the same level of steatosis may be present), while pathologists define pancreas steatosis as the ectopic deposition of fat within the pancreatic parenchyma.

What is evident from the results is that macroscopic assessment performed either by eye or digital image analysis of photographs is inadequate in ascertaining pancreas parenchymal steatosis, and the macroscopic fatty appearance has no correlation with histological steatosis. The EIT results also showed good inverse correlation with the macroscopic exterior but like visual assessment, also failed to delineate true parenchymal steatosis. The reason for this inverse correlation may be that as external capsular fat increases, the electrical fields' ability to penetrate the pancreas parenchyma is impaired, explaining the poor correlation with parenchymal steatosis. Further analysis using EIT may be considered through variations in frequencies and voltages to see whether improved penetration through the layering technique can be achieved. Additionally, the technique may have value as an alternative method to histology when assessing cross-sectional biopsies, as tissue thickness is significantly less and circumferential external adipose tissue would not be an issue.

Current clinical practice self-selects pancreases that have minimal external fat, with the majority of external adipose tissue removed through the workbench procedure. The remaining adipose tissue is often regarded as avascular and associated with the capsule often embedded into the surface of the pancreas. The assumption that external

appearances correlate with unseen internal steatosis is incorrect, as is calling the external appearance steatotic.

Pancreatic steatosis has been historically regarded as abnormal, but these results demonstrate the appearance of ectopic steatosis in the parenchyma is a common factor and begins as early as seven years old. All donors in this series were shown to an element of steatosis, with the majority of donors (aged 6-53 years) being found to have deposits up to 5%, while some adult donors had focal deposits as high as 20% when examined via biopsy and up to 40% via MRI.

Donors were non diabetic anddeemed metabolically normal, passing screeing criteria to be able to donate their pancreas. This suggests that convention of defining the presence of parenchymal pancreatic steatosis as pathological needs to be re-evaluated. Although pancreas steatosis may be exaggerated by other pathologies such as steroids, viral infections or inflammatory processes, its presence appears to be part of a spectrum. These results naturally raise the question that if pancreatic steatosis is regarded as a normal occurrence, at what threshold does the transition occur from innocuous to pathological?

Further work is needed to understand an acceptable threshold of pancreas steatosis in transplantation, and to define what level should be classed as NAFPD. The current NAFLD classification in liver steatosis considers an accumulation of fat in at least in 5% of hepatocytes, which may be translational to the pancreas. With the median steatosis level in this series of 66 pancreases being roughly 5%, it may be hypothesised that this may be the applicable threshold for NAFPD.

The next key finding demonstrated in these results is the heterogeneity of the steatosis distribution. Within a single cross sectional biopsy, the distribution of steatosis is uneven, and the discordance between biopsies is very high. This suggests that tissue biopsy in the assessment of steatosis is not the most accurate test, and may explain why the confidence interval of the MRI scan was so broad (CI 0.645-1.45) as the correlation of MRI was plotted against histology.

Analysed MRI images were linked to biopsy sites, but an examination of the whole organ MRI has clearly demonstrated the focal nature of the steatosis deposits as shown in Figure 3.5.6.1. These results now provide some insight into why conflicting results have been published in regard to pancreas steatosis, particularly when tissue biopsy was used in quantification. The results provide evidence that single site biopsies and studies using small amounts of tissue may provide misleading assessments, as these do not accurately represent the entire organ.

The use of BMI as a surrogate for steatosis has effectively no value within the range considered for donation. Chapter two identified a significant number of pancreases were being discarded despite BMI meeting the donation criteria. Yet these results show that the use of BMI to screen for steatosis, when comparing matched donors with equal BMI, can lead to very different levels of steatosis as seen in Figure 3.5.5.3. The poor utility of BMI explains why conflicting results about steatosis and pancreas transplant outcome exist, where BMI has been used as a surrogate.

When further considering the links between NAFLD and NAFPD, data from the rs738409 C/G polymorphism work identified that roughly a third of donors carried the polymorphism. However, only donors >40 years who carried the polymorphism had significantly more

steatosis (median steatosis 11.3% IQR 5.7-18.4) than donors <40 years (median steatosis 4.8 IQR 3-6.2) p = 0.02. No difference was seen in younger donors with and without the rs738409 polymorphism. This data supports the concept of 'nurture over nature', where the combination of lifestyle choices (perhaps with genetic predisposition) and other factors such as age, may contribute to the overall amount of pancreatic steatosis. While the rs738409 polymorphism alone does not seem to induce significant pancreas steatosis, it may increase the overall risk.

When using the rs738409 polymorphism as a surrogate for steatosis and comparing it with one-year graft survival, no difference was observed. This result generates the hypothesis of whether rs738409 polymorphism is an appropriate surrogate marker for parenchymal steatosis and whether steatosis really does have an adverse impact on outcome. The suggestion from this small experiment is that steatosis may not be associated with adverse outcome, but again highlights the need for an accurate method to quantify steatosis and allow prospective data to be collected.

Although the rs738409 polymorphism is unlikely to be clinically relevant in the screening of donors in the immediate future, another question that comes from this work it whether allografts that contain this polymorphism are more likely to develop steatosis following implantation than those which are not. It may be that the development of allograft pancreas steatosis following transplantation is a risk factor for graft loss, rejection or delayed post-transplant morbidity.

It is important and interesting to speculate whether pancreas steatosis has any impact at all in recipient outcome following transplantation. It seems apparent that all donors will have some deposits of steatosis, albeit heterogeneously distributed and in relatively low

amounts, which although may promote biochemical inflammation does not result in all transplant recipients developing poor outcomes. Further evidence to help understand at what point steatosis becomes pathological and leads to adverse outcomes is lacking.

MRI fat fractions correlated strongly with both macroscopic external fattiness and internal histological steatosis ($p<0.001$), with MRI-FF providing a more reliable estimate of steatosis by taking the entire pancreas into account. One suggestion to allow prospective investigation based on this data would be to use MRI to quantify heterogeneous fat distribution across the entire pancreas. The technique has been shown to be feasible, reproducible and enables quantification of in a non-invasive method.

Other new methods of assessment of steatosis included adiponectin. Median donor serum adiponectin levels were lower for discarded pancreases than implanted organs (see Figure 3.5.8.1). Although the differences were not statistically significant, the results are in line with previous findings, suggesting that adiponectin may correlate negatively with pancreatic steatosis. As the ELISA process is easy to replicate, and could be performed in hospital laboratories, a larger study might be feasible in order to assess whether the correlation of adiponectin and outcome might be clinically valuable as a discriminatory tool when assessing donors, or whether the results observed here are due to confounding variables.

CHAPTER 4:

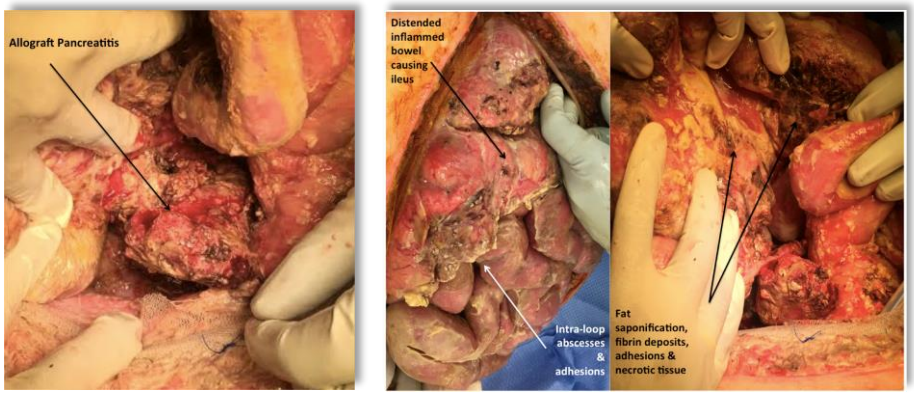
Assessment of allograft pancreatitis

This chapter outlines the relevant immunology and mechanisms of pancreatitis which are initiated following IR and then focuses on the leucocyte profile of donor allografts. The latter part of the chapter covers two common clinical markers of allograft pancreatitis that have been studied using retrospective data in an attempt to understand how allograft pancreatitis may be better identified and quantified.

4.1 Pancreatitis and allograft pancreatitis overview

Transplant surgeons commonly describe features of allograft oedema, saponification of fat and release of enzyme and inflammatory mediator-rich fluid as allograft pancreatitis, features that are also the observed in native pancreatitis [113]. The literature describes a wide range in the incidence of graft pancreatitis, from 6-60%, suggesting that either there is little consensus regarding the diagnosis or classification of the disorder, or there is indeed a large variation in incidence [114] [115] (see Figure 4.1.1).

Figure 4.1.1: Two photographs showing the manifestation of allograft pancreatitis in a patient who required further surgery due to severe inflammation.



In SPK patients Fertmann et al. identified that up to 50% of early pancreas graft failures are caused by allograft pancreatitis which is associated with acute graft loss and increased mortality (up to 9%) [116]. Troppmann et al. identified allograft pancreatitis as the second most common post-transplant complication, thrombosis being the commonest. However,

thrombosis may occur as a consequence of pancreatitis (or sometimes vice versa), making it difficult to interpret the literature consistently [117].

The lack of definition of the underlying process means not only that the true incidence of transplant pancreatitis is difficult to determine, but also that there is no consistent endpoint to assess the effect of interventions.

Nadalin et al. attempted to categorise acute and chronic graft pancreatitis (A-GP and C-GP), distinguishing A-GP further into: physiological (P-AGP), early (E-AGP) and late AP (L-AGP). However, non-specific symptoms including abdominal pain, fever and SIRS, following repeat laparotomy continue to make the diagnosis difficult [118].

4.2. Ischaemia reperfusion (IR) injury

Static cold storage (SCS) is the standard method of transplant pancreas preservation. The combination of hypothermia and specialist preservation solutions such as UW solution, reduces cellular energy consumption (two-fold for every 10°C drop in temperature) and reduces cell swelling [119].

It has been shown that selective accumulation of succinate (kreb cycle intermediate) is a universal metabolic signature of tissue ischaemia, and responsible for mitochondrial ROS production during reperfusion. Upon reperfusion, the accumulated succinate is rapidly re-oxidised by succinate dehydrogenase, driving extensive ROS generation by reverse electron transport (RET) at mitochondrial complex I. Decreasing ischaemic succinate accumulation

by pharmacological inhibition has been shown to be sufficient to ameliorate in vivo IR injury in murine models [120] [121].

4.2.1 NF-κβ

The NF-κB transcription factor family in mammals consists of five proteins, p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2) that associate with each other to form distinct transcriptionally active homo- and heterodimeric complexes [122]. The predominant form is a p50/p65 heterodimer, which is attached to Iκβs proteins that prevent NF-κβ transcription. An important feature of the NF-κβ signalling pathway is the diversity of extracellular agents and mediators that induce NF-κβ activation via Iκβ degradation. Genes for pro-inflammatory cytokines including IL-1β, IL-6, TNFα, and chemokines, C-C motif ligand CCL-2 and CCL-3 are activated following NF-κβ transcription. NF-κβ activation has been observed in experimental models of pancreatitis paralleling trypsinogen activation in time course, suggesting it can independently trigger pancreatic inflammation [123].

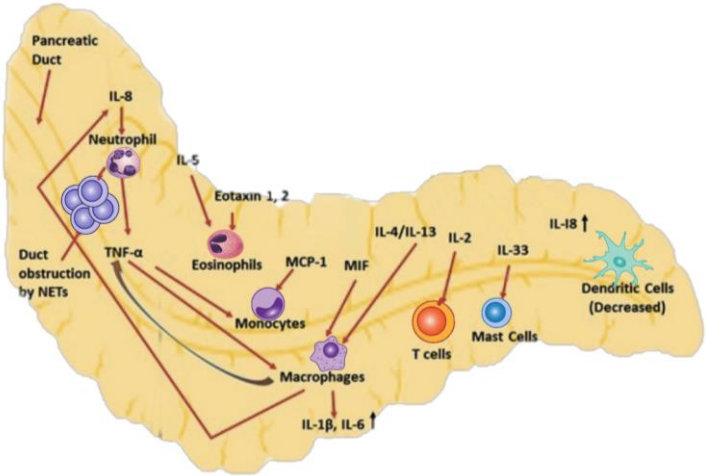
4.2.2 Neutrophil Extracellular Traps (NETs)

Neutrophil extracellular traps (NETs) mediate microbial killing and are secreted from neutrophils when activated. They are made up of chromatin, elastase and cathepsin G, and also produced in response to acute inflammation, such as pancreatitis. NETs are mediated by ROS/reactive nitrogen species (RNS) production involving NADPH-oxidase and myeloperoxidase (MPO). NADPH-oxidase generates superoxide radicals forming hydrogen peroxide, which is utilised by MPO to form hypochlorite and leads to lipid peroxidation and membrane damage. Nitric oxide (NO) can also react with superoxide radicals and generate peroxynitrite radicals that cause cellular damage. Inflammatory mediators and cytokines including IL-1β, IL-6, TNFα and macrophage migration inhibitory factor (MIF) which all alter

microvascular homeostasis and blood flow. This is associated with thrombus formation and multi-organ dysfunction. Preventing neutrophil adherence blunts ischaemia reperfusion and remote organ injury, suggesting neutrophil activation is an important cause of multiple organ dysfunction [124][125].

Summarising, these key processes provide a snapshot into the complexities of allograft pancreatitis. It is evident that with a plethora of mediators, cytokines, chemokines and cell types, inflammation is very quickly amplified from triggers at multiple entry points. The intricate sequence of events leading to tissue damage is well documented elsewhere and is summarised in Figure 4.2.3.

Figure 4.2.3: Diagrammatic overview of some of the known inflammatory mediators involved in the process of pancreatitis.



4.3 Pancreas leucocyte flush study

Having shown the importance of certain types of leukocytes and their roles initiating injury in pancreatitis, understanding the behaviour of the resident leucocyte population within the pancreas allograft will further identify potential targets that may be used to prevent allograft pancreatitis and systemic inflammation seen following implantation. Pancreases

are routinely flushed with preservation solution during and following procurement and during the workbench procedure. The purposes for which are, to ensure all blood has been eliminated from the pancreas (preventing vascular thrombosis), to cool the organ, to ensure there are no major leaks prior to implantation, and to assess vascular flow. The volumes used to achieve this, in general, are not large [127]. This is because the pancreas is a relatively small organ (as shown in chapter three, only weighing on average 200 grams) and has multiple arterial supplies, appearing adequately flushed following procurement. Another rationale for using small flush volumes is augmentation of the arterial supply during the workbench preparation (ligation of the splenic artery at the tail and gastroduodenal artery at the head). The avoidance of unnecessary handling with concerns about possible deleterious effects of high pressure perfusion are additional reasons not to flush large volumes of preservation solution [127].

The objective of this work was to assess the nature of passenger leucocytes that come out of the donor organ during the cold flush process (based on the assumption that these would otherwise be flushed out of the organ into systemic circulation following reperfusion). Although this is not a fully representative profile of what circulating leucocyte cells are potentially transferred following transplant, it may inform a hypothesis through identifying what type of cells are being transferred into the recipient circulation and how these possibly may contribute to inflammation. Immunophenotyping was performed using flow cytometry, labelling the different leucocyte populations with appropriate antibody tags.
The work was performed in collaboration with Dr. James Fildes at Manchester Collaborative Centre for Inflammation Research (MCCIR).

4.3.1 Material

25 pancreases (17 implanted and 8 discarded) undergoing workbench preparation were flushed with 250mls of UW solution, once the Y graft arterial conduit was attached and other pre-implantation preparation completed. Effluent fluid from the portal vein was collected as the flush sample of the pancreas. This effluent contained cells from within the vasculature, including those that had migrated out of the tissue into the vessels following pancreas retrieval. Flush samples were mixed with 10% dimethylsulfoxide (DMSO) and Hanks Buffer, stored at -80°C, and then later analysed by flow cytometry.

4.3.2 Methods

Immunophenotyping and fluorescence-activated cell sorting (FACS) of the pancreas effluent was performed on a BD LSR II flow cytometer (Becton Dickinson, Oxford, UK). Leukocytes were rapidly thawed at 37°C and centrifuged at 700g for 5 minutes at 4°C. The supernatant was decanted and the cell pellet re-suspended in 100µl PBS and stained with 1µl Zombie UV viability dye (BioLegend, San Diego, USA). Following a 30-minute incubation in the dark, the samples were washed in 2mls of flow staining buffer (PBS supplemented with 2% foetal calf serum) then centrifuged at 700g for 5 minutes at 4°C. The supernatant was removed and the cell pellet re-suspended in 100µl PBS. Cells were then stained with the following antibodies (full fluorochrome antibody profiles can be seen in Appendix 1).

Tube 1 Antibody	Volume	Tube 2 Antibody	Volume
PerCPCy5.5 CD14	2.5µl	APC lin cocktail	10µl
PE-CF594 CD56	2.5µl	FITC CD123	5µl
PE-Cy7 CD19	2.5µl	PE CD16	5µl
APC-Cy7 CD8	2.5µl	BV605 CD11c	5µl
APC CD3	5µl	BV711 CD45	2.5µl
PE CD16	5µl	AF700 HLA-DR	2.5µl
FITC CD4	5µl	PerCPCy5.5 CD14	2.5µl
BV711 CD45	2µl		

Tube 1 included a pan-leukocyte marker (CD45) and markers to detect monocytes (CD14/CD16), B cells (CD19), cytotoxic T cells (CD3/CD8), helper T cells (CD3/CD4), NK cells (CD16/CD56) and NKT cells (CD3/CD16/CD56). Tube 2 was focused on identifying different subsets of dendritic cells.

After a 10-minute incubation in the dark, 2mls 1x red blood cell lysing solution (BD Biosciences, UK) was added and incubated for a further 10 minutes in the dark at room temperature. Cells were then washed via centrifugation at 700g for 10 minutes at 4°C in 2mls flow staining buffer. The supernatant was removed and the cell pellet re-suspended in 0.3ml flow staining buffer. Finally, 20µl e123count beads (eBioscience, CA, USA) were added to allow absolute cell counts to be performed using the equation below:

Absolute cell count = (cell count x eBead volume) ÷ (eBead count x cell volume) x Bead concentration

Samples were acquired at a high flow rate for 2 minutes on an LSR II flow cytometer (Becton Dickinson, Oxford, UK). All gating strategies and analysis were performed using FlowJo version 10.1.

4.3.3 Gating strategy

Beads that were added to the sample were first identified by their intense fluorescence at both 530nm and 575nm and then selected in a gate to allow quantification of the number of cells in the sample. These beads were subsequently removed from further analysis through the use of a 'make not gate', which left only recorded events of the cells not beads.

Single cells were then gated (avoiding doublets) on the basis of their forward scatter recordings of height and area. Any deviation from the straight diagonal positive correlation was regarded as a doublet.

Viability was then assessed on the basis of lack of UV fluorescence. From the viable cell population, CD45+ cells were gated to isolate the total leukocyte population. From here, different leukocyte subset populations were gated using the following parameters:

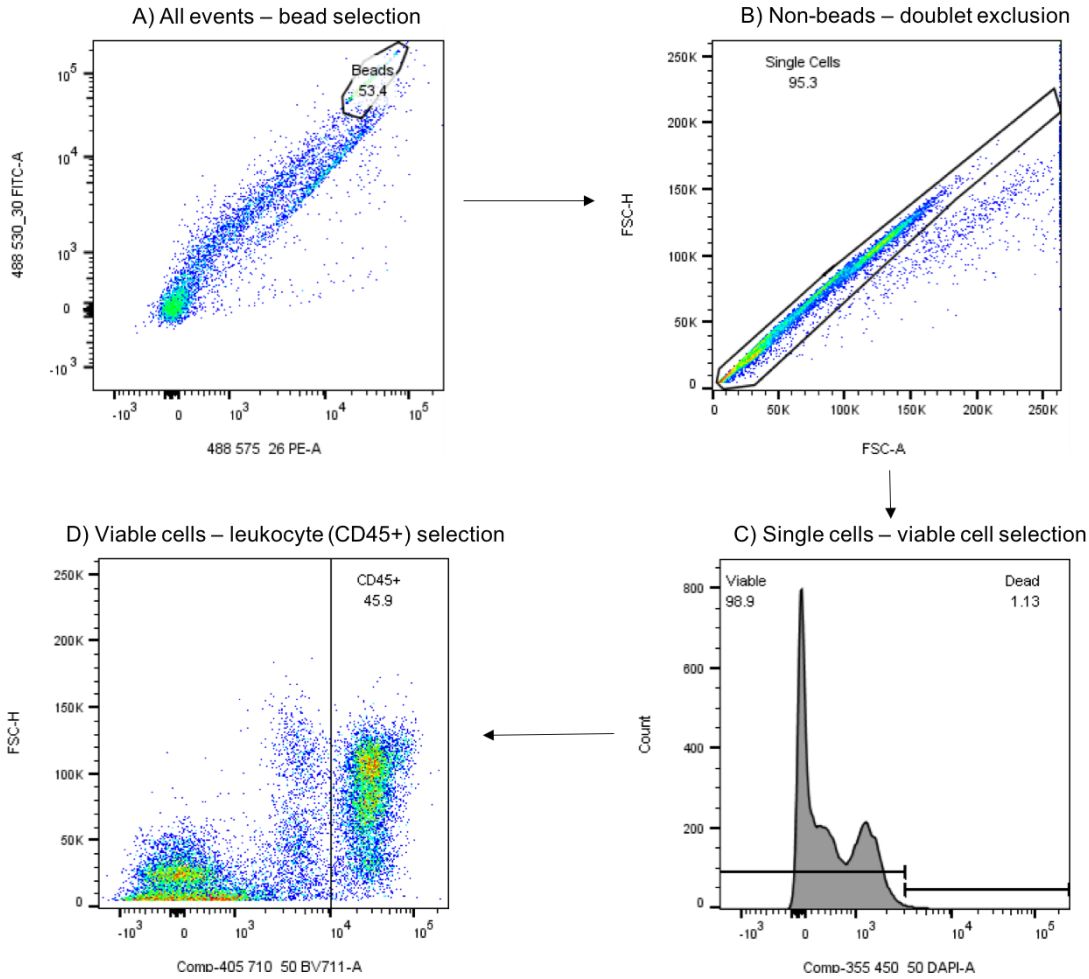
- Dual screen for CD14 and CD16 to identify monocytes (both classical and non-classical).
- Cells identified to lack CD14 but to express CD16 were gated against CD56 to identify NK cells.
- Selecting CD3 positive cells provided pan T cells with sub-analysis of:

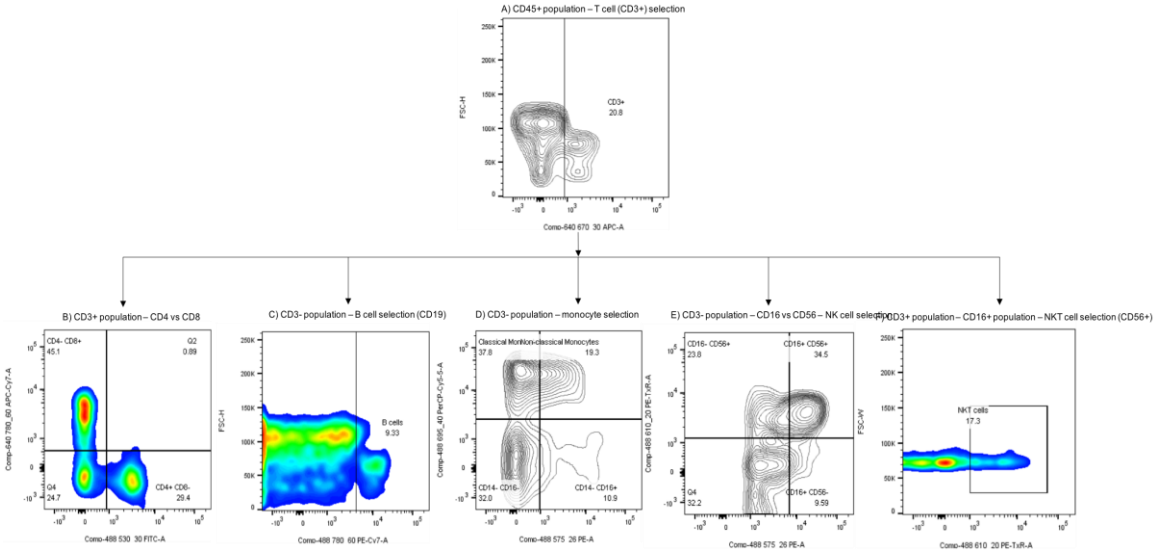
  a) CD4 against CD8 to identify helper and cytotoxic T cells respectively

  b) CD56 to get NKT cells
- Using the 'not gate' on the CD3 population, T cells were removed from the analysis, leaving the plot of B cells, confirmed with CD19 expression.

Figure 4.3.3.1 shows the output from the gating strategy.

Figure 4.3.3.1: FACS output outlining method A) Absolute count beads are selected based on their intense fluorescence in both FITC and PE channels B) Single cells are selected in a diagonal gate to remove any events with higher area relative to height, suggesting doublets C) Dead cells are removed from the analysis due to their non-specific binding of antibody that could interfere with the interpretation of results. Only cells with background level fluorescence in the DAPI channel are selected as viable D) The distinct CD45+ leukocyte population is selected as the initial gate from which other sub-populations are characterised.

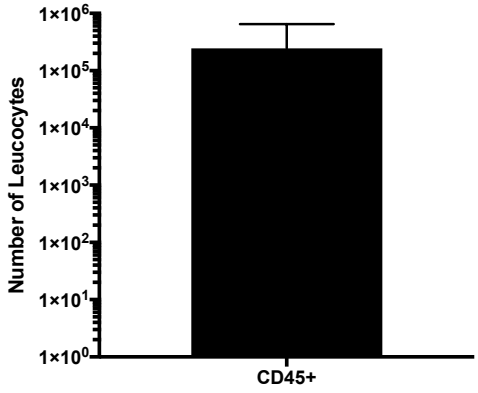




4.3.4 Results

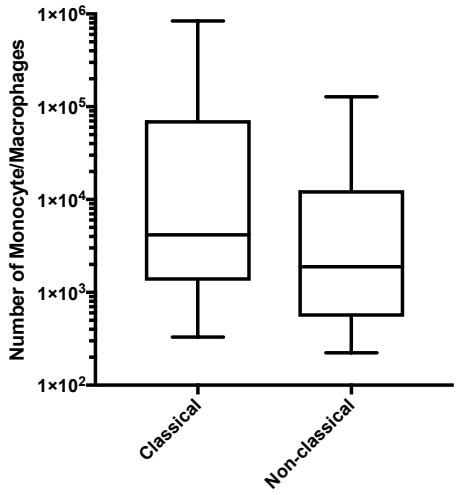
A median 10.1x10^5 cells (interquartile range 2.3x10^3 – 1.7x10^6) of viable CD45$^+$ leucocytes were identified from the effluent of 25 pancreases (see Figure 4.3.4.1). All further analysis of pancreas leucocyte populations is expressed with median and IQR values unless stated otherwise.

Figure 4.3.4.1: Median number of CD45$^+$ leucocytes isolated from 25 human pancreases.



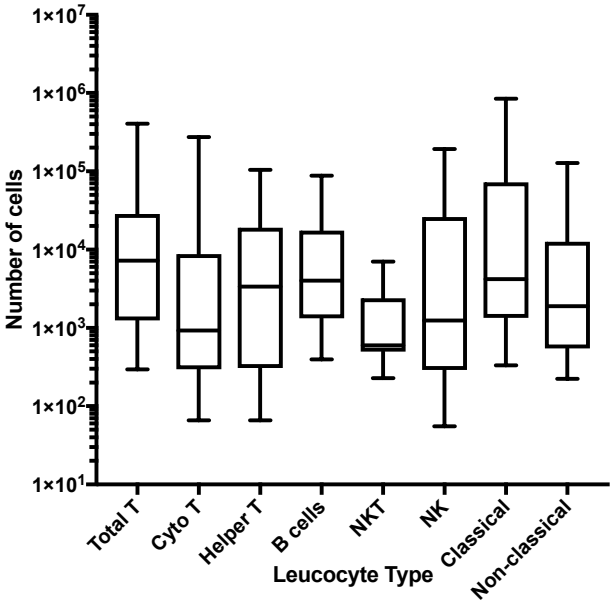
Within the total leucocyte population, there was a predominance of monocytes with a median of 6.1x10^4 cells (5.4x10^3 – 1.3x10^5). The ratio of classical monocytes to non-classical was roughly two to one (4.4x10^4 and 1.9x10^4 respectively) (see Figure 4.3.4.2).

Figure 4.3.4.2: Box and whisker plot showing the median and IQR of monocyte populations.



The second most frequent leucocytes isolated were T helper cells: $3.4x10^4$ ($3x10^2$-$1.04x10^5$) with the remaining cell populations identified as cytotoxic T cells $9.x10^3$ ($6.5x10^1$-$8.8x10^4$), B-cells $4x10^3$($1.3x10^3$-1.7 $x10^4$), NKT cells $5.9x10^2$($5x10^2$ -$2.4x10^3$) and NK cells $1.2x10^3$ ($2.9x10^2$ –$2.6x10^4$). The results of these cells populations have been summarised in Figure 4.3.4.3 below.

Figure 4.3.4.3: Box and whisker plot of the different types of leucocytes isolated from pancreatic effluent.



Overall, the cell populations isolated from the pancreases effluent using the technique described were lower than the kidney which has been previously reported in the literature. This may be due to the low levels of flush used, or due to some of the results that came from discarded pancreases (non-haemostatically benched) and so, the effluent volumes were reduced due to leakage.

4.3.5 Flushed leucocytes in pancreatitis

The two largest cell populations isolated from the flush samples (monocytes and T-helper cells) have both been implicated in the initiation of pancreatitis. Donor pancreases seem to possess a significant repertoire of leucocytes which, if transferred into the recipient during transplantation, would be capable of initiating acute injury through direct allo-recognition and promotion of inflammation.

I have demonstrated large numbers of monocytes reside within the pancreas, which following revascularisation, may potentially mobilise to recipient lymph nodes and self-present donor antigens to naive T cells. This may result in alloantigen-specific clonal expansion and be a potential cause of graft infiltration and injury.

The main limitation of this study is the fact that cells were only collected during flushing of the organ rather than at the time of reperfusion. Further work collecting samples following reperfusion would provide more evidence in an attempt to develop this hypothesis further.

4.4 Common pancreatitis biomarkers

Some of the cellular mechanisms (potential leucocytes involved and cytokines associated with allograft pancreatitis) have been discussed, yet the challenges in the diagnosis and

quantification of severity raise a need for a more robust and reproducible definition. Additionally, an accurate, easy to measure surrogate marker for allograft pancreatitis would provide a clear benefit in allowing early identification of patients who may be candidates for intervention therapies, as well as in quantifying the response to those interventions.

Native pancreatitis is associated with raised amylase and/or lipase but these are not criteria that define the condition. Serum lipase at a value of three times the upper limit of normal (ULN) has a range sensitivity and specificity of (64-100%) and (99-100%), respectively. However, the range for amylase sensitivity is lower at (50-78.6%) and specificity (99-100%) [128]. In the transplant setting, differentiating between IR from those patients developing allograft pancreatitis, is more challenging. The objective of this next study was to assess whether lipase was useful in the early diagnosis of allograft pancreatitis.

4.5 Lipase physiology: release and clearance

Lipase is a 48 kilodalton (kDa) digestive enzyme mainly secreted by the exocrine pancreas (other sites include oesophagus, duodenum, stomach, colon as well as liver, heart, lungs and leukocytes). During acute pancreatitis, serum lipase increases within 4-8hr, peaking at 24 hours and remains elevated for one to two weeks. It has a half-life between 7 and 14 hours (longer than amylase) and is finally excreted by the kidneys [129]. Having impaired renal function has been shown to lead to an increased level of lipase [130].

Lipase elevations above the ULN are seen in a wide range of conditions, other than pancreatitis, including peptic ulcer disease and other hepatobiliary disorders. Hence, knowledge of alternative raised lipase causes is essential for clinicians because such levels may be erroneously interpreted as indicative of pancreatitis. This is especially important as

lipase is advocated as the preferred test for the diagnosis of pancreatitis by various guidelines and much weight is given to levels of >3xULN [131].

4.5.1 Lipase in pancreas transplantation

In 1993, Busing et al. identified 14 patients who underwent pancreas transplantation and patients had increased serum amylase and lipase levels following implantation. The mean peak levels occurred during the first 36 hours following reperfusion. The amylase level was increased 3-15-fold and that for lipase 4-30-fold, but within the first week about two-thirds of patients had normal serum pancreatic enzyme levels [132]. This initial rise following transplant was attributed to ischaemia reperfusion, which was confirmed by Redfield et al. In 2015 authors reported in the first 45 days following transplantation, reasons for elevated lipase levels other than ischaemia reperfusion injury, included enzyme leak, infected fluid/abscess, graft thrombosis, ileus or acute rejection. This broad differential confirms that lipase is a marker of pancreatic injury, but its usefulness to identify patients with early allograft pancreatitis may be limited, with post-transplant hyperlipasemia being common [133].

4.5.2 Utility of Lipase in Oxford data

To assess the clinical usefulness of lipase in the diagnosis of allograft pancreatitis, a retrospective analysis of 69 SPK recipients was performed. Patients had weekly lipase levels measured over the first 28 days (four weeks) of transplant.

4.5.3 Lipase assay

Samples were analysed in the main biochemistry lab in the John Radcliffe Hospital, Oxford. All samples were taken on a clinical basis. The assay used by the lab was an automated

enzymatic colorimetric assay with 1, 2 O dilauryl-rac-glycero-3-glutaric-acid (6 methyl-resorufin) ester as substrate (Roche Cobas platform). The biological reference range or cut off was 13-50 U/L with a turnaround time of four hours [134].

4.5.4 Methods

Retrospective analysis of 69 recipients of SPK was performed on patients transplanted in Oxford between 2009 and 2017. All organs were retrieved by NORs, and pancreases transported using SCS and UW solution for preservation. Donor characteristics from the NHSBT EOS were recorded and combined with recipient characteristics (see Table 4.5.5.1). Weekly lipase results over the first 28 post-operative days were examined.

The incomplete data set was the main limitation of this analysis, so only sets that had a minimum of one value a week for the first four weeks were included as well as those with CT imaging postoperatively (within 14 days of transplant). Where more than one value in the week was available, the mean value was taken. Clinical symptom profiles were recorded and included: pain, nausea, core body temperature >38°C, heart rate >90 bmp, respiratory rate >20/min, hyperglycaemia >7 and elevated white cell count >12.

Pancreatitis was quantified using the CTSI score. Scoring was done specifically for this study rather than as part of clinical care. CTSI was originally developed to assess the severity of acute native pancreatitis (last modified in 2004) [135]. CTSI sums up two scores: Balthazar score and pancreas necrosis [136]. The maximum score obtainable being 10 and anything other than 0 defines allograft pancreatitis (see Table 4.5.4.1). Scoring was performed by two radiologists who were blinded to the clinical outcomes; where scores differed, scans were

rescored to reach an agreed value. The coefficient of variance between radiologists was 7%, showing good concordance between assessors.

Table 4.5.4.1: CTSI score, being the maximum score 10. 0-3: mild acute pancreatitis, 4-6: moderate acute pancreatitis, 7-10: severe acute pancreatitis.

Balthazar score	**Points**
A: normal pancreas	0
B: Enlargement of pancreas	1
C: Inflammatory changes in pancreas and peri-pancreatic fat	2
D: Ill-defined single peri-pancreatic fluid	3
E: Two or more poorly defined peri-pancreatic fluid collections	4
Pancreatitis Necrosis	
None	0
≤30%	2
>30-50%	4
>50%	6

4.5.5 Lipase results

Table 4.5.5.1 shows the demographic donor and recipient details of patients with and without allograft pancreatitis. Of the 69 patients, only 67 patients had complete data sets (2 absent CT scans) meaning two patients were excluded from analysis.

Table 4.5.5.1: Demographic details of 67 recipients included for analysis with related donor information.

Donor	**Pancreatitis**	**Non-Pancreatitis**
Total Number of Organs	26	41
Age (Median and IQ Range)	42.5 (29-52)	35 (22-45)
Male:Female	11:15 (42%:58%)	19:22 (46%:54%)
BMI (Median and IQ Range)	22.5 (21.1-25.91)	22.9 (20.3-24.9)
Girth in cm (Median & IQ Range)	81 (73.2-85.4)	85 (75.1-90.5)
DBD:DCD	23:3	38:3
Ethnicity (Caucasian:Other)	26	37:4
Median CTSI score	3	0
CIT (Median and IQ Range)	10.2 (9.1-12.1)	10 (8.5-11.3)

Recipient		
Total Number of Patients	26	41
Age (Median and IQ Range)	43 (35-53)	45 (33-47)
Male:Female	13:13 (50%:50%)	20:21 (49%:51%)
BMI (Median and IQ Range)	24.6 (23-28)	25.2 (23-28)
Haemo/peritoneal dialysis	15:11 (58%:42%)	24: 17 (58%:42%)

Of the 67 patients analysed, 61 patients (91%) had elevated lipase levels during the first 28 days following pancreas transplantation, with 27 patients (40.2%) being diagnosed with pancreatitis based on CT imaging (CTSI>0, median CTSI score 3). Results demonstrate that serum lipase results only became significantly associated with pancreatitis in post-operative week three and four. The results are presented in Figure 4.5.5.2 and Table 4.5.5.3.

Figure 4.5.5.2: Box and whisker plot showing the difference in lipase levels between SPK patients with and without pancreatitis during the first four weeks following transplant.

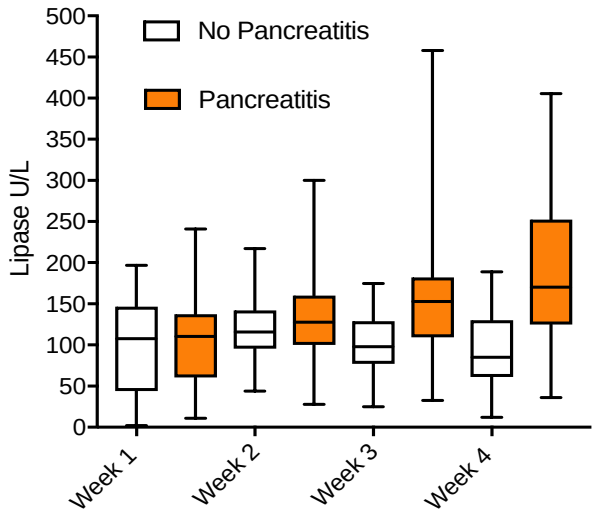
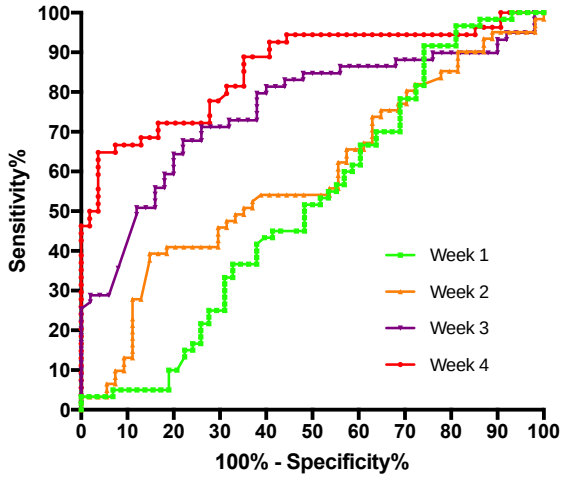


Table 4.5.5.3: Data supporting Figure 4.5.5.2, showing the median value with IQR of patients with and without pancreatitis during the first four weeks following transplant.

Post op	No Pancreatitis	Pancreatitis	P Value
Week 1	107.7 (44.1-146.8)	110.6 (60.5-137.4)	0.737
Week 2	115.7 (95.5-142.1)	127.6 (100.1-160)	0.117
Week 3	97.9 (77.2-129)	152.8 (109.1-182.2)	<0.001
Week 4	84.9 (60.9-130.1)	170.1 (124.7-252.2)	<0.001

Statistically significant differences in lipase did not signify a clinically useful differentiator as there was too much overlap in results. In order to further assess the usefulness, a ROC analysis measuring sensitivity and specificity was performed (see Figure 4.5.5.4).

Figure 4.5.5.4: ROC analysis of lipase results 3xULN (>150U/L).



Lipase 3xULN (150U/L)	Sensitivity% (95% CI)	Specificity% (95% CI)	Area (95% CI)	Likelihood Ratio
Week 1	16.7 (8.3-28.5)	75.8 (62.8-86.1)	0.5 (0.4-0.6)	0.7
Week 2	39.3 (27.1-52.7)	83.3(70.7-92.1)	0.6 (0.5-0.7)	2.4
Week 3	52.5 (39.1-65.7)	84 (70.9-92.8)	0.8 (0.7-0.8)	3.3
Week 4	66.7 (52.5-78.9)	88.9 (77.4-95.8)	0.9 (0.8-0.9)	6

4.5.6 Lipase discussion

Many advocate lipase as the preferred biochemical test for the diagnosis of pancreatitis at the cut-off level of 3 x ULN, often considering it as a criterion for diagnosis. Yet although the results of this chapter did identify a correlatation between allograft pancreatitis until three weeks post operatively. Lipase was found to have a poor sensitivity throughout the first four weeks following transplant but had higher specificity (75-88%). The findings support that lipase is a specific marker for pancreas injury, but not a sensitive one in the diagnosis of

allograft pancreatitis. This result supports the work of Busing and Redfield, where the authors have shown early allograft pancreatitis may be masked by elevations following IR [132][133].

Although results were negative, they need to be considered in the context of the small sample size and number of results recorded per patient (median = two results per week but ranged from 1-4). Another consideration is the type of transplant assessed, as SPKs are enterically drained. Repeating the study using bladder drained PTA patients would have allowed more accurate measurement of the exocrine enzymes. This analysis was not performed due to the limited number of PTA transplants performed at Oxford, but should be a consideration for future assessment of lipase to clarify the findings of this work. Small et al. reported a series of 79 SPKs transplants from 1995-2007 and found 14% of recipients (also based on CT imaging) experienced graft pancreatitis. Patients presented with pain, peritonitis, fever, and findings compatible with pancreas graft inflammation on CT or US imaging. Authors concluded that the diagnosis of graft pancreatitis was clinical, suggesting that the results of lipase study may not alter, even with these improvements, to the study design [137].

4.6 Rational for the utility of C-reactive protein as a marker of allograft pancreatitis

C-reactive protein (CRP) is an annular, pentameric protein found in plasma, where levels rise in response to inflammation [138]. In pancreatitis, CRP seems to be a useful marker with many pancreatitis management guidelines, including the British society of gastroenterology, choosing to include CRP (values >150) in their guidelines for the diagnosis and management of acute native pancreatitis [139].

CRP has a plasma half-life of 19 hours, so the major factor that affects blood levels is its rate of production. Stirling et al. retrospectively identified 337 cases of native acute pancreatitis over a 5-year period and found that the interval change in CRP is a comparable measure to the absolute CRP level in the evaluation of severity. This study suggested that a rise of >90 mg/L from admission or an absolute value of >190 mg/L at 48 hour predicts disease [140].

Chhabra et al. analysed post-operative CT scans of 109 SPK transplants to quantify the severity of allograft pancreatitis. Authors found that the CT score correlated to the length of post-operative stay and to the levels of serum inflammatory markers measured within 24 hours of each scan. They concluded that CRP and white cell count were useful quantitative markers of graft pancreatitis and may reduce the need for repeated radiological investigations to assess graft inflammation [141]. However, the exact value of CRP and how it should be used was not clear.

Wullstein et al. concluded that peak serum CRP during the first 72 hours is a helpful parameter in predicting pancreas graft-related complications and pancreas graft survival. Mean peak CRP was higher in patients needing re-laparotomy (n=31) due to graft pancreatitis ($p = 0.03$) and in patients with graft loss (n=19; $p<0.01$) compared with patients without these complications. This study was not specifically designed to assess CRP utility in allograft pancreatitis and was only a small sample of 31 patients [142].

These studies demonstrate the need for further evidence to clarify the rationale of using CRP as a surrogate marker in the context of allograft pancreatitis, which was performed using retrospective data from the Oxford transplant centre. The methods outlined below

mirror those for the lipase work discussed earlier, and duplications have been omitted from the following section.

4.6.1 CRP Methods

A retrospective analysis of 397 recipients was performed, whom underwent SPK or PTA transplantation, performed at Oxford between 2009 and 2017. 366 patients of this sample wereused in the analysis for CRP.

All organs were retrieved by the National Organ Retrieval Service (NORS), and pancreases were transported using SCS and UW solution for preservation. Donor characteristics from the NHSBT EOS were recorded and combined with recipient characteristics (see Table 4.6.4.1). Daily CRP on the day of transplant and first 7 post-operative days was examined.

Data sets that had two successive days of missing values were omitted from the analysis, while those that had a single value missing were included. Not all patients had CT imaging postoperatively (within 14 days of transplant), as scans were performed per protocol between 2009 and 2012, and only for clinical indications thereafter. Clinical symptom profiles were recorded and included: pain, nausea, core body temperature >38°C, heart rate >90 bmp, respiratory rate >20/min, hyperglycaemia (>7) and elevated white cell count (>12).

Pancreatitis was quantified using the CTSI score where CT scan was available. Scoring was done specifically for this study rather than as part of clinical care. CTSI assessment used was the same as the one described in section 4.5.4.

The presence of allograft pancreatitis was additionally determined at re-look laparotomy in 18 patients where CT scan result was unavailable. Pancreases were visually assessed by the operating surgeon, where visual appearance criteria for inclusion included the same criteria as that used by CTSI (enlarged pancreas due to oedema, inflammatory changes of the pancreas and peri-pancreatic fat, peri-pancreatic fluid or collections, pancreatic necrosis).

4.6.2 Statistical analysis

Statistical analysis was performed using graph-pad prism version 7 and IBM SPSS version 25. Appropriate non-parametric tests were applied: Kruskall-Wallis, Mann-Whitney U test or Wilcoxon Rank were used with a $p<0.05$ deemed significant. ROC curves were plotted for sensitivity and specificity, and Kaplan-Meier estimates were used for survival data. A multivariate regression analysis was used for donor and recipient risk factors. Data was presented with median values and interquartile ranges (IQR) where appropriate.

4.6.3 Limitations of data

Reasons to exclude patients from the analysis included missing CRP values and lack of CT scan evidence, in the absence of diagnosis via laparotomy. Hospital lab assays used to measure CRP prior to 2014 reported results at a maximum of 156 mg/L, with higher levels reported as “>156mg/L”. Since changing to Sigma Aldrich high sensitivity CRP assay at the start of 2014, levels were reported without this capping. A sub-analysis of the capped and uncapped values was performed to assess whether this change introduced bias.

With allograft pancreatitis being a spectrum, binary categorisation was known to miss a proportion of patients with mild pancreatitis who were not investigated with CT (e.g.: mild abdominal pain, slightly elevated inflammatory markers but self-limiting and settle with

conservative management). This would underestimate the incidence of pancreatitis rather than overestimate it, so any positive association demonstrated in the results would remain valid.

4.6.4 CRP Results

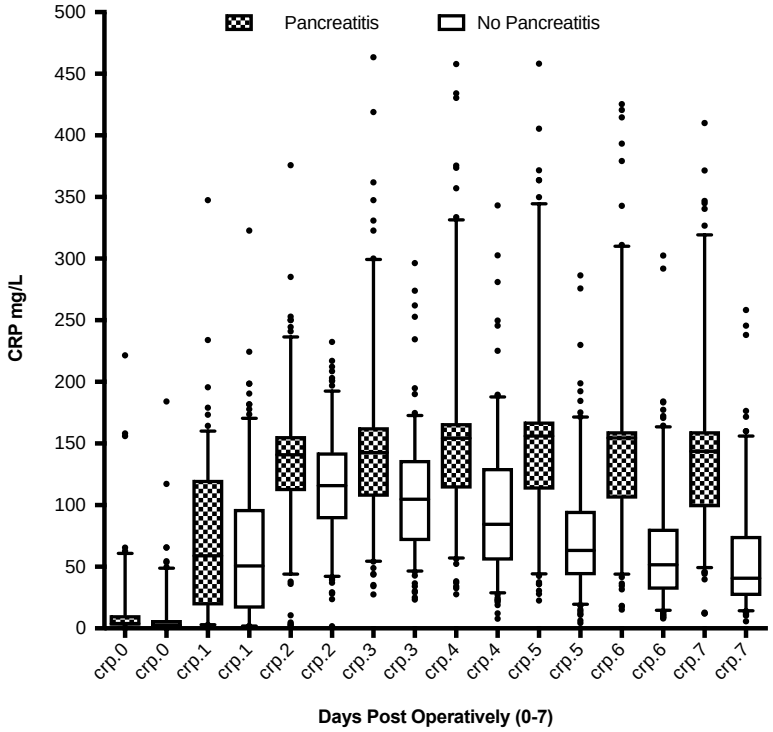
In total, 366 of 397 pancreas transplants provided enough data for inclusion in the analysis. Demographic characteristics for donors and recipients are described in Table 4.6.4.1. Of the 366 patients, 249 patients (68%) had CT scans, of which 146 showed features of pancreatitis (58%). 18 patients had a diagnosis of pancreatitis made during surgery therefore, a total of 164 patients had visually or radiologically diagnosed allograft pancreatitis, showing an overall incidence of 44.8%.

Table 4.6.4.1: Demographic details of recipients with and without pancreatitis with corresponding donor details. Values expressed as median and IQ Range where appropriate.

Donor	**Pancreatitis**	**Non-Pancreatitis**
Total Number of Organs	164	202
Age	43.5 (29-52)	36 (22-45)
Male: Female	77:87 (47%:53%)	107:95 (53%:47%)
BMI	23.5 (21.1-25.9)	22.9 (20.3-24.9)
Girth	70 (60-77.7)	83 (75-89)
DBD:DCD	135:29	166:36
Ethnicity (Caucasian: Other)	149:15	184:18
Median CTSI score	3	0
CIT	10.2 (9.1-12.1)	10 (8.5-11.3)
Recipient	**Pancreatitis**	**Non-Pancreatitis**
Total Number of Patients	164	202
Age	43 (35-51)	44 (36-49)
Male: Female	84:80 (51%:49%)	94:108 (47%:53%)
BMI	26 (23-28)	25 (23-28)
Ethnicity (Caucasian: Other)	144:20 (88%:12%)	191:11 (94%:6%)
Pre-dialysis (Y:N)	89:75 (54%:46%)	103:99 (51%:49%)
HD:PD	49:40 (55%:45%)	63:40 (61%:39%)
Years of diabetes	29.5 (23-38)	29 (22-36.5)

Serum CRP levels from all 366 patients for the first 7 post-operative days are demonstrated in Figure 4.6.4.2.

Figure 4.6.4.2: Box and whisker plot of CRP levels over the first 7 days in all 366 patients with and without pancreatitis (capped and uncapped values).



Results demonstrated that CRP levels were significantly higher in patients who were diagnosed with graft pancreatitis. Day 0 CRP showed a statistically significant difference between patients with and without pancreatitis ($p = 0.001$). This may be due to outliers that skewed the data, as when examining the median values there was considerable overlap between groups, and so was further evaluated in the ROC curve. The difference in CRP between pancreatitis and non-pancreatitis groups remained significant throught the first 7 days, showing a persistent rise in CRP in patients with allograft pancreatitis, while those without pancreatitis had a falling CRP from day 3 onward (see Table 4.6.4.3).

Table 4.6.4.3: Showing the median CRP values with interquartile ranges and associated p-value for the first 7 days following transplantation.

Variable	Pancreatitis	No pancreatitis	p-value
Day 0 CRP	3.9 (2.15-10.80)	2.5 (1.20-6.80)	0.001
Day 1 CRP	58.9 (18.6-120.6)	50.7 (16-97.1)	0.046
Day 2 CRP	141.9 (111.6-156)	115.8 (88.4-142.8)	<0.0001
Day 3 CRP	142.7 (106.9-163.2)	104.8 (70.7-136.8)	<0.0001
Day 4 CRP	154.3 (113.5-166.7)	84.5 (55.10-130.2)	<0.0001
Day 5 CRP	156.0 (112.5-167.9)	63.25 (43.08-95.4)	<0.0001
Day 6 CRP	154.6 (105.3-160)	51.6 (31.40-80.8)	<0.0001
Day 7 CRP	143.7 (98.3-160)	40.70 (26.3-75)	<0.0001

Figure 4.6.4.4 shows the sub-analysis using only uncapped CRP values (i.e. without an assay upper limit) of 172 patients from 2014 onwards. 95 patients were found to have pancreatitis (55.2%; 85 diagnosed via CT and 10 via laparotomy.) The supporting data in Table 4.6.4.5 shows the same pattern with significant differences in CRP observed from day 0. However, both day 0 and day 1 values, although statistically significant, have considerable overlap in the IQR which was considered in the ROC analysis. The difference between pancreatitis and non-pancreatitis groups from day 2 onward remained statistically different ($p<0.0001$). Falling CRP values were noted in patients without pancreatitis from day 3 onward, while the median value of CRP in patients with pancreatitis was more pronounced reaching 185mg/L on day 5.

Figure 4.6.4.4: Box and whisker plot of uncapped CRP levels over the first 7 days in patients with and without pancreatitis.

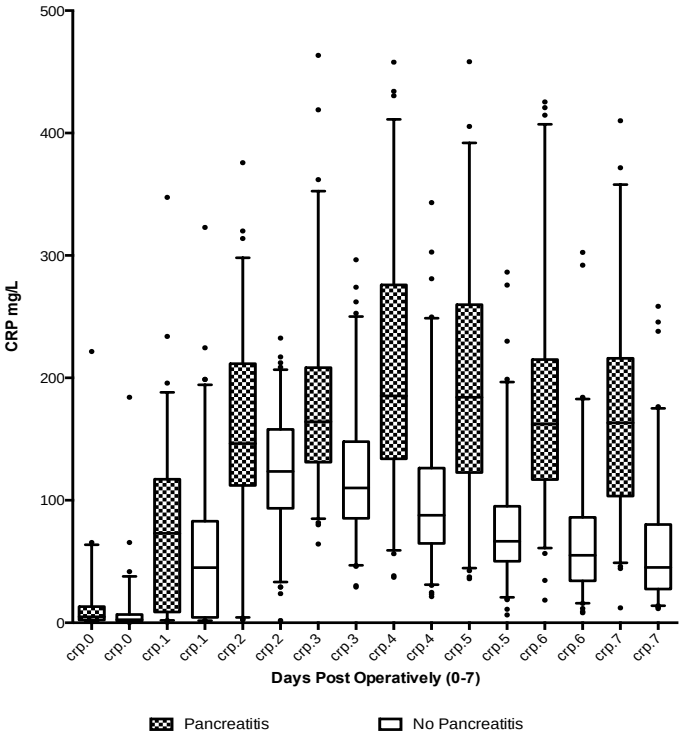
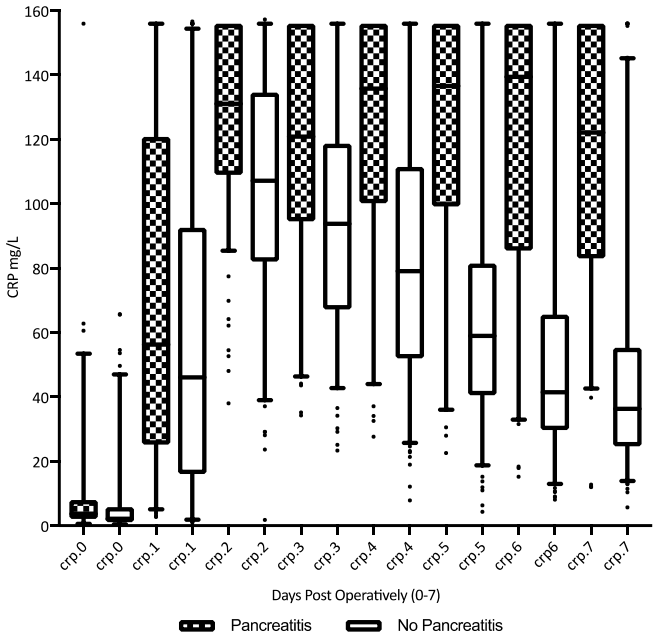


Table 4.6.4.5: Showing the median uncapped CRP values with interquartile ranges and associated p-value for the first 7 days following transplantation.

Variable	Pancreatitis	No pancreatitis	p-value
Day 0 CRP	4.8 (2.2-13.2)	2.4 (1.4-6.7)	0.038
Day 1 CRP	73 (8.7-117.1)	44.8 (4.3-82.8)	0.0373
Day 2 CRP	146.4 (112.1-211.6)	123.6 (93.5-158)	<0.0001
Day 3 CRP	164.2 (131.2-208.4)	110.1 (85.3-147.9)	<0.0001
Day 4 CRP	185.3 (133.9-275.9)	87.80 (64.8 -126.4)	<0.0001
Day 5 CRP	184.2 (122.7-259.8)	66.6 (50.1-95.1)	<0.0001
Day 6 CRP	162.2 (116.9-215.1)	55.1 (34.2-86)	<0.0001
Day 7 CRP	163.3 (103.4-216)	45.2 (27.5-80.3)	<0.0001

When analysing the capped values alone of 194 patients where all CRP<156mg/L, the results confirmed that the capping of CRP did not introduce bias into the pattern (see Figure 4.6.4.6).

Figure 4.6.4.6: Box and whisker plot of capped CRP levels over the first 7 days in patients with and without pancreatitis.



No significant differences in CRP values from day 0 were observed, with large statistical differences between groups seen from day 2 onward p<0.0001. Falling CRP values were noted in patients without pancreatitis from day 3 onward (p<0.0001). The results show median peak of CRP was less pronounced reaching 136mg/L on day 5. These results are summarised in Table 4.6.4.7.

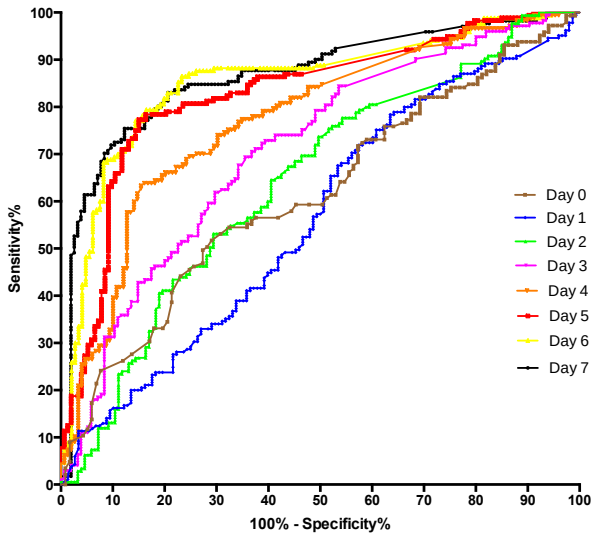
Table 4.6.4.7: Showing the median capped CRP values with interquartile ranges and associated p-value for the first 7 days following transplantation.

Variable	Pancreatitis	No pancreatitis	p-value
Day 0 CRP	3.70 (2.1-8.0)	2.2 (1.10-5.80)	0.067
Day 1 CRP	56.3 (25.2-120.8)	46.1 (16.1-92.55)	0.0171
Day 2 CRP	131.1 (109-156)	107.2 (82.5-134.6)	<0.0001
Day 3 CRP	120.9 (94.6-156)	93.8 (67.2-118.7)	<0.0001
Day 4 CRP	135.8 (100-156)	79.1 (52-111)	<0.0001
Day 5 CRP	136.6 (99.2-156)	59 (40.53-81.45)	<0.0001
Day 6 CRP	139.5 (85.48-156)	41.5 (29.7-65.6)	<0.0001
Day 7 CRP	122.1 (83.5-156)	36.3 (24.7-55.3)	<0.0001

4.6.5 Sensitivity and specificity

In order to assess the predictive value of CRP level at different time-points to diagnose graft pancreatitis, ROC curves were plotted for each post-operative day using all the data (see Figure 4.6.5.1). The results above identified a statistical difference on day 0 and day 1, so understanding whether these were correct or due to erroneous outliers was essential.

Figure 4.6.5.1: ROC analysis of all CRP results in day 0-7.



Area under ROC curve at day 5	0.83
Std Error	0.02
95% confidence interval	0.8 - 0.9
p-value	<0.0001

When assessing the area under the curve for days 0-4, results did not demonstrate any discriminatory power, suggesting that the differences seen in results from this time period are not of clinical value and may be due to outliers. Results from day 5 CRP, using a threshold value of 100 mg/L, showed a considerable increase in sensitivity and specificity; sensitivity = 82.35% and specificity = 82.95% (see Table 4.6.5.2). The area under the ROC curve on day 5 was 0.8281 (95% CI of 0.789-0.874), $p<0.0001$. Day 6 and 7 were also shown to be significant $p<0.0001$.

Table 4.6.5.2: Sensitivity and specificity of CRP at days 3 to 5 post operatively.

	CRP	Sensitivity %	95% CI	Specificity %	95% CI	Likelihood Ratio
Day 3	>99.8	53.4	44.5-62.1	77.9	70-84.6	2.4
Day 4	>99.8	63.8	56.1-70.9	81.9	76.9-88.4	3.9
Day 5	>99.8	82.4	75-88.3	82.9	74.4-88.1	4.4

4.6.6 Relook laparotomy

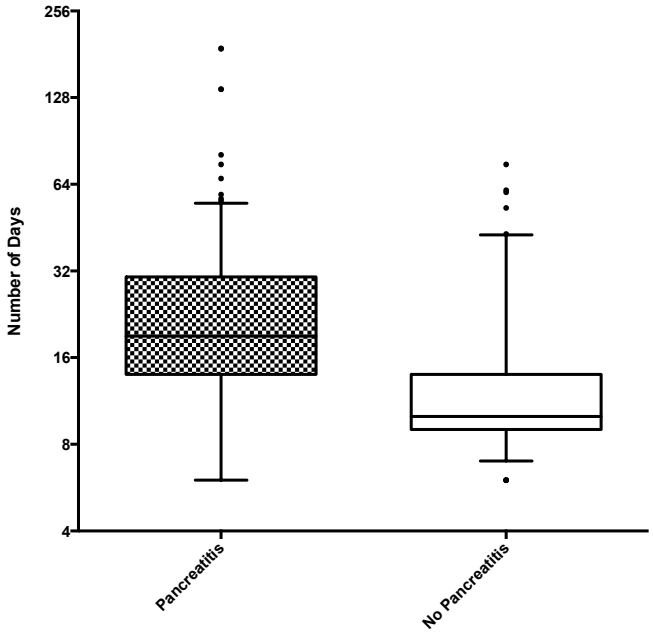
Of the 366 patients, 109 patients had a relook laparotomy (30%) within the first 30 days following transplant. Indications for laparotomy included fluid collections on CT, drops in haemoglobin, poor perfusion of allograft on CT, rises in blood sugar, raised WCC and CRP suggesting sepsis and persistent or worsening abdominal pain. The timing of the first relook laparotomy was significantly later in patients with allograft pancreatitis, with the median at day 7 (IQR 1-42) while non-pancreatitis had a median of day 3 (IQR 1-10) ($p<0.01$). This result was expected, as early laparotomy occurred in response to technical problems such as bleeding or graft thrombosis, with documented procedures performed during laparotomy including washout of fluid collection, haemostasis for recurrent bleeding, evacuation of haematoma, and pancreas explant (often with secondary thrombosis).

The rate of pancreatectomy was significantly different between patients with and without pancreatitis; with 26/164 (16%) in the pancreatitis group versus 7/202 (3%) in the non-pancreatitis group, ($p<0.001$). This result suggests that allograft pancreatitis associated acute inflammation is a risk factor for early graft loss.

4.6.7 Length of stay

The overall length of hospital stay was significantly longer in patients with allograft pancreatitis compared to patients without pancreatitis, with a median length of stay of 19 days for pancreatitis patients (IQR 14-30.5) versus 10 days (IQR 9-14) in non-pancreatitis patients ($p<0.001$) (see Figure 4.6.7.1). When using Spearman rank, a positive correlation was identified with day 5 CRP values and length of stay ($p = 0.01$) but with an r^2 value of 0.3 and wide 95% CI 0.1-0.4, the clinical usefulness of this latter result is questionable.

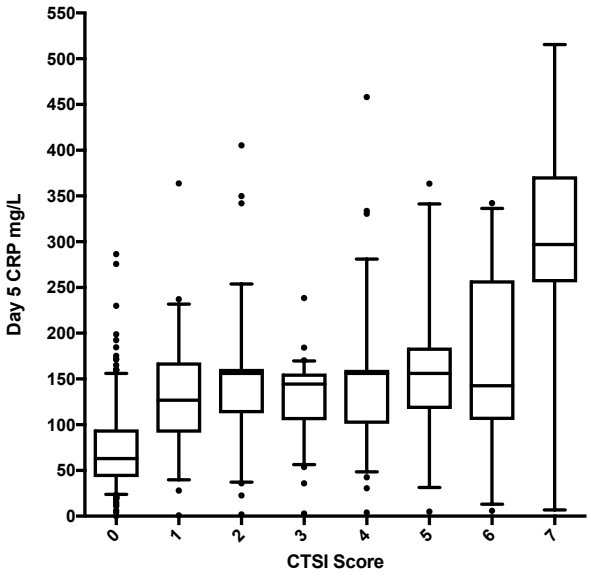
Figure 4.6.7.1: Box and Whisker plot of the median length of stay in patients with and without pancreatitis.



4.6.8 Computed Tomography Severity Index (CTSI) score

CTSI scores were compared with CRP and found to have a positive correlation using the Kruskall-Wallis test. The median CTSI score for patients with pancreatitis was 3 (IQR 1-7) and when correlated with day 5 CRP was also found to have a positive correlation ($p = 0.01$) (see Figure 4.6.8.1).

Figure 4.6.8.1: Box and whisker plot showing CTSI score as a measure of pancreatitis severity compared against day 5 CRP as a surrogate marker.



4.6.9 Donor factors

Donor factors including eGFR, BMI, age, girth, alcohol use, days on ITU, cardiovascular downtime, warm ischaemia times, anastomotic times, cold ischemia time and specific blood parameters including WCC, amylase, liver biochemistry, glucose and donor insulin use were analysed using a multivariate linear regression to eliminate any bias and take account for all variables. No donor variable was found to independently predict the risk of pancreatitis ($p = 0.7$). However, the absence of association does not exclude an effect and may be due to the sample size analysed.

4.6.10 Recipient factors

Recipient variables were also analysed using multivariate regression. Variables included type of pancreas transplant (PTA or SPK), recipient eGFR, number of years of diabetes, daily insulin requirement, age, pre-dialysis or on dialysis, HbA1c and recipient BMI. However, no significant patterns were identified ($p = 0.87$).

4.6.11 Pancreas Donor Risk Index (PDRI)

In 2010, Axelrod et al. constructed the Pancreas Donor Risk Index Score (PDRI) based on roughly 8000 transplants between 2000 to 2006 using US registry data, with the objective to improve organ allocation. The PDRI included 10 donors and two transplant characteristics. Donor factors included donor sex, age, black race, Asian race, BMI, cause of death, creatinine, height and DCD status. Transplant factors included pancreas preservation time and the interaction between PAK and donor cause of death [143].

BMI and DCD status had the greatest impact on risk of graft loss, with Mittal et al. validating the PDRI on a UK cohort. Authors found it predicted 1-year graft survival following SPK transplantation, but not after PTA or PAK transplantation. However, as differences in survival between highest and lowest risk groups were small, authors failed to recommend PDRI assessment as part of clinical assessment [144]. Blok et al. suggested that the PDRI also had some predictive merit but should be used with caution, highlighting good results from high-PDRI donors [145]. While Salamanca-Bustos et al. has since reported that PDRI had no correlation in their Spanish cohort [146]. Calculating PDRI scores based on the sample used in this chapter, results found no correlation between the PDRI score and allograft pancreatitis ($p = 0.77$) or graft loss outcome ($p = 0.41$), suggesting PDRI is not useful in the assessment of allograft pancreatitis.

4.6.12 Survival

Survival data was examined from 2012 to 2017 to assess the impact of pancreatitis. Kaplan-Meier survival curves were plotted and compared using the log rank test. Allograft pancreatitis defined on CT (scored CTSI>0) was found to significantly reduce graft survival; with a hazard ratio of 2.1 (CI 1.2-3.5) $p = 0.013$ (see Figure 4.6.12.1 and Table 4.6.12.2).

Figure 4.6.12.1: Kaplan-Meier Curve showing the number of graft survival days in patients with and without pancreatitis (p = 0.013).

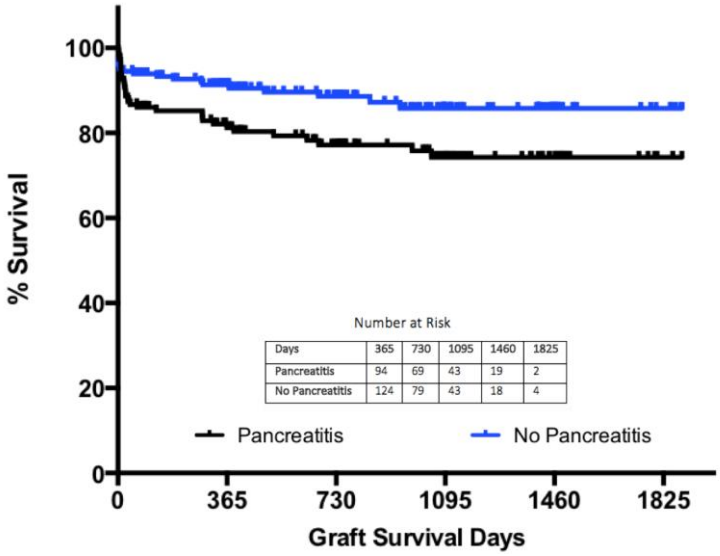


Table 4.6.12.2: Hazard ratio for graft survival in patients with and without pancreatitis.

	Pancreatitis	**No Pancreatitis**
Hazard Ratio	2.1	0.5
95% Confidence Interval	1.2-3.5	0.3-0.8

Graft survival was reduced by approximately 10% when compared to patients without pancreatitis over the first 3 years following transplant (see Table 4.6.12.3).

Table 4.6.12.3: Trend in graft survival years in patients with and without pancreatitis.

Graft Survival Year	**1**	**2**	**3**
Pancreatitis	81.2%	77.2%	74.2%
No Pancreatitis	91.3%	88.6%	85.8%

Figure 4.6.12.4 shows a further Kaplan Meier curve demonstrating the graft survival difference when using day 5 CRP at the threshold value of 100mg/L. A significant difference was observed (p = 0.003) with Table 4.6.12.5, showing the hazard ratio of 1.2 (CI 1.3-3.7) when comparing day 5 CRP at the threshold value of 100mg/L.

Figure 4.6.12.4: Kaplan-Meier Curve showing the number of graft survival days in patients with and without CRP levels >100 mg/L (p = 0.003).

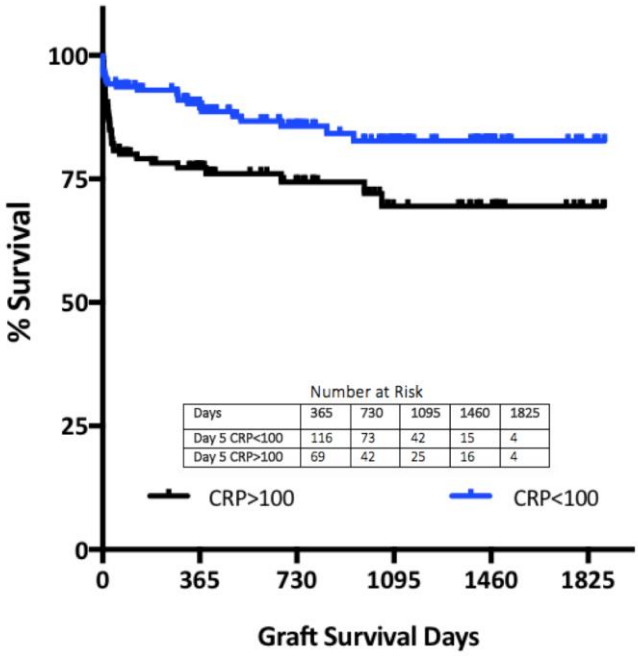
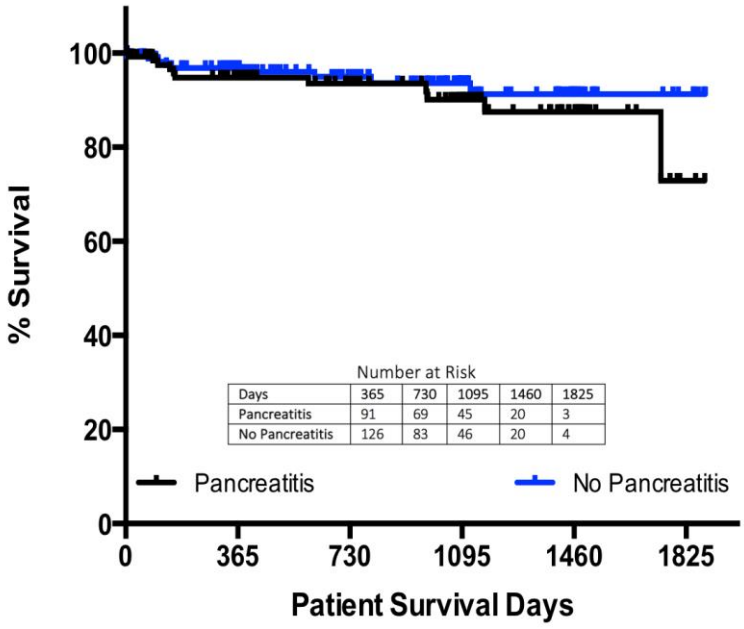


Table 4.6.12.5: Hazard ratio of day 5 CRP at the threshold value of 100mg/L.

	Day 5 CRP<100	**Day 5 CRP>100**
Hazard Ratio	0.4	1.2
95% Confidence Interval	0.3-0.8	1.3-3.7

Patient survival was not affected by CRP or pancreatitis, with no significant differences observed (p = 0.32) (see Figure 4.6.12.6).

Figure 4.6.12.6: Kaplan-Meier Curve showing the number of patient survival days in patients with and without pancreatitis (p = 0.32).



4.6.13 CRP discussion

The observed incidence of allograft pancreatitis was recorded as 44.8%, which lies near the upper end of the documented literature range (6-60%). One reason for this high incidence may be the widened range of acceptable organs deemed suitable to transplant in the UK compared to other global programs.

Some pancreas programs use continuous steroids following transplant, while others give five days as part of an early steroid withdrawal protocol. This series only had a single dose given at reperfusion and may account for the relatively higher incidence of pancreatitis. However, what is apparent from the results is that allograft pancreatitis is a common and important cause of morbidity affecting graft loss.

Wullstein et al. identified CRP rises within 72 hours following transplantation correlated with graft outcomes [142]. The results of this study partially support this finding, showing statistically significant differences in CRP between pancreatitis and non-pancreatitis groups: Day 1 ($p = 0.04$) Day 2 ($p<0.0001$) and Day 3 ($p<0.0001$). However, the sensitivity and specificity of these results was poor, suggesting earlier correlations with the outcome may not be due to allograft pancreatitis.

When examining Day 0 CRP values, a statistically significant difference was identified between patients with and without pancreatitis ($p = 0.004$), with poor sensitivity and poor specificity on the ROC curve. It is possible that this result may be due to outliers that have skewed the data but if this result is correct, another explanation for the difference may be that the inflammation attributable to pancreatitis is recipient-related rather than driven solely by the donor pancreas. There is some evidence to show that subtle variations in the CRP gene sequence, mostly single nucleotide polymorphisms, predictably and strongly influence the blood level of CRP [147]. The importance of gene polymorphisms in inflammation has not been examined and with other patients shown to be genetically predisposed to developing inflammation [148], the difference in CRP at day 0 warrants further investigation.

What is clear from the data is that the sensitivity and specificity of CRP became strongly significant (>80%) from day 5 onward, at a threshold value >100mg/L. This appears to be clinically useful at identifying patients early, while prior to this time-point, a raised CRP remained non-discriminatory. The results support evidence seen from native pancreatitis guidelines, which following the Santorini consensus, identified the use of CRP after 48 hours of symptoms at a cut off of 150mg/l as an indicator of severity [149] [139]. CTSI scores when

compared with CRP, were also found to have a positive correlation supporting idea. The consideration of using CRP as a surrogate marker for the diagnosis of pancreatitis and potential marker to assess severity should be considered. It is important to note that CRP is a non-specific marker of inflammation, and one of the clear limitations of using this marker is that it does not indicate the organ affected. Since CRP can also be produced by non-hepatic cell types such as macrophages, PBMCs, smooth muscle cells and other parts of the GI tract, the source of high CRP levels must be considered [150].

Additionally, a further limitation in design was its assessment using SPK patients as there is evidence in the literature to indicate that CRP levels are inversely related to renal function with increasing CRP levels correlating with decreasing eGFR. If the study were redesigned, using PTA patients only to assess the effect of allograft pancreatitis would be more suitable. However, the results of such a study may not be translatable to the SPK situation. When analysing the data, no significant difference in eGFR between pancreatitis and non-pancreatitis populations was identified ($p = 0.87$) and no correlation between eGFR and CRP found suggesting the pattern of CRP in these results may not be affected by DGF.

Benz et al. observed 17 patients undergoing pancreas transplantation who had continual monitoring of tissue PO_2 using an electrode implanted into the tail of the pancreas and a catheter into the distal splenic vein. Results identified that decreasing tissue PO_2 after primary re-oxygenation is associated with the degree of damage and directly related to impaired microcirculation. This study suggests that grafts from older donors may have a higher susceptibility to develop allograft pancreatitis due to increased microcirculatory disturbance [151]. However, multivariate analysis demonstrated that potential confounding variables (donor and recipient), did not identify any association with allograft pancreatitis.

These results did not support previously published evidence, which suggested high donor BMI and old donors are associated with increased allograft pancreatitis.

The reasons for this finding may be due to the bias of performing univariate versus multivariate analysis, as pancreatitis does not have a single causative risk factor. Alternatively, the absence of association may be due to the need for a combination or accumulation of risk factors, rather than any individual risk factor in isolation. Another consideration is that significant differences may have been missed due to the limitations in patient numbers.

Results demonstrated that inferior graft survival was observed in patients with allograft pancreatitis, ($p = 0.013$). The presence of allograft pancreatitis was found to double the risk of graft loss (HR 2.1 95% CI 1.3-3.5), and using day 5 CRP at the threshold of 100mg/L, was a significant predictor in identifying this loss $p = 0.0033$. The lack of correlation between PDRI and allograft pancreatitis is surprising given the close relationship shown between pancreatitis and graft survival. However, as multivariate analysis failed to identify any positively associated factors, this may be the reason why PDRI assessment of allograft pancreatitis remained insensitive.

Unsurprisingly, length of stay increased rates of relook laparotomy and rates of pancreatectomy were significantly associated with allograft pancreatitis, confirming the increased morbidity associated with the condition. It may be that if the effects of pancreatitis could be abrogated then pancreas graft survival might become comparable to kidney transplant alone.

4.7 Initial conclusions

Donor risk factors had surprisingly little value in determining allograft pancreatitis and rather than restrict the donor pool to exclude many potentially useful donor organs, the focus should be on developing treatments to abrogate allograft pancreatitis.

In the immediate post-operative period, CRP provides better utility than lipase, with the compelling evidence in favour of using CRP as a biomarker to identify patients with allograft pancreatitis. Day 5 CRP was shown to be a sensitive 82.4% (CI 75-88.3) and specific 82.9% (74.4-88.1) marker of allograft pancreatitis diagnosed using CT. Therefore, one major conclusion from this study is that CRP might be valuable as the primary endpoint of clinical trials testing potential interventions in the treatment of allograft pancreatitis such as steroids or other anti-inflammatory agents.

CHAPTER 5:

The assessment and characterisation of graft pancreatitis using FACBC PET - CT, Luminex cytokine analysis and FACS

5.1 Overview and objective

The previous chapter identified allograft pancreatitis as an important, common and significant morbidity affecting graft survival. Being able to assess and quantify graft pancreatitis early, would allow the identification of those patients who are at risk of deterioration. Chapter four highlighted the use of CT and CRP to help achieve this and the focus of this chapter is on the use of *anti*-1-amino-3-fluorine-18-fluorocyclobutane-1-carboxylic acid (FACBC) as an alternative method to help further quantify inflammation associated with allograft pancreatitis.

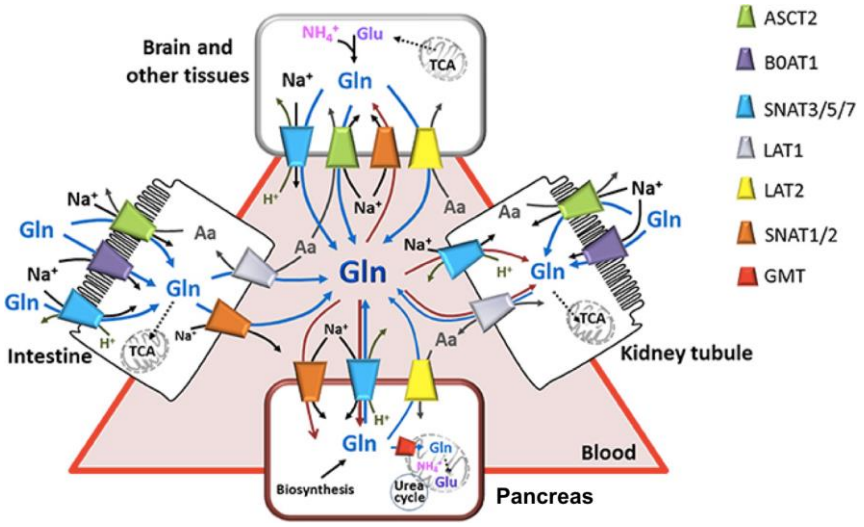
5.2 Amino acid transportation in pancreatitis

The human pancreas produces approximately 1-1.5L of fluid daily containing between 5-20g of protein which are predominantly digestive enzymes, secreted upon hormonal stimulation by pancreatic acinar cells and released into the ductal system. Therefore, amino acid uptake into the pancreas to meet and support protein synthesis is essential [152]. Pancreatic acinar cells accumulate amino acids against a marked concentration gradient to synthesise these digestive enzymes hence, expectantly, this central process is thought to be affected by pancreatitis if acinar cells become damaged [153.] Understanding how pancreatic amino acid transporter (AAT) expression and cellular localisation changes in the context of allograft pancreatitis is critical as currently is unclear [154] [155].

Rooman et al. screened the mouse pancreas for the expression of genes encoding AATs. It has been shown that the most highly expressed transporters, namely sodium dependent SNAT3 (Slc38a3 gene) and SNAT5 (Slc38a5 gene) and sodium independent neutral amino acids transporters LAT1 (Slc7a5 gene) and LAT2 (Slc7a8 gene), are expressed in the baso-lateral membrane of acinar cells. SNAT3, SNAT5, LAT1 and LAT2 have also been shown to be

expressed in human acinar cells and are involved in the transportation of glutamine and leucine (see Figure 5.2.1) [156].

Figure 5.2.1: Glutamine amino acid transportation across human tissues including the pancreas. Image source: [157]

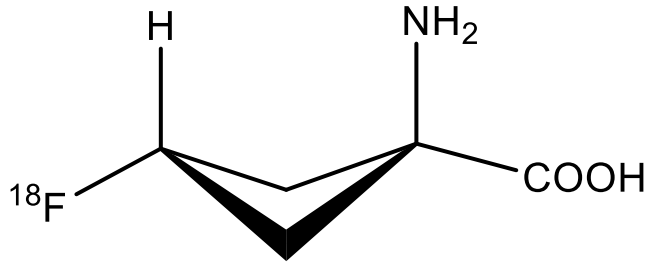


Rooman el al. found that in the acute phase of pancreatic injury, acinar cell injury was observed and SNAT3 and SNAT5 were found to be strongly down-regulated while LAT1 and LAT2 were also down-regulated but only in the in-vitro model (clearly, if the cells die there is an overall reduction as no further transportation would occur). Additionally, expression of other amino acid transporters was also modified in both murine and human pancreatitis [156]. These findings provide insight into pancreatic amino acid transport in healthy pancreas and during acute pancreatitis injury, and assessing their importance in the transplant setting will help build further evidence.

5.3 FACBC

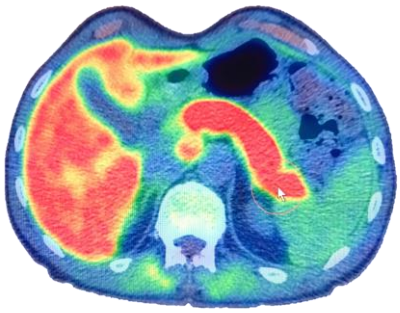
FACBC is a synthetic amino acid analogue used in the staging prostate carcinoma (see Figure 5.3.1). The uptake of FACBC is mediated through a combination of amino acid transport proteins where the radiotracer is not metabolised [158].

Figure 5.3.1: Chemical structure of FACBC.



The concept of using radio-labelled amino acids for the imaging of cancer is based on the increased metabolic demand of tumour cells. Enhanced channelling of amino acids into select metabolic pathways, increases expression of amino acid transporters to acquire these substrates [159]. FACBC is a glutamine and leucine analogue (with regard to function and structure) taken up predominantly by AATs (ASCT2 and LAT1). The normal bio-distribution of FACBC has shown to include relatively intense uptake in the liver and pancreas, which is hypothesised to act similarly to Carbon 11 (^{11}C) acetate, targeting fatty acid metabolism [160]. Using this evidence, the uptake of FACBC using PET-CT was used to assess, and potentially quantify, the severity of allograft pancreatitis testing the hypothesis that AATs are altered in allograft pancreatitis (see Figure 5.3.2).

Figure 5.3.2: An axial FACBC PET-CT image, showing high levels of uptake in the native human pancreas and liver. Areas of high FACBC uptake are coloured red and areas of low activity are coloured blue. Each pixel (voxel) represents a 3D cube, which using computer software will allow a numerical value to be associated with it. In that way, the FACBC uptake in the whole pancreas can be measured to provide an overall number which may allow quantification of the FACBC uptake and inflammation.



5.4 Study design and methods

SPK transplant recipients were recruited (11 in total). Following surgery, patients underwent a PET-CT scan within the first seven post-operative days as FACBC scans could only be performed on Wednesdays. Patients were informed about the study through the waiting list and consented to participate in the trial at the same time as for transplant (once organs had been checked and the transplant was confirmed to proceed). Inclusion criteria included male and female patients aged 18 to 60 years who had an SPK with no previous transplant.

During transplantation, 5 mL of whole blood was taken from a central venous catheter and stored in a purple EDTA blood tube, then transported in a cool box (5°C) and processed within 30 minutes of taking the sample. Samples were collected at the following time points: baseline sample before surgery started, time point zero - reperfusion of the pancreas (once clamps were removed and blood circulated through pancreas into systemic circulation) and

post reperfusion samples at 1hr, 2hr, 6hr, 12hr 24hr, 48, 72hr, 96hr and 336hr (14 days post-transplant).

Sample processing involved the separation of 1mL of blood in order to isolate cells, while the remaining 4mL was centrifuged to isolate plasma, which was then aliquoted and frozen at -80°C for later analysis. Cell pellets were isolated for flow cytometry to analyse the reperfusion leucocyte profile using the same techniques described in chapter four.

Each PET-CT scan required injecting 0.1mls of tracer providing 50 MBq of FACBC activity. Participants were scanned in the supine position, with the arms remaining positioned by their side following FACBC intravenous administration. The PET scan consisted of a 20-minute acquisition performed over two bed positions centred over the pancreas transplant, each lasting five minutes and then repeated, starting three minutes post-injection (bed 3-13min and 13-23min). The pancreas uptake of FACBC was characterised by the standardised uptake value (SUV). This was evaluated by drawing a threshold (40% of SUV_{max}) volume-of-interest (VOI) around the organ and recording the mean SUV within the VOI.

The second and final PET-CT scan was performed 14 days after the first, using the same protocol. The original study was designed to assess 20 patients but following an interim analysis, it was stopped after 11 patients. No patients withdrew consent. The commercial manufacturer of FACBC who funded the study put a hold on the study based on the analysis of 10 patients as they were assessing whether there is sufficient commercial benefit to warrant continuing to 20 patients.

5.4.1 FACBC analysis

FACBC was characterised by SUV, which has subsidiary components that include (a) SUV_{max}, a significant parameter for predicting clinical outcomes from cancer studies, providing the highest metabolic activity point, and (b) $SUV_{mean,}$ which provides the average metabolic activity over the entire organ. Another parameter, total pancreas FACBC uptake (TPF), encompasses both volume and mean FACBC activity and so, both SUV and total lesion glycolysis (TLG) were analysed. The FACBC scans were interpreted using Hermes Hybrid Viewer (Hermes Medical Solutions AB).

5.4.2 Cytokine analysis using Luminex

The level of cytokines and chemokines within the perfusate was assessed using a human 23-plex magnetic bead panel (R&D Systems Luminex assay, Abingdon, UK). All reagents used were supplied within the kit. Plasma samples were thawed and a 96-well plate was prepared by adding 200µl assay buffer before being shaken for 10 minutes at room temperature. Samples underwent a 2-fold dilution using calibrator diluent RD6-52.

1. 50µL of sample was added per well.
2. The diluted micro particle cocktail was re-suspended by vortexing.
3. 50µL of the micro particle cocktail was then added to each well of the microplate.
4. The plate was next securely covered with the foil plate sealer and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12” orbit) set at 800 ± 50 rpm.
5. Using the magnetic microplate washer, the plate was washed by applying the magnet to the bottom of the microplate.
6. The plate was allowed to stand for 1 minute before the liquid was removed.

7. Each well was then filled with wash buffer (100µL) and allowed to stand for a further 1 minute before the liquid was again removed.
8. The plate was washed three times.
9. 50µL of diluted Biotin-Antibody cocktail was added to each well and the plate securely covered with the foil plate sealer, then left to incubate for 1 hour at room temperature on the shaker set at 800 ± 50 rpm.
10. The plate was then washed again as in step 8.
11. 50µL of diluted streptavidin-PE was then added to each well and the plate was securely covered with the foil plate sealer and incubated for a further 30 minutes at room temperature on the shaker set at 800 ± 50 rpm.
12. The plate was then washed again as in step 8.
13. 100µL of wash buffer was added to each well to re-suspend the micro particles. The plate was then incubated for 2 minutes while being agitated on the shaker set at 800 ± 50 rpm.
14. The plate was then read using Luminex® analyser.

5.4.3 Flow cytometry

Immunophenotyping and FACS of the venous blood samples was performed on a BD LSR II flow cytometer (Becton Dickinson, Oxford, UK). The method used was the same as that described in sections 4.3.2 and 4.3.3. Not all the leucocyte immunophenotype samples were analysed due to cost implications and so, five time points were selected to provide an overview of potential patterns: baseline (prior to surgery), 2hours, 24 hours, 96 hours, (4 days) and 336hrs (14 days) post reperfusion.

5.4.4 Statistical analysis

Data normality was determined by assessing the descriptive statistics, mean standard deviation, skewness and kurtosis. A non-parametric data distribution was identified and so the Mann-Whitney U test, Wilcoxon Rank and Kruskall Wallis tests were utilised. Data was considered significant at $p<0.05$.

5.5 Results

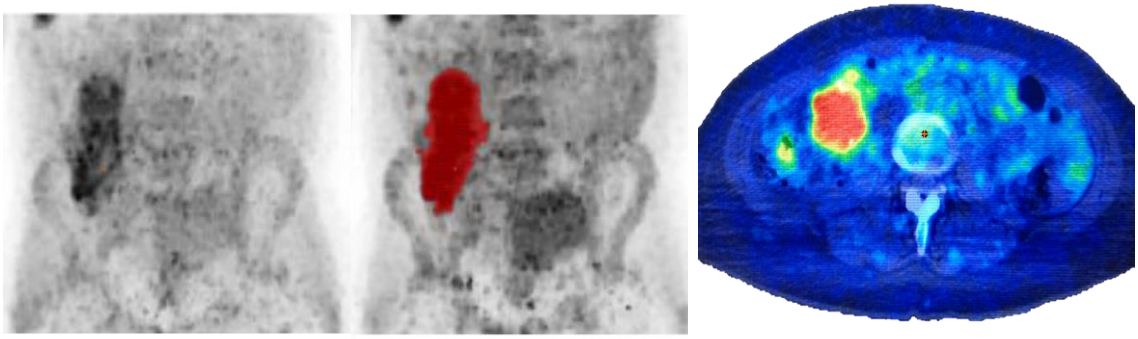
10 patients completed the study, having both scans and all blood tests performed. 1 patient did not have the second scan due to a technical failure and the data was not analysed. Demographic data of the patients enrolled has been included in the following table:

Donor	**Pancreatitis**	**Non-Pancreatitis**
Total Number of Organs	5	5
Age (Median and IQ Range)	47.5 (43.5-50.3)	22.4 (16.7-37.3)
Male: Female	3:2	2:3
BMI (Median and IQ Range)	23.7 (21.7-26.8)	23.9(15.3-26.2)
CIT (Median and IQ Range)	10.25 (8.3-13.4)	9.2 (8.7-11.9)
Recipient		
Total Number of Patients	5	5
Age (Median and IQ Range)	42.6 (35.7-47.7)	35.9 (31-50.5)
Male: Female	1:4	3:2
BMI (Median and IQ Range)	21.67 (19.8-24.6)	21.21 (19.2-26.6)

5.5.1 Feasibility

The results show that the administration and recording of images of the allograft following transplantation using FACBC is possible. All 10 patients had images of the pancreas allograft that were suitable for assessment. Figure 5.5.1.1 shows the images of the pancreas in the right iliac fossa, which has been highlighted in red to assess its volume using Hermes.

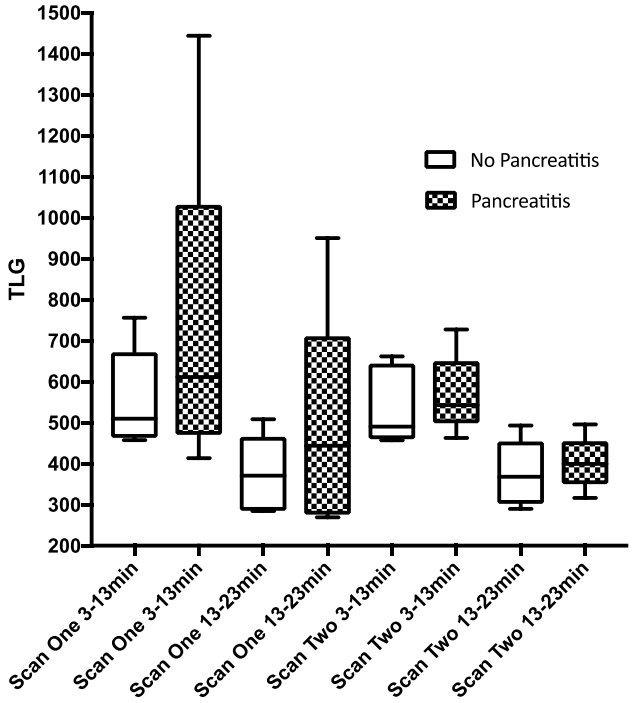
Figure 5.5.1.1: SPK FACBC Images. This two-part image shows the coronal PET-CT image of a transplanted pancreas in the right iliac fossa - highlighted section in red using Hermes (image on the left), and an axial cross-section showing the transplanted pancreas with increased FACBC uptake (image on the right).



5.5.2 Total Lesion Glycolysis (TLG)

TLG combines volumetric and FACBC uptake information. Results identified activity in patients with pancreatitis (n=5) had a much larger range than those without pancreatitis (n=5) in the first scan, while no differences were seen between groups in the second scan. Although the median values in patients with pancreatitis were slightly higher than those without, in this first scan over both bed positions, the results were not found to be statistically different ($p = 0.79$) (see Figure 5.5.2.1).

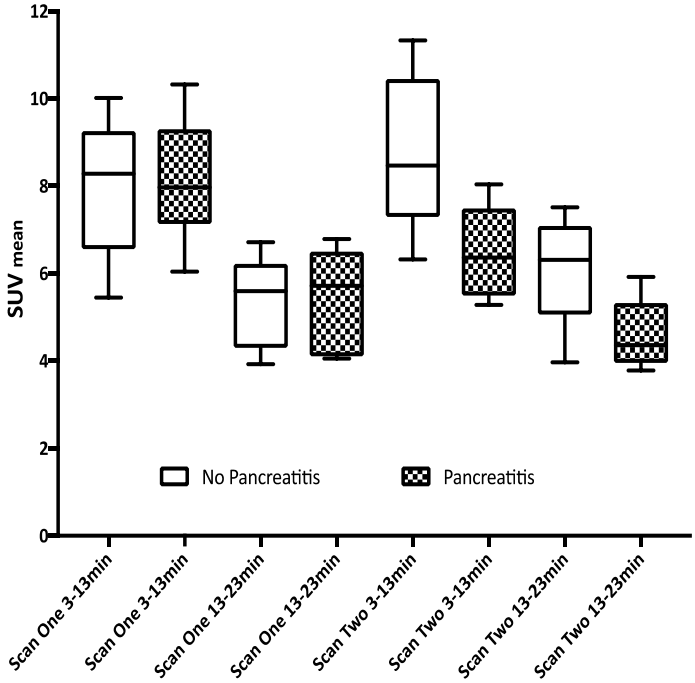
Figure 5.5.2.1: Box and whisker plot showing the TLG values for patients with and without pancreatitis over the two scans. Each scan has two imaging time points and the data was separated accordingly.



5.5.3 SUV mean

SUV_{mean} provides the average FACBC activity for the entire pancreas. Results demonstrated no difference in the first scan but a reduced SUV_{mean} in patients with pancreatitis. This suggests significantly reduced amino acid transportation in the presence of inflammation when the second scan was taken two weeks later ($p = 0.043$) (see Figure 5.5.3.1).

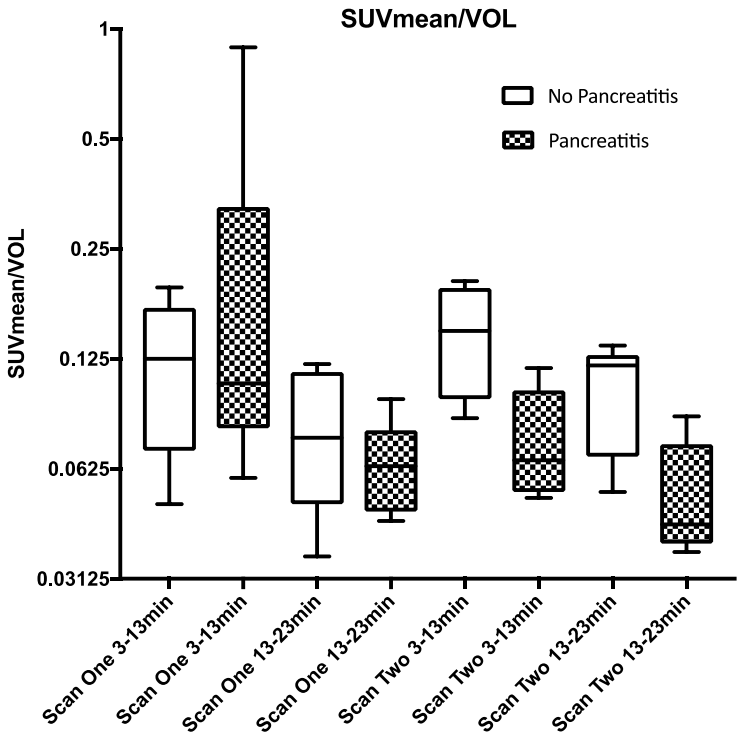
Figure 5.5.3.1: Box and whisker plot showing the SUV_{mean} for patients with and without pancreatitis over the two scans. Patients with pancreatitis were found to have reduced SUV_{mean} compared to non-pancreatitis patients on the second scan.



5.5.4 SUV mean/volume

To further confirm the difference seen in SUV_{mean}, the activity per unit volume was measured and results confirmed its reduction in the second scan ($p = 0.047$), with no difference observed in the first scan. The results suggest that there may be increased volume due to oedema (inflamed pancreas) in addition to reduced protein synthesis and reduced AAT in the presence of inflammation (see Figure 5.5.4.1).

Figure 5.5.4.1: Box and whisker plot showing the activity per unit volume in patients with and without pancreatitis.



5.5.5 Cytokine analysis

Of the 23 cytokines tested with Luminex, eight showed differences between patients with and without pancreatitis. The four-part panel shown in Figure 5.5.5.1 demonstrates the results of IL-4 ($p = 0.001$), IL-6 ($p = 0.001$), IL-18 ($p = 0.03$) and gp130 ($p = 0.05$), all which were found to be reduced in patients with allograft pancreatitis (see Figure 5.5.5.1). Wilcoxon Ranks were used to compare differences. Reduced expression of IL-4 and IL-6 was persistent across the two weeks following reperfusion, while the reduction in IL-18 was more prominent over the first four days only. gp130 remained reduced for the first 72hours following reperfusion, then began to increase matching levels seen in patients without pancreatitis.

Figure 5.5.5.1: Shows the median values with IQR of four cytokine concentrations measured pg/ml in patients with and without allograft pancreatitis (n=5 for each group). A: Shows the reduced concentration of IL-4 in patients with pancreatitis. B: Identifies the reduced concentration of IL-6 in patients with allograft pancreatitis. C: Differences in IL-18 concentration. D: Differences in gp130 concentration.

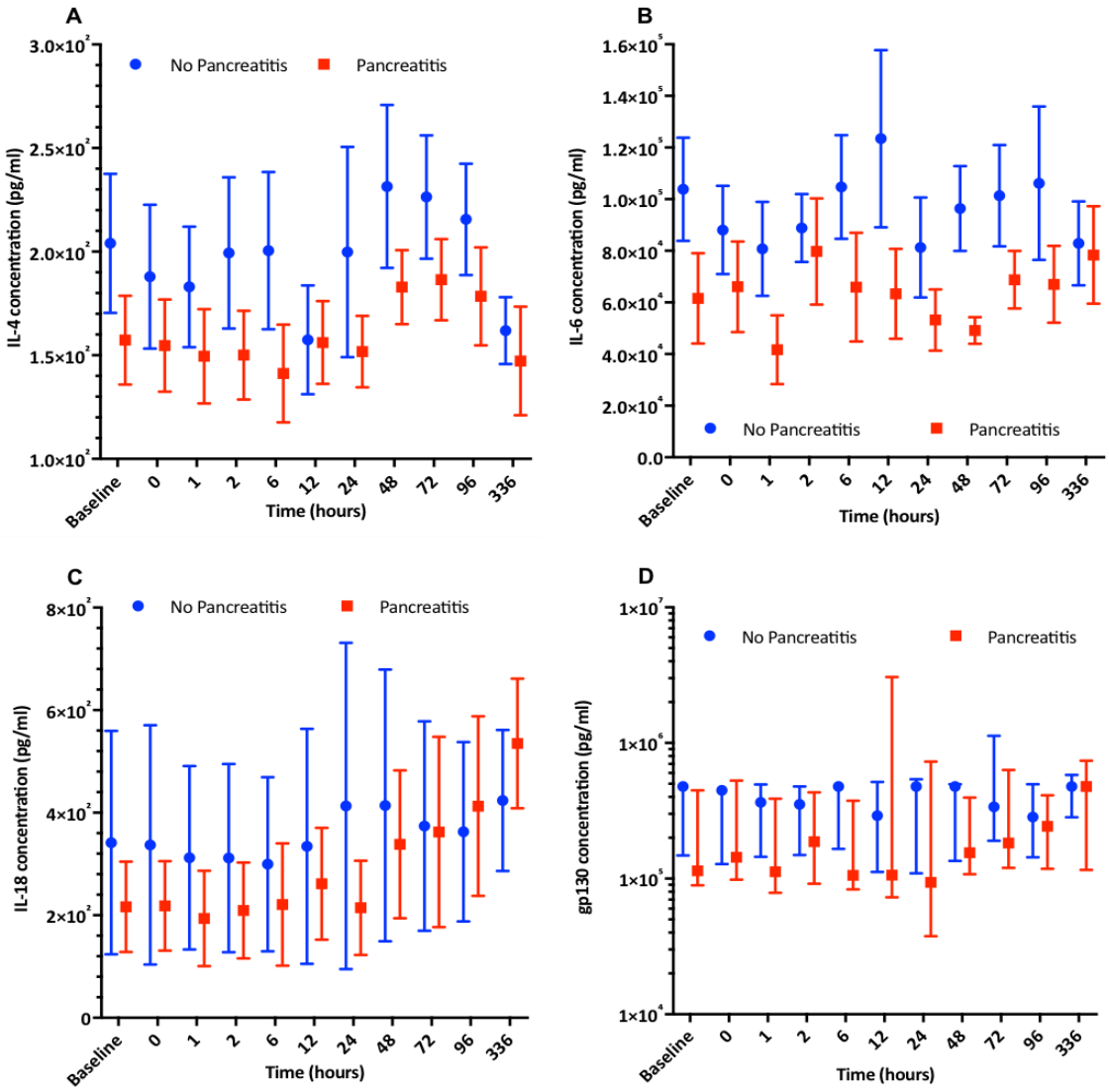
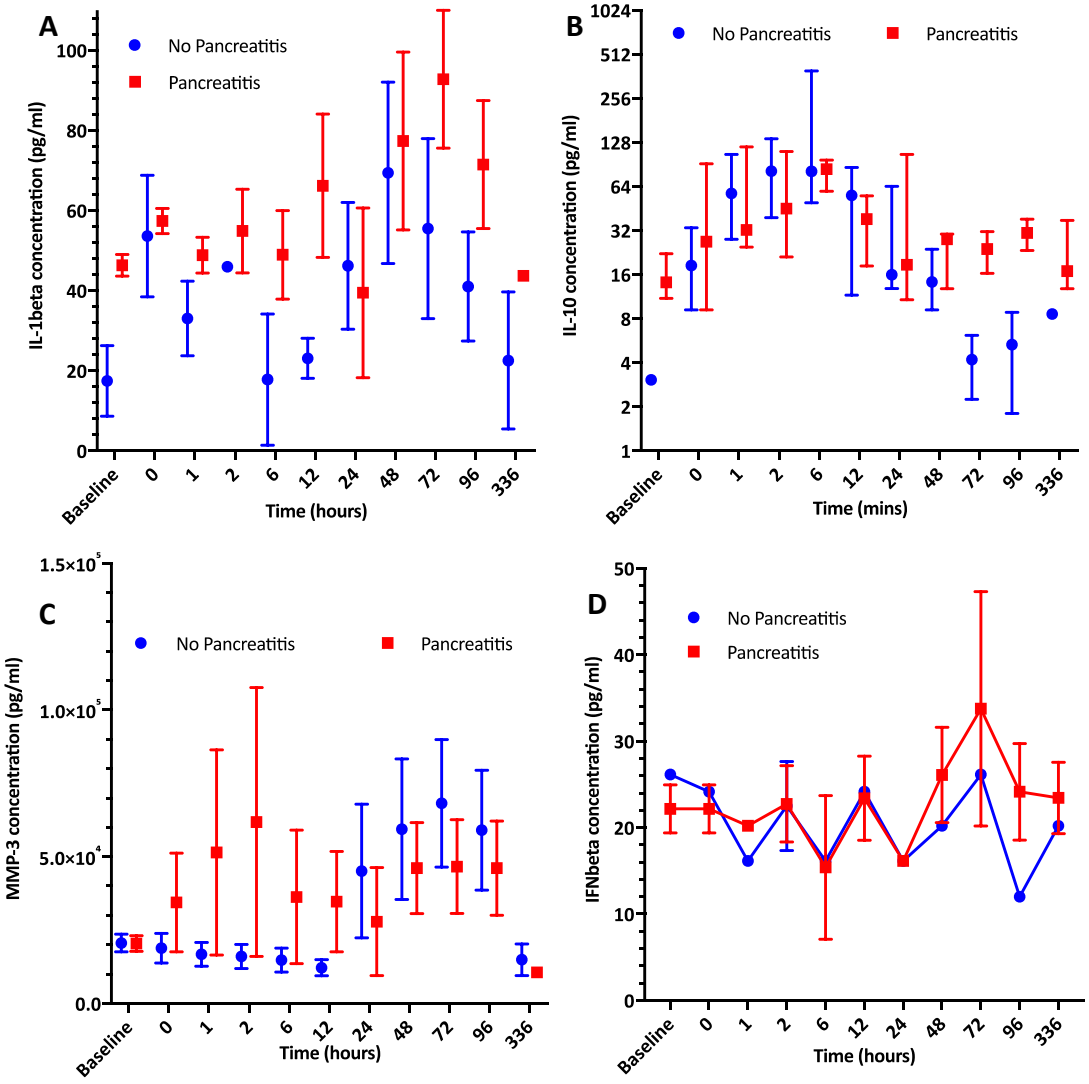


Figure 5.5.5.2 shows the results of IL-1ß, IL-10, IFNß and MMP-3. IL-1ß was higher in patients with allograft pancreatitis (p = 0.003), while IL-10 and IFNß were found to increase only after 24hrs and then remained elevated in patients with pancreatitis, while levels reduced in patients without pancreatitis. The changes in MMP-3 levels also appeared to be short-lived

with an initial 12hr increase in concentration in patients with pancreatitis compared to those without, with no significant difference seen in concentrations between groups seen after 24 hours (see Figure 5.5.5.2).

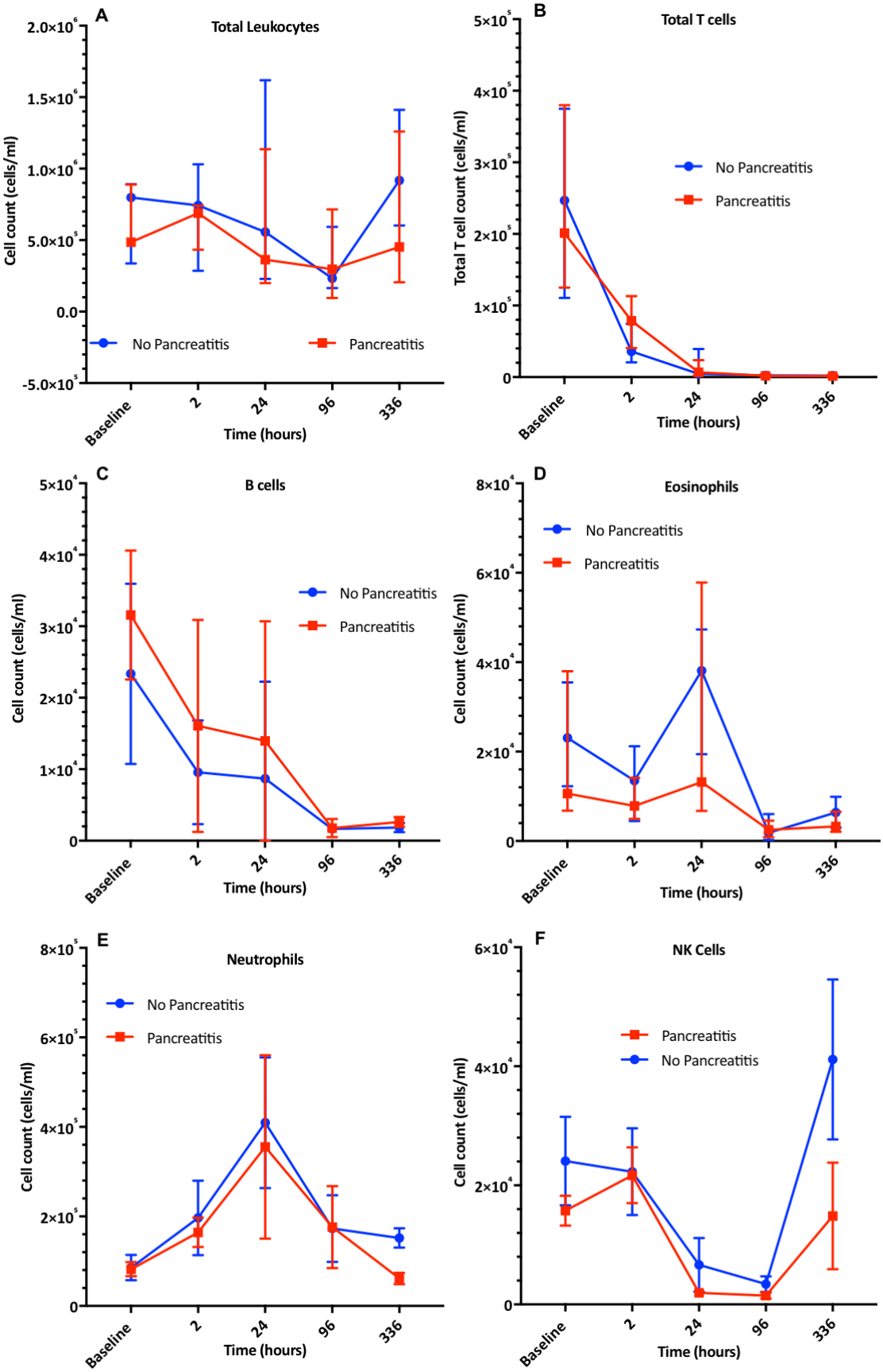
Figure 5.5.5.2: Shows the median values with IQR of four cytokine concentrations measured in pg/ml in patients with and without allograft pancreatitis. A: Shows the increased concentration of IL-1ß in patients with pancreatitis. B: Shows the rising concentration of IL-10 following 24hours in patients with allograft pancreatitis. C: Shows the early rise of MMP-3 over the first 12hours following reperfusion. D: Shows the rising concentration of IFNß following 24hours in patients with allograft pancreatitis.



5.5.6 Flow cytometry results

Figure 5.5.6.1 demonstrates the results of the flow cytometry, comparing the differences in the population of leucocytes isolated between patients with and without pancreatitis. There was no difference in the overall number of leucocytes between patients with and without allograft pancreatitis and as expected, both groups saw depletion of both T and B cells following administration of alemtuzumab. Neutrophils were observed to increase over the first 24 hours following surgery and then decreased but again, no difference was seen between patients with or without pancreatitis. Mast cells, NKT cells and a sub analysis of $CD4^+$ and $CD8^+$cells were also analysed but with no statistical differences observed between groups (graphs were not plotted). NK cells did show a difference at two weeks, where levels were reduced in patients with pancreatitis ($p = 0.03$). However, prior to this point no difference was seen.

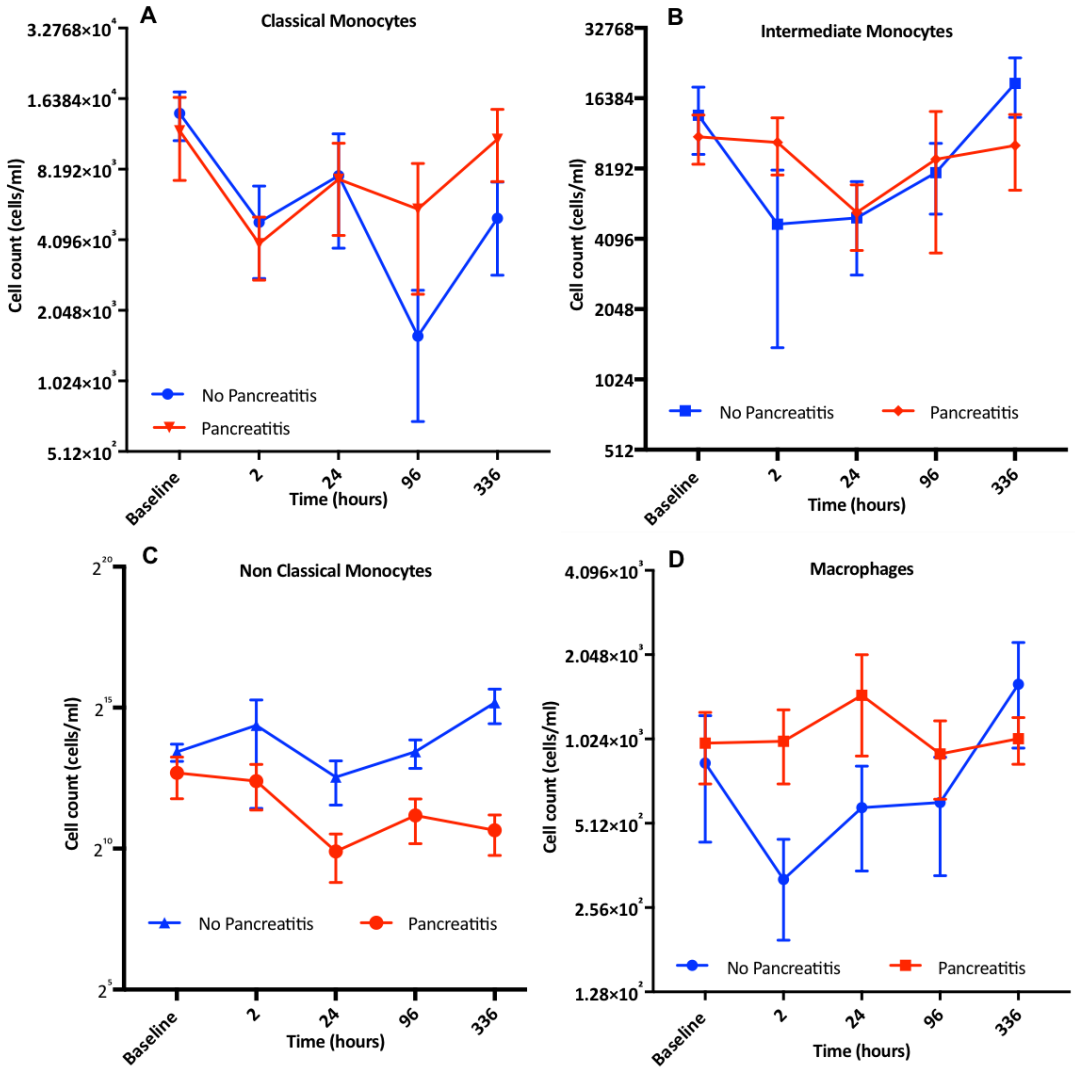
Figure 5.5.6.1: Leucocyte flow cytometry results following reperfusion. A: Shows the difference in total leucocyte populations between patients with and without allograft pancreatitis. B: Shows the depleting number of T cells following reperfusion. C: Shows the depleting number of B cells following reperfusion. D: Shows populations of eosinophils in patients with and without allograft pancreatitis. E: Shows populations of neutrophils in patients with and without allograft pancreatitis. F: Shows the populations of NK cells in patients with and without allograft pancreatitis.


A
Total Leukocytes
Cell count (cells/ml)
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
-5.0×10^5
No Pancreatitis
Pancreatitis
Baseline
2
24
96
336
Time (hours)
B
Total T cells
Total T cell count (cells/ml)
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
No Pancreatitis
Pancreatitis
Baseline
2
24
96
336
Time (hours)
C
B cells
Cell count (cells/ml)
5×10^4
4×10^4
3×10^4
2×10^4
1×10^4
0
No Pancreatitis
Pancreatitis
Baseline
2
24
96
336
Time (hours)
D
Eosinophils
Cell count (cells/ml)
8×10^4
6×10^4
4×10^4
2×10^4
0
No Pancreatitis
Pancreatitis
Baseline
2
24
96
336
Time (hours)
E
Neutrophils
Cell count (cells/ml)
8×10^5
6×10^5
4×10^5
2×10^5
0
No Pancreatitis
Pancreatitis
Baseline
2
24
96
336
Time (hours)
F
NK Cells
Cell count (cells/ml)
6×10^4
4×10^4
2×10^4
0
Pancreatitis
No Pancreatitis
Baseline
2
24
96
336
Time (hours)

Chapter four identified monocytes as the predominant resident population within the pancreas allograft during the cold phase of workbench preparation, where I hypothesised these cells may be flushed into circulation following reperfusion. The results of the monocyte analysis following reperfusion shown in Figure 5.6.6.2 demonstrate an elevated number of classical monocytes in patients with pancreatitis after 24 hours. Although results were not statistically different ($p = 0.08$), this may be due to the sample size.

No statistical difference was seen when examining intermediate monocytes in patients with and without pancreatitis, while non-classical monocytes were found to be reduced in patients with pancreatitis compared to patients without pancreatitis. No statistically significant difference was observed before 24 hours ($p =0.06$), but after 24 hours populations of non-classical monocytes were signficantly reduced ($p=0.01$). Higher levels of macrophages were observed in patients with pancreatitis over the first four days following reperfusion, but the difference was not found to be significant, ($p= 0.07$) (see Figure 5.6.6.2).

Figure 5.5.6.2: Monocyte and macrophage flow cytometry results following reperfusion. A: Shows the difference in classical monocyte populations between patients with and without allograft pancreatitis. B: Shows the number of intermediate monocytes following reperfusion between patients. C: Shows the number of non-classical monocytes following reperfusion between patients with and without pancreatitis. D: Shows the number of macrophages following reperfusion between patients with and without pancreatitis.



5.6 Discussion

The first question when designing the study was whether the acquisition and interpretation of FACBC images was possible. The background signal noise from recent surgery combined with less mobile patients was a consideration however, results have shown that the concept of using FACBC in the assessment of allograft pancreatitis is technically feasible and safe.

A major limitation of this discussion is the sample size of 10 patients. Having only five patients in each group limited any conclusions and so, the results were used to generate a hypothese for future evaluation. The reason for the termination of the study at 10 patients was a commercial decision by the tracer manufacturer.

During acute pancreatitis pathogenesis, ROS has been shown to have deleterious effects but are known to be neutralised by glutathione (GSH), an intracellular antioxidant. GSH is synthesised from glutamate, glycine and cysteine (all AAT dependent). Kuster et al. identified glutamate, glycine and cysteine concentrations rapidly deplete in murine acinar cells during pancreatitis, with levels of free GSH (oxidised and reduced) diminished. Gene expression levels of the acinar glutamine transporters LAT1 (slc7a5), LAT2 (slc7a8), SNAT2 (slc38a2), SNAT3 (slc38a3) and SNAT5 (slc38a5), as well as the glycine transporter GlyT1 (slc6a9) were found to be impaired during pancreatitis [161].

The results from this chapter support previously cited evidence, but there was insufficient evidence to suggest FACBC is more sensitive or provided more information than standard CT. Using a larger sample size and use of a control organ to generate a baseline for activity may improve the sensitivity and utility of this imaging technique, but the current data show no differences between groups within the first 7 days post-transplant.

One consideration for the lack of difference in this first scan may be the introduction of uncontrollable confounders such as the day of transplant. As FACBC scanning was limited to once a week (restricted by the need to manufacture the radio-isotope), this had the effect of patients being scanned from day one to day seven post-transplant. Eliminating this variation may show improved utility of the first scan and so should be considered for future repetition.

Another limitation was not having background activity to act as a control and allow comparison. It became evident that variability between allografts from different donors may also account for differences in AAT. For future studies, using a native organ benchmark to

help identify changes would be helpful in assessing changes more easily. Potentially, repeating the scan to either include the native pancreas or liver in the images which are not expected to be inflamed may be a useful control however, it would require a larger radiation dose.

Oka et al. studied FACBC uptake mechanisms in inflammatory and tumour cells using rat leucocytes (T-cells, B-cells, granulocytes, macrophages), prostate cancer (MLLB2) cells, and glioma (C6) cells in vitro. FACBC uptake ratios of T/B cells to tumour cells were comparable, while granulocytes/macrophage uptake was lower. Authors concluded that low FACBC accumulation in granulocytes/macrophages may be advantageous in discriminating inflamed regions from tumour regions and that significant FACBC uptake in T/B cells may cause false-positives in patients who undergo FACBC-PET [160]. With flow cytometry results showing an increase in classical monocyte after 24 hours following reperfusion and higher macrophages in patients with allograft pancreatitis, this may explain why FACBC results showed reduced activity on the second scan in patients with pancreatitis.

The humanised monoclonal antibody alemtuzumab (Campath-1H) is directed against the cell surface antigen CD52, a 12-amino-acid glycoprotein tethered to the outer layer of the cell membrane via a glycosylphosphatidylinositol anchor. CD52 is expressed at high levels on the surface of T and B lymphocytes and at lower levels on natural killer cells, monocytes, macrophages and dendritic cells. There is little or no CD52 on neutrophils, plasma cells or bone marrow stem cells [162].

Alemtuzumab was used as part of induction immunosuppression, with results showing depletion of T-cells and B-cells within 24 hours of reperfusion, while the effect on neutrophils and NK cells was less dominant. The transient rise of neutrophils seen within

the first 24 hours may be due to post-surgical changes or the single dose of steroids given during transplantation causing transient neutrophilia. However, with no change observed between patients with and without pancreatitis, the role of neutrophils in the initiation of allograft pancreatitis may be less important and requires further study.

NK cells levels were higher in patients without pancreatitis while being reduced in patients with allograft pancreatitis following 96 hours reperfusion. There is some evidence to suggest that recipient NK cells can reduce the inflammatory burden immediately following transplantation by killing donor leucocytes and stimulating graft tolerance (recipient NK cell killing of donor dendritic cells) [163] [164]. One hypothesis to be considered is whether allograft pancreatitis may be due to the presence of low NK cell populations – this warrants further investigation.

Patients with allograft pancreatitis were found to have increased number of macrophages and classical monocytes while numbers of non-classical monocytes were reduced, compared to patients without pancreatitis. Human monocytes comprise a heterogeneous population that can be classified into three subsets based on CD14 and CD16 expression. Classical monocytes ($CD14^{+}CD16^{-}$) express high levels of the chemokine receptor CCR2 and can migrate to sites of injury and infection where they differentiate into inflammatory macrophages. Non-classical monocytes ($CD14^{low}CD16^{+}$) have high levels of the adhesion-related receptor CX3CR1 and exhibit a unique ability to patrol the resting vasculature and remove debris. In addition, the third subset, intermediate monocytes $CD14^{+}CD16^{+}$, have been identified and possess some inflammatory characteristics [165] [166].

Ong el al. assessed the basal cytokine secretion profile of the three monocyte subsets, where the classical subset secreted the lowest levels of many inflammatory cytokines [167]. If the predominance of classical monocytes is important in allograft pancreatitis, the effect of alemtuzumab on the various subsets of monocytes may be variable, with classical monocytes possibly being resistant to the effects of immunosuppression. This may explain the varying allograft pancreatitis incidence in the literature, where alternative induction agents such as thymoglobulin (ATG) that has monocyte activity were used.

Mayer et al. prospectively measured 51 native pancreatitis patients (16 mild, 35 severe) ,based on Atlanta classification, admitted within 72 hours after symptom onset. Results identified IL-10, and IL-6 were significantly elevated in patients with distant organ failure, while an imbalance in IL-1β /IL-1RA was found in severe AP [168]. Native pancreatitis cytokine concentration ranges have been compared with those found in allograft pancreatitis and are summarised in the table below (see Table 5.7.1).

Table 5.7.1: IQR values taken from Tables 4 and 5 (rows 1-4) combined with IQR values from allograft pancreatitis results. Source: [168]

	IL-1β (pg/ml)	**IL-6 (ng/ml)**	**IL-8 (pg/ml)**	**IL-10 pg/ml)**
Severe AP (n= 35)	3.6-8.2	347-1680	73-158	11-55
Mild AP (n=16)	0.1-4.4	107-443	59-678	0.2-4.9
Survivors (n= 38)	1.1-5.8	237-1100	46.9-138	4.3-14.9
Non-survivors (n=13)	4.9-16.9	983-1760	152-585	21.9-64.6
Graft Pancreatitis (n= 5)	61-93	41050-62410	19-51	18-81
No Graft Pancreatitis (n= 5)	20-51	80300-123030	20-52	4-10

Further cytokine comparison between native and allograft cytokine results have been made using data from Kilciler et al. [169] (see Table 5.7.2).

Table 5.7.2: IQR taken from Table 2 (rows 1-3) combined with IQR values from allograft pancreatitis results. Source: [172]

	IL-2(pg/ml)	IL-4(pg/ml)	TNF-α (pg/ml)
Post ERCP Pancreatitis	12-19	29-63	19-40
Non ECRP Pancreatitis	11.4-13	37-50	18-50
No Pancreatitis	12-15	30-90	19-45
Allograft Pancreatitis (n= 5)	156-181	100-111	40-74
No Allograft Pancreatitis (n= 5)	200-224	92-143	42-82

Allograft pancreatitis demonstrates a different cytokine profile to that reported in native pancreatitis, with significantly elevated concentrations compared to native disease (IL-1β IL-2, IL-4, IL-6, IL-10 and TNF-α), suggesting that the inflammatory stimulus is much larger. But if high cytokine levels suggest increased severity and more injury, why do only a proportion of patients go on to develop allograft pancreatitis?

When comparing results of patients with and without allograft pancreatitis, the results demonstrated no difference in CXCL-1, IL-2, CXCL-8, IFN γ, IL-7, IL-11, IL-12, IL-19, IL-21, IL-22, IL-27, IL28a, IL-34 or TNF-α. Additionally, significantly reduced concentrations of IL-4 ($p = 0.001$), IL-6 ($p = 0.001$), IL-18 ($p = 0.03$) and gp130 ($p = 0.05$) were observed in patients with allograft pancreatitis.

IL-1β (also known as mononuclear cell factor, and produced by classical monocytes) was found to be significantly elevated in patients with allograft pancreatitis ($p = 0.003$), while MMP-3 was found to rise within the first 24 hours, with IFNβ rising after 24 hours in patients with allograft pancreatitis. The evidence below attempts to explain these patterns and build a hypothesis.

MMP-3 degrades collagen, proteoglycans, fibronectin, laminin and elastin as well as activates other MMPs such as MMP-1, MMP-7, and MMP-9, rendering MMP-3 crucial in connective tissue remodelling [170]. MMP-3 has been implicated in exacerbating traumatic brain injury (TBI) through disruption of the blood-brain barrier (BBB), where MMP-3 production is increased. This stimulates membrane permeability allowing more cell infiltrate into the brain and spinal cord at sites of inflammation [171]. MMP-3 has also shown some evidence of disrupting pancreas membrane permeability in native pancreatitis [172]. IFNβ also promotes monocyte infiltration into tissue in the context of multiple sclerosis [173]. Considering high MMP-3 and IFNβ in allograft pancreatitis, this may increase membrane permeability allowing cell infiltration (classical monocytes) causing pancreas injury.

Anti-inflammatory IL-4 was reduced in patients with allograft pancreatitis. IL-4 induces differentiation of naïve helper T cells to TH_2 cells, resulting in positive feedback. It also promotes macrophage activation into M2 while inhibiting classical M1 activation. M1 macrophages produce pro-inflammatory cytokines initiating an immune response, while M2 macrophages are associated with wound healing and tissue repair [174]. T-cell depletion following immunosuppression would prevent positive feedback amplification of IL-4 and would remove the inhibition against M1 activation. This may further contribute to the elevated number of macrophages seen in allograft pancreatitis and requires further study.

The relationship between IL-1β and IL-6 is complex. There is evidence to show the pro-inflammatory stimulus of both IL-6 and IL-1β, with IL-6 increasing IL-1β, and also IL-6 reducing IL-1β through anti-inflammatory effects on IL-1ra [175]. At the same time, IL-1β can increase IL-6 [176], while high level expression can also reduce IL-6 [177].

Patients with allograft pancreatitis had reduced IL-6 concentrations (about half the concentration) compared to those without pancreatitis. But all transplant patients had 40 -100 fold more IL-6 than severe native pancreatitis patients. IL-6 uses gp130, also known as IL-6 signal transducer (IL6ST), in its signalling complexes. Other cytokines that signal through gp130 include IL-11 and IL-27 [178], both of which showed no change between patients with and without allograft pancreatitis. Interestingly, gp130 concentrations were also reduced (maybe expectantly in the context of reduced IL-6) in patients with allograft pancreatitis.

IL-6 is known to switch differentiation of monocytes from dendritic cells to macrophages. The amplification of IL-1β secreting monocytes and macrophages may negatively inhibit further IL-6 production leading to the lower levels observed, explaining why patients with allograft pancreatitis have increased macrophages but reduced IL-6 [179].

A key component of the inflammatory reaction seen in pancreatitis is the assembly of cytosolic sensor proteins (Nod-like receptors) forming the 'inflammasome' complex. This structure includes caspase-1, which cleaves pro-forms of cytokines leading to the subsequent release of IL-18 [180]. IL-18 concentrations were reduced for the first 24 hours in patients with allograft pancreatitis and then began to rise, becoming higher in patients with pancreatitis by two weeks. IL-18 is a member of the IL-1 family of cytokines. Similar to IL-1β, IL-18 is synthesised as an inactive precursor requiring processing by caspase-1 before its active. However, unlike IL-1β the IL-18 precursor is present in nearly all human cells [181].

Wereszczynska-Siemiatkowska et al. evaluated the profiles of IL-18, glutathione peroxidase (GPx), and selenium concentrations in serum of 61 patients with pancreatitis compared with 15 healthy controls. In this study, patients with pancreatitis were found to have

significantly elevated IL-18 concentrations and significantly lower GPx concentrations, with ROC analysis showing the high prognostic accuracy of IL-18 and GPx concentrations in the determination of AP severity [182].

The results of this chapter suggest that IL-18 plays a significant role in patients with allograft pancreatitis after 24 hours, which may be due to the time needed to form the inflammasome complex and activate IL-18. This may also be another reason why concentrations of IL-4 and IL-6 were lower, as also require activation, or it may be possible that the reverse is happening and allograft pancreatitis enzyme release may be promoting cytokine degradation. What is evident is that the high levels of IL-18 seen at two weeks, when the second FACBC scan was performed (showing reduced activity in patients with allograft pancreatitis) making it possible that GPx concentration, although not measured, may also be decreased (supporting the concept seen by Wereszczynska-Siemiatkowska et al).

IL-10 has shown a variable association with tissue injury and severity of pancreatitis. Pezzilli et al. identified healthy subjects as having undetectable IL-10 levels while in AP, serum IL-10 levels were increased on the first day of the disease and then progressively decreased in the following days. This implies that an effective anti-inflammatory response early on in the course of the disease may help in reducing its severity [183]. It has also been shown that IL-10 inhibits activated macrophages to produce cytokines [184]. Results in this chapter identified a rise of IL-10, which remained elevated in patients with allograft pancreatitis, may suggest that the elevation of IL-10 may be in response to early inflammation to increased macrophage number.

Current Banff criteria for grading pancreas allograft rejection also highlights inflammatory changes as part of the process of rejection [185]. The pattern of high classical monocyte, high macrophage and low NK cell numbers seems to be present in patients who suffer from allograft pancreatitis and is associated with a unique cytokine profile revealed in this work. Chapter four identified that patients with allograft pancreatitis are twice as likely to experience early graft loss compared to those without allograft pancreatitis. Considering these results, graft loss may be partly due to increased risk of allograft rejection as a result of early monocyte/macrophage inflammatory infiltration.

CHAPTER 6:

Future avenues for research and conclusions

6.1 Future work

There are key challenges facing pancreas transplantation. First, the morbidity of pancreas transplantation needs to be low enough to justify its application in a much larger cohort of patients with severe complications of diabetes (including those who have not yet developed renal failure). This requires new therapeutic strategies to reduce the incidence/severity of allograft pancreatitis following reperfusion. Second, strategies are needed to monitor the pancreas in the long-term, based on real-time data that informs clinicians of the state of the pancreas and allows effective assessment of these strategies.

Novel strategies are needed for both quantifying the allograft and associated pancreatitis as well as looking for solutions to reduce its burden. Some of the tools identified in the thesis create the potential for new prospective studies to help answer some of the questions raised in this work.

6.1.1 What is the threshold of acceptable pancreas steatosis?

Given that all pancreases have some degree of steatosis, it is important to identify a threshold above which the risk of post-transplant morbidity becomes excessive (whether or not this is deemed to be 'pathological' in the non-transplant setting).

The association between liver and pancreatic steatosis (and that between NAFPD and NAFLD) may be important, as well as the positive association of the rs738409 C/G polymorphism in donors >40years. A prospective study to compare liver and pancreas steatosis will help build evidence on this issue. This might be based upon liver and pancreas biopsies. To support this, 25 concordant liver samples through the QUOD biobank have been identified for potential correlation for potential future analysis based on the results in this

thesis. However, in view of the heterogeneous distribution seen using histology, considering a prospecitive imaging study may be more appropriate.

6.1.2 How to quantify steatosis and relate it to outcome

The use of MRI gives the opportunity to perform a prospective series of pancreas transplants, where steatosis can be quantified accurately and linked to outcome (this was a limitation of this thesis). Being able to assess whether and at what level of steatosis is actually adversely associated with the outcome following pancreas transplantation, might revolutionise clinical practice and organ utility, potentially allowing more transplants to be performed as well as considerably reducing waste compared to current methods of assessment.

6.1.3 Evaluation of a trial end point to assess allograft pancreatitis

CRP was shown to be a useful biomarker in chapter four but it has limitations. Repeating studies using a model of pancreas transplant alone (rather than SPK) and limiting cofounders to confirm findings is important. The consideration of donor derived cell free DNA (dd-cfDNA) is also a future study that may be of use. There is extensive evidence showing the rise of dd-cfDNA being associated with allograft injury and rejection in kidney and heart transplantation [186]. Quantification of free DNA fragments in plasma samples may show that increased inflammation is associated with increased allograft damage hence, being able to quantify dd-cfDNA may provide very useful insights into allograft pancreatitis.

6.1.4 How to further assess the immunology of allograft pancreatitis

Many of the mechanisms and pathways identified for allograft pancreatitis were based upon assumptions from native disease and animal models, but lack of evidence for allograft

pancreatitis makes it difficult to postulate whether the pathways are similar. Results from this thesis have identified predominant cell populations (such as macrophages and classical monocytes) which may be responsible for injury as well as a unique cytokine profile, which may be the key in inflammation. Limitations on sample size prevent a definite conclusion and therefore, further investigation on a larger population is needed.

6.1.5 Assessment of allograft pancreatitis tissue

Another potential avenue for research is to consider the examination of explanted pancreas tissue where organs have been removed due to pancreatitis. Assessing whether allografts contain high proportions of classical monocytes and macrophages that are infiltrated into the organ would help confirm the mechanism of injury, or provide new information into what tissue changes are occurring in allograft pancreatitis. A series of 45 explanted organs have been identified and a study is currently being designed to continue work in this area.

6.1.6 Design of future study – safety and efficacy of tofacitinib in ameliorating ischaemia reperfusion injury and allograft pancreatitis

This proposed study has been approved by the MHRA, REC, OUH hospital R&D team and has radiology IRMER approval. The study has received funding through the Oxford BRC (Diabetes and Metabolism theme) and is planned to begin recruitment shortly. Copies of the protocol and relevant approvals for this trial are provided in the appendix (see Appendix 3 and 4). The implementation of this study was beyond the time-scope of this thesis but its rationale has been described below.

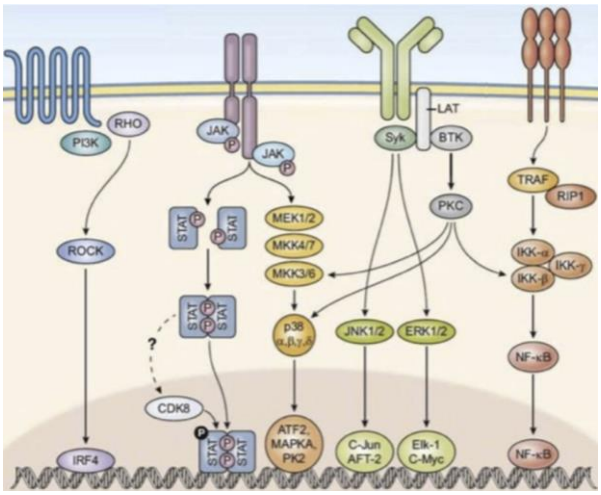
6.1.6.1 Kinase overview

Kinases are critical in metabolism, cell signalling, protein regulation, cellular transport, secretory processes, and many other cellular pathways, which makes them important to human physiology [187]. There are 518 kinases in the human genome, functioning widely across many cellular processes. Tyrosine kinases are one major subgroup of protein kinases while others, attach the phosphate moiety to other amino acids (serine and threonine) [188]. Tyrosine kinases have attracted the greatest attention so far as potential therapeutic targets, especially in oncology and rheumatology, and can be divided into two sub-groups:

a) The receptor kinases (RK) contain an extracellular ligand-binding domain that is connected to the cytoplasmic catalytic domain by a transmembrane helix, and are activated by various growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) [189].
b) Non-receptor cytoplasmic kinases (NRK) lack the extracellular component and are located in the cytosol, nucleus or the inner surface of the plasma membrane. Major NRKs are those linked to sarcoma (SRC), Janus-associated kinase (JAK) and tyrosine kinase (from the oncogenic transcript, which results from the fusion of the ABL gene and breakpoint cluster region gene (BCR-ABL)) [190].

An overview of the main kinase signal pathways can be seen in Figure 6.1.6.1.1.

Figure 6.1.6.1.1: Overview of kinase signal pathways, including protein kinases such as SYK, PKC, MAPK, JNK, ROCK and JAK. Image source [191].



6.1.6.2 Kinase inhibitors in ischaemia reperfusion

The various mechanisms participating in the development of IR have been already described earlier in this thesis but appreciating the ubiquitous presence of kinase-controlled mechanisms involved in cytokine driven inflammation, makes this an attractive target for pharmacological intervention. Several kinase family members have been identified as potential pharmacological targets for the treatment of IR injury, with much of the work done in this area being experimental using animal models. Some of the key studies have been outlined below.

Studies within myocardial ischaemia have shown that Rho-kinase inhibitors reduce IR injury, are cardio protective and reduce cytokine production. However, the full role of Rho-kinase in leukocytes during IR injury is not well understood [192]. Kitano et al. studied mice that were subjected to 30min IR injury through arterial clamping. Rho-kinase activity was found to be significantly increased in leukocytes subjected to IR injury compared to the sham

controls. Administration of fasudil, a Rho-kinase inhibitor, significantly reduced IR expression of the pro-inflammatory cytokines interleukin IL-6, C-C motif chemoattractant ligand 2 (CCL2), and TNF-α in leukocytes, compared with saline [193].

In liver transplantation, non-alcoholic steatohepatitis (NASH) was shown to aggravate IR injury possibly by activating various kinases and subsequently, releasing cytokines and chemokines. Yang et al. demonstrated that sorafenib, an inhibitor of several TKs, protected NASH rats from IR injury by inhibiting inflammation, necrosis, and apoptosis. The hepato-protective effects of sorafenib are thought to work through the inhibition of the Rho-kinase-dependent Raf/MEK/ERK pathway, which is up-regulated during IR injury in the livers of NASH rats and show the value of considering kinase inhibitors in the treatment of IR [194].
In lung IR animal models, mitogen-activated protein kinases (p38, c-jun N-terminal kinase, extracellular signal–regulated kinase) are activated after oxidative stress. Wolf et al. has shown using an experimental rat model, that the inhibition of p38 and c-jun kinase provided significant protection from injury [195].

Similar animal studies have been performed in liver transplant rat models where another kinase inhibitor called sotrastaurin, prevented T lymphocyte activation via a calcineurin-independent pathway. This is of particular appeal as it has also been documented that the role of activated T cells in the inflammatory cascade leading to liver IR injury is critical. This study explored the cytoprotective functions of sotrastaurin in a clinically relevant rat model of hepatic cold ischemia, followed by orthotopic liver transplantation (OLT). Livers from Sprague-Dawley (SD) rats were stored for 30 hours at 4°C in UW solution and then transplanted to syngeneic recipients. Sotrastaurin treatment of liver donors/recipients or recipients, only prolonged OLT survival to >90% (vs. 40% in controls), decreased

hepatocellular damage and improved histological features of IR injury. Additionally, sotrastaurin treatment decreased activation of T-cells and diminished macrophage/neutrophil accumulation in OLTs. These beneficial effects were accompanied by diminished NF-κB/ERK signalling, depressed pro-apoptotic cleaved caspase-3, yet upregulated anti-apoptotic Bcl-2/Bcl-xl, and hepatic cell proliferation [196].

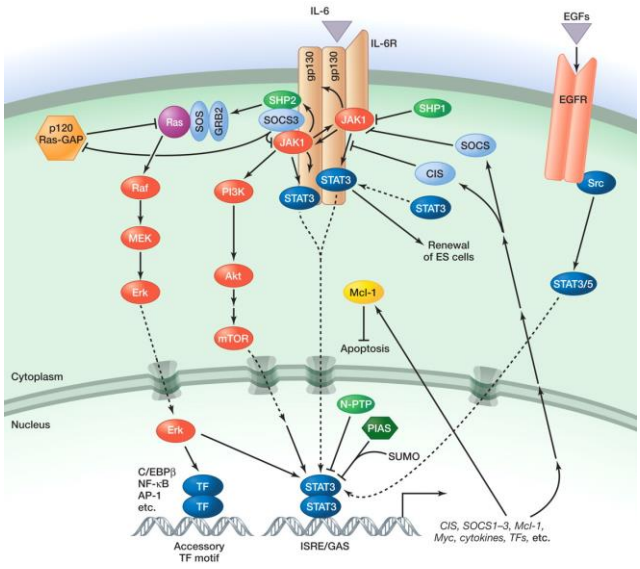
Young Dong et al. investigated the use of nafamostat mesilate, which inhibits various serine proteases and mitogen-activated protein kinase (MAPK) generated during inflammation, and has recently made the transition from experimental animal studies to a clinical single arm pilot study. Nafamostat meslilate is widely used in Asia as an anti-inflammatory and anticoagulant agent. In this study, 10 human livers were flushed with a solution containing nafamostat mesilate following graft perfusion using standard Histidine-tryptophan-ketoglutarate (HTK) solution, and then transplanted into 10 patients who underwent deceased donor liver transplantation. A reduction in peak AST and ALT levels was observed compared to standard practice, suggesting the impact of IR may have been reduced [197].

6.1.6.3 Janus Kinase (JAK) as a potential therapeutic target

The JAK family in humans consists of four members (JAK1, JAK2, JAK3 and tyrosine kinase 2(TYK2)). The activation of JAK occurs by a ligand-receptor interaction that results in signalling through the phosphorylation of cytokine receptors and the creation of docking sites for a family of signalling proteins known as signal transducers and activators of transcription (STATs) [198]. Phosphorylated STATs dimerise and then migrate to the nucleus where they become part of transcriptional regulatory machinery and lead to transcription of responsive genes. Under normal physiological conditions, the two phosphate-transferring

domains ensure that ligand-dependent activation of the JAK/STAT signalling is transient and tightly regulated [199] (see Figure 6.3.1).

Figure 6.1.6.3.1: The JAK-STAT pathway. Image source [200].



The importance of JAKs in vivo was first identified in knockout mice models and through demonstration of JAK3 mutations in patients with immunodeficiency. Mutation of JAK3 results in a severe combined immunodeficiency (SCID) characterised by an almost complete absence of T cells, NK cells and defective B cells [201]. In contrast with other ubiquitously expressed JAKs, JAK3 is primarily expressed in haematopoietically derived cells where it is associated with the IL-2 receptor common γ chain and mediates signalling by IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (these being the cytokines that are critical for the development and maturation of T cells) [202]. Importantly, Yarilina et al. have shown that synovial macrophages in rheumatoid arthritis are downregulated through the use of JAK inhibition, suggesting it may be beneficial to reduce the effect of classical monocyte and macrophage as shown in chapter five [203].

6.1.6.4 Tofacitinib clinical transplant studies

In 2008, a dose-escalation study in human renal allograft recipients recruited 28 patients; six patients received tofacitinib 5mg twice daily, six patients received tofacitinib 15mg twice a day, 10 patients received tofacitinib 30mg twice a day, and six patients received placebo. The most common adverse events observed in the study included infection and gastrointestinal symptoms (abdominal pain, diarrhoea, dyspepsia, and vomiting). Higher doses of tofacitinib were associated with anaemia and decreased natural killer cell counts (50%), mirroring what had been observed in earlier primate studies, yet numbers of neutrophils, platelets and T helper cells remained unchanged [204].

A subsequent randomised pilot study comparing tofacitinib (15mg and 30mg, n = 20 in each group) vs tacrolimus (n = 21) in de novo renal allograft recipients was then initiated in 2009. Patients, additionally, received an IL-2 receptor antagonist, MMF and corticosteroids, which are all part of current standard treatment regimes in kidney transplantation.

Tofacitinib doses were reduced following 6 months of therapy due to the high incidence of BK virus nephropathy in patients treated with higher-dose of tofacitinib, and MMF was also discontinued in this group. The 6-month biopsy-proven acute rejection rates were one out of 20, four out of 20, and one out of 21 for 15mg tofacitinib, 30mg tofacitinib and tacrolimus groups respectively. Results demonstrated that low dose tofacitinib had equivalent efficacy to tacrolimus, but that the higher dose was associated with clinically relevant infections. This data suggested that the co-administration of 30mg BD tofacitinib with MMF was associated with over-immunosuppression, with the incidence of infection being higher and more significant as tofacitinib doses were escalated. At the 15mg twice-daily dose, the efficacy and safety profile produced equivalent results to the current standard [205].

6.1.6.5 Subsequent tofacitinib use

Despite being originally intended for transplant patients, its use as a primary immunosuppressant in transplantation was not pursued due to a commercial decision by Pfizer. The FDA has since licensed tofacitinib under the trade name of Xeljanz for the treatment of rheumatoid arthritis [206] and since that time, its role as an anti-inflammatory has been exploited in the treatment of psoriasis [207], inflammatory bowel disease [208] as well as other autoimmune disorders such as vitiligo [209] and alopecia [210].

6.1.6.6 Kinase inhibition and tofacitinib in allograft pancreatitis

Solid organ pancreas transplantation is a highly relevant clinical environment in which to examine the potential therapeutic benefit of abrogation of IR injury and allograft pancreatitis, as shown in chapter four.

Balachandra et al. investigated the use of the tyrosine kinase inhibitors, tyrphostin AG126 and AG556 in a murine model of acute pancreatitis. After the induction of pancreatitis through intraperitoneal injection of cerulein, mice were treated with tyrphostin AG126 and AG556. Significantly reduced inflammation and tissue injury, reduced cerulein-induced nitrotyrosine formation and PARP activation within the pancreas was observed, suggesting that kinase inhibition reduced the development of acute pancreatitis [211].

Oxidative stress during pancreatitis has shown to activate the JAK/STAT pathway and cytokine expression. High serum levels of cholecystokinin (CCK) and cerulean (CCK analogue) have been reported in patients with acute pancreatitis, with cerulean also shown to induce acute pancreatitis in a rodent model [212]. Studies have shown that cerulein-activated NADP oxidase elicits ROS, which trigger the phosphorylation of the JAK1, STAT1, and STAT3

proteins. This then induces the production of inflammatory cytokines, including TNFα, interleukin IL-1β, and IL-6, in pancreatic acinar cells. The JAK/STAT pathway is also known to influence cell proliferation and inhibit apoptosis in the pancreas, which may also be important during pancreatitis [213].

Of the four JAK family members, JAK1, JAK2 and TyK2 are ubiquitously expressed and found within the pancreas [213]. A summary of the broad effect of tofacitinib can be seen in Figure 6.1.6.6.1.

Figure 6.1.6.6.1: Key cytokines signaling through JAK/STAT combinations. Type I and II cytokine receptors lack intrinsic enzymatic activity and utilise associated JAK combinations to signal. γ-chain cytokines: IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Type II cytokine receptors such as those for the gp130 subunit sharing receptors IL-6 and IL-11 as well as IL-10, IL-19, IL-20 and IL-22 mainly signal through JAK1 but also associate with JAK2 and TYK2. Other abbreviations in image: EPO: Erythropoietin; GM-CSF: Granulocyte/macrophage colony stimulating factor; TGF: Transforming growth factor; TPO: Thrombopoietin. Image source [214].



Tofacitinib has been tested extensively in the context of transplantation and in many other inflammatory conditions since its inception, at doses between 5mg and 30mg given twice daily to patients. Having considered all the evidence relating to dosing, side-effects and safety, an intermediate dose (10mg BD) restricted to 7 days was settled upon for this pilot study. The proposal for short-term administration is based on the hypothesis that the inflammatory stimulus in allograft pancreatitis following reperfusion is relatively short-lived, and by avoiding long-term administration should prevent the side-effects seen in other trials associated with higher dose longer term administration regimes.

Based on the results from chapter four and five, day five CRP is the preferred primary end point for intervention, to be used in combination with day five CT imaging. Further cytokine analysis dd-cfDNA and flow cytometry results are planned to be used as secondary end points to further build on the evidence of chapter five.

This pilot study plans to enrol 20 patients undergoing SPK transplantation who will be given tofacitinib twice a day. The outcomes will be compared to the 10 control patients who were included in the FACBC PET CT study from chapter five. (REC ref: 17/SC/0133). A copy of the trial protocol is available in Appendix 3.

6.2 Thesis summary

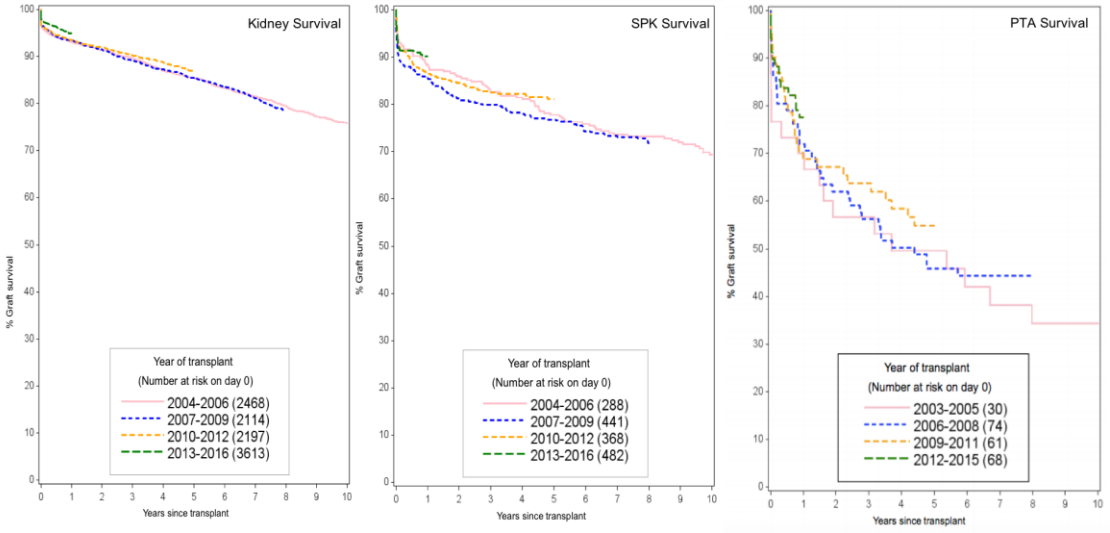
Pancreas transplantation (solid organ or islet) is currently the only way to completely reverse the metabolic effects of diabetes. With the most sophisticated insulin pumps and feedback control mechanisms having yet to prove equal efficacy and currently do not yet address the issue of glucagon. To date, the application of transplantation in the management of diabetes has been restricted to those patients with two of the most severe

and life-threatening complications of diabetes, namely renal failure and hypoglycaemia-unawareness. The limiting factor that has restricted transplantation of these patients with diabetes is the risk of the procedures themselves first and secondly, the effect of long-term immunosuppression.

The morbidity associated with solid organ transplantation is therefore a major factor in the decision as to which patients should undergo transplantation. Pancreas transplant clinicians have struggled for decades to achieve the best balance of clinical risk/benefit, but there remains a fundamental paradox: to achieve the maximum benefit of a functioning pancreas transplant, the procedure should be carried out early, before the complications of diabetes have progressed to the point where these are irreversible (e.g. retinopathy, neuropathy, nephropathy) and before the risk of surgery increases. However, current morbidity rates do not justify placing patients at risk at an earlier phase of their disease, especially whilst current graft survival rates are inferior to those of kidney alone transplantation. When comparing national one year graft survival data (median and IQR), statistics from NHSBT have highlighted the difference between kidney and pancreas transplantation.

Current DBD kidney alone one year graft survival is currently 95% (IQR 94-96%) compared to one year DBD SPK graft survival at 88% (IQR 85-91%) and 78% (IQR 66-86%) for DBD PTA grafts. NHSBT Kaplan Meyer curves in Figure 6.7.1 show that almost all of the difference occurs within the first 6 months following transplant, confirming that early morbidity has a large impact on outcome (see Figure 6.7.1). Additionally, chapter four highlighted the difference in graft survival between patients with and without pancreatitis, with one year SPK survival reduced to 81.2% in patients with pancreatitis compared to 91.3% in patients without, showing the detrimental impact early morbidity has on graft survival.

Figure 6.2.1: Analysis of NHSBT survival graphs of kidney and pancreas transplants show clearly that there is a difference in graft survival between kidney, SPK and PTA transplants. Image source [215].



In chapter two, I showed the falling transplant rate observed in the UK that seems to mirror findings seen elsewhere. This has been associated with increasing donation rates (including of donor organs within nationally agreed parameters) and a national transplant waiting list which has declined in recent years, implying that the transplant rate is primarily driven by clinical demand and rate of referral rather than donor supply.

The current practice and the threshold to offer pancreas transplantation is based on a balance of risk: short-term complications such as allograft pancreatitis outweigh the long-term benefits. In order to mitigate short-term risk, transplant teams are anxious to avoid organs that they believe are more likely to develop pancreatitis with the severe morbidity that this incurs.

Donated pancreases are much less likely to be transplanted than any other abdominal organ, and for every discarded pancreas there is a trail of time, effort and cost. Importantly,

the increase in offers and increased rate of organ decline in recent years has escalated the workload but not the utilisation.

There is an urgent need to better understand what criteria are important when considering pancreas offers, especially when considering ways to reduce the incidence of allograft pancreatitis. Improving documentation and the recording of more relevant information to NHSBT is essential. Only then will the combination of donor/recipient factors that should influence the decision to accept or decline pancreases be uncovered.

There is a consensus that allograft pancreatitis is heavily influenced by the characteristics of the donor, with a number of donor factors having been implicated in poor outcomes, and incorporated in the various donor risk indices that have been published. Although logically, it is likely that there are both donor and recipient factors involved in determining the severity of an inflammatory response.

The issue of pancreatic steatosis is one such parameter, and has been the basis of a lot of the work described in this thesis. Both chapter one and two, touched on the conflicting published data regarding BMI and pancreas transplant outcomes. The suggestion that donors with higher BMIs are more likely to have increased pancreas steatosis, causing allograft pancreatitis remains contradictory. This highlights the need for a more robust, accurate and quantifiable assessments of steatosis.

Further to this, the imprecise use of the term steatosis and confusion as to the relevance of intra-pancreatic (parenchymal) fat versus extra-pancreatic fat, has contributed to the indecision when considering how to tackle the topic. Chapter three, clearly demonstrated

that the use of BMI as a surrogate marker for steatosis is inaccurate and inappropriate. Firstly, visual inspection only estimates external fat and not internal fat which would represent true steatosis. Secondly, there is considerable heterogeneous distribution of steatosis within pancreases offered for transplantation, as well as the large variations between organs from donors with similar demographics. These findings confirm that current assessments of steatosis and the interpretation of the data are not fit for purpose.

Even the previously presumed gold standard of tissue biopsy (not viable for organs to be transplanted) does not fully capture the focal nature of steatosis within the human pancreas. What was evident was that the presence of pancreas steatosis is common and is a spectrum.

When attempting to find alternative methods to assess steatosis, adiponectin although having a relationship with steatosis did not provide information regarding its distribution. Electrical impedance tomography (EIT) like macroscopic assessment, failed to differentiate internal steatosis from external fat with only MRI able to providedany quantitative measure.

Another striking outcome of this work, after uncovering these findings and re-examining the literature, is to consider whether pancreatic steatosis truly causes allograft pancreatitis. This remains uncertain and the thesis provides a strong rationale to analyse further the true significance of pancreatic steatosis.

Chapter four identified that allograft pancreatitis was associated with inferior graft survival, supporting already published work by Baidal et al. in islets and more recently by Mittal et al. in whole organ pancreas transplant. These publications highlighted early graft

dysfunction being associated with impaired oral glucose tolerance tests and early HbA1c impairment, having increased graft loss in the longer term [216] [217]. If allograft pancreatitis is also a cause of early pancreas impairment, the change in HbA1c may be another possible marker to consider when monitoring effectiveness of intervention studies.

The data presented builds a case for further research on pharmacological strategies to prevent or ameliorate allograft pancreatitis. Currently no identified donor or recipient risk factors have prognostic value in predicting graft pancreatitis and the current scoring systems such as PDRI have very low utility, making an effective to identify patients at risk of pancreatitis valuable.

It is my hypothesis that effective suppression of allograft pancreatitis, through suppression of inflammation using tofacitinib would have a beneficial effect on reducing the early loss of transplanted organs. This would improve graft survival and patient outcomes, putting pancreas transplanttion in line with kidney transplantation.

In line with previous publications, lipase was found not to be useful in the early post-operative period, possibly because there is much complexity and redundancy within the host inflammatory response. However, CRP did appear to be a clinically useful marker that may represent a useful surrogate for allograft pancreatitis despite it being a non-specific inflammatory marker. The positive correlation, high sensitivity and specificity observed from the day five measurement demonstrates the value of using CRP as a tool to help identify patients.

Chapter four and five described the use of flow cytometry to identify which of the key cell types may be involved in initiating injury as well as providing some evidence into what cytokine pattern could be involved. Given the small sample size, the conclusions from these studies were limited, however a number of new questions were raised around the importance of classical monocytes and why did these appear not to be affected by the induction immunosuppression. Additionally whether the the cytokine profiles observed may be linked to the pattern of leucocytes seen remains a question for further study. Considering the work in these chapters had small samples, it is possible that the profiles observed could well be very different if repeated on a larger sample and so further study is necessary.

Chapter five also explored the utility of PET-CT using FACBC, and although this suggests that there is little to be gained from this alternative scan compared to standard CT, the hypothesis that AAT is altered in pancreatitis was shown to be correct and may be useful in future assessments. With evidence to show reduced serum GPx in patients with pancreatitis, the measurement of this should be considered as another potential way to assess effectiveness of future interventional studies. Understanding the importance of augmented AAT and FACBC uptake in the presence of allograft pancreatitis requires further investigation.

The ability to detect and then quantify pancreatitis is vital if new interventions are to be tested: new methodology is needed to provide the endpoints for clinical trials of novel therapies in this field. This methodology may then prove to be of broader value in the wider field of native (i.e. non-transplant) pancreatitis, an area in which specific therapies are lacking and badly needed. My selection of the JAK-inhibitor tofacitinib was based on a study

of its characteristics. Tofacitinib is a drug with a broader spectrum of activity than some of its competitor agents and should have a diverse effect against several targets that may be involved in allograft pancreatitis. The ability to abrogate inflammation will be pivotal in the future success of pancreas transplantation and it is my hope that the clinical trial that I have set up, but not been able to complete will be able to show the value of tofacitinib in this role.

Clearly, suppression of the inflammatory response has some dangers (inflammation is an essential stage in the recovery from surgery). In regards to the dose, I have studied the available clinical evidence in respect to the use and dose-response of tofacitinib in other patient groups and based on current evidence, I have selected a regimen that has been used in transplant patients without a major incidence of treatment-related complications.

6.3 Conclusions

6.3.1. Utilisation of organs

Pancreas organ shortage is not a vital issue, but optimising organ offers to reduce waste is essential. A large amount of information is not being captured through current NHSBT coding regarding offers and reasons for non use. Overhauling this system, improving granularity and focusing on strategies to standardise the decision making process between centres is essential.

6.3.2. Method of fatty and steatotic organ assessment

Fattiness and steatosis are not the same, and fattiness should not be used to infer steatosis. Current assessments of organs are unfit and BMI should not be used as a surrogate for

steatosis. Quantification of steatosis using MRI should become the new gold standard of assessment.

6.3.3. Recommendation for allograft pancreatitis identification

Day 5 CRP should be considered when identifying patients with allograft pancreatitis in combination with CT. This should perhaps become the endpoint of future trials.

6.3.4. Immunology of allograft pancreatitis and potential targets

The significance of monocyte, macrophages and NK cells in the context of allograft pancreatitis needs further evaluation. Considering their changes in potential trials aimed at abbrogating inflammation essential to improving outcomes and reduce graft loss.

6.4 Personal perspective

The work in this thesis is new, and although much of the scientific methodology is well established, in the context of pancreas transplantationit has generated new results, some of which have been negative. I believe will this thesis will help to provide a greater insight into the field of solid organ pancreas transplantation.

Over the last three years I have gained a new found respect for the work that goes into the ability of raising a number of in depth questions and considering various study designs when postulating how best to test and prove a hypothesis. Proving something is not always an easy task and as straight forward as it may seem, and this is something I have appreciated even more through this thesis. The work that goes into data collection, although very laborious, is just the beginning of a long journey where the analysis and interpretation continues to raise further questions. Finally, divingdeep into the outcomes to find a

reasonable meaning requires time and consideration and at times, leads to more questions than answers.

The learning in this jou rney has been very enriching; from learning many lab skills such as immunohistochemistry, flow cytometry, ELISA and DNA extraction, to manipulating technology in the interpretation of images including CT, MRI and PET, to learning how to scan slide sections and photographs harnessing the technology of digital pathology. Likewise, using the ideas from the scientific bench, I have attempted to develop and mature my understanding of these concepts through designing clinical pilot trials and collecting data from real patients. The work I have undertaken at Oxford has been the most challenging and most rewarding, providing a huge opportunity to develop a network of friends and colleagues across the country, and to share and develop new ideas. I have learnt from my entire Oxford experience, the importance of creating a strong scientific foundation on which I can build information and, at the same time, the power of thinking outside the box.

I have learnt the importance of staying curious and the patience needed to hold the course, or scrap the idea and start again. Using these skills to navigate the unknown over the last three years, I have become my own judge, and asked the dreaded question of 'so what', which has helped me steer a course to the next step.

APPENDIX 1:

NHSBT discard audit evaluation criteria

*delete non applicable terms ** tick as appropriate

Pancreas evaluation proforma

1. Macroscopic aspect

*Minimal/Moderate/Excessive fat infiltration in the pancreas

*Minimal/Moderate/Excessive fat infiltration around the pancreas

2. Consistency (as reported by examining consultant surgeon):

**Soft ☐ Hard ☐ Normal ☐ Abnormal ☐

3. Capsular damage

*Focal/Localised/Generalised

4. Length of portal vein to nearest millimeter (mm) ☐

**Adequate ☐ **Inadequate ☐ **Venous extension required ☐

5. Arterial damage

*None/Mild/Moderate/Severe **(unusable) ☐ **(can be reconstructed) ☐

**Arterial Traction damage ☐

**Arterial Dissection ☐

**SMA damage Splenic artery damage ☐

6. Arterial conduit damage

*None/Mild/Moderate/Severe **(unusable) ☐ **(can be reconstructed) ☐

7. Mesenteric staple level

**Adequate length ☐ **Too close to the root of mesentery ☐

8. Other comments: ☐

9. Would you use this graft for:

For chosen recipient *YES/NO
For someone else *YES/NO
Not suitable for anyone *YES/NO

If not reasons additional comments here:

☐

APPENDIX 2:

Flow cytometry fluorochromes

Flurochrome	Excitation Range	Emission Range	Source Emission	Description
Percp (Peridinin Chlorophyll Protein)	490nm max. absorption	677 m max. emission	Argon Ion LASER (488nm).	PerCP is a component of the photosynthetic apparatus found in the dinoflflagellate, Glenodinium sp. Molecular Weight is 35kDa. Fluorescence emission maximum is around 677nm. Usually 2-9 PerCP molecules per antibody. PerCP is highly sensitive to photobleaching.
FITC (Fluorescein Isothiocyanate)	468-509nm	504-541nm	Argon Ion LASER (488nm)	Molecular Weight of 389 daltons. Fluorescence emission maximum is around 520nm. Using a 530 + /- 15 nm band pass filter will give optimal detection. Usually 2-9 FITC molecules per antibody.
PE-Texas Red (PhycoerythrinTexas Red-X) – also known as ECD	486-575nm	610-635nm	Argon Ion LASER (488nm)	Texas Red is a sulfonyl chloride derivative of sulforhodamine 101. Molecular Weight is 625kDa. Excitation of Texas Red is about 595 (same as PE emission)

				and has a fluorescence emission maximum around 620nm. Using a 620 +/- 10nm band pass filter will give optimal detection.
APC (Allophycocyanin)	600-640 nm	660nm max.	Helium Neon LASER (635nm)	APC is an accessory photosynthetic pigment found in bluegreen algae. Molecular Weight is about 105kDa with 6 phycocyanobilin chromophores (which are similar in structure to the chromophore in R-PE) per molecule. Fluorescence emission maximum is around 660nm. Using a 660 + /- 10 n m band pass filter will give optimal dectection.
PE-CY5 (PhycoerythrinCyanin 5.1) – Also known as PC5, Cy-Chrome and Tricolor	486-575nm	660-680nm	Argon Ion LASER (488nm) Usually 1 PE-CY5 molecule per antibody.	PE-CY5 is a tandem conjugate system, combining R-PE (240 kDa) and a cyanine dye (1.5kDa). Very efficient energy transfer between R-PE and the Cyanine dye (little is lost as 576nm).

				Fluorescence emission maximum is around 670nm. Using a 650nm long pass filter will give optimal detection. Usually 1 PE-CY5 molecule per antibody.
PE-CY7 (Phycoerythrin Cyanin 7)	486-575nm	750-810nm	Argon Ion LASER (488nm)	PE-CY7 is a tandem conjugate system, combining R-PE and a cyanine dye. CY 7 has a fluorescence emission maximum around 767nm.
PerCP-CY 5.5	490nm max. absorption	695nm max.	Argon Ion LASER (488nm)	PerCP-CY 5.5 is a tandem conjugate system, combining PerCP and a cyanine dye. Excitation of CY 5.5 is about 675 (same as PerCP emission) and has a fluorescence emission maximum around 695nm.
APC-CY7	600-640nm	750-810nm	HeliumNeon LASER (635nm)	APC-CY7 is a tandem conjugate system, combining APC and a cyanine dye. CY 7 has a fluorescence emission

				maximum around 767nm.
BV711(Brilliant Violet)	407nm	711nm	Violet LASER 405 nm	Brilliant Violet family of dyes, offers an additional bright choice for the violet laser. The brightness of the dye makes it particularly useful in multicolor applications, in which it can be used to better resolve dim populations.
Alexa Fluor 700 Probes.	696nm	719nm	Helium-Neon LASER (635nm)	Alexa Fluor is a series of dyes from Molecular Probes. Ligand receptor CD3/TCR, CD4.

APPENDIX 3:

Safety and efficacy of tofacitinib in ameliorating ischaemia reperfusion injury and allograft pancreatitis – Trial Protocol

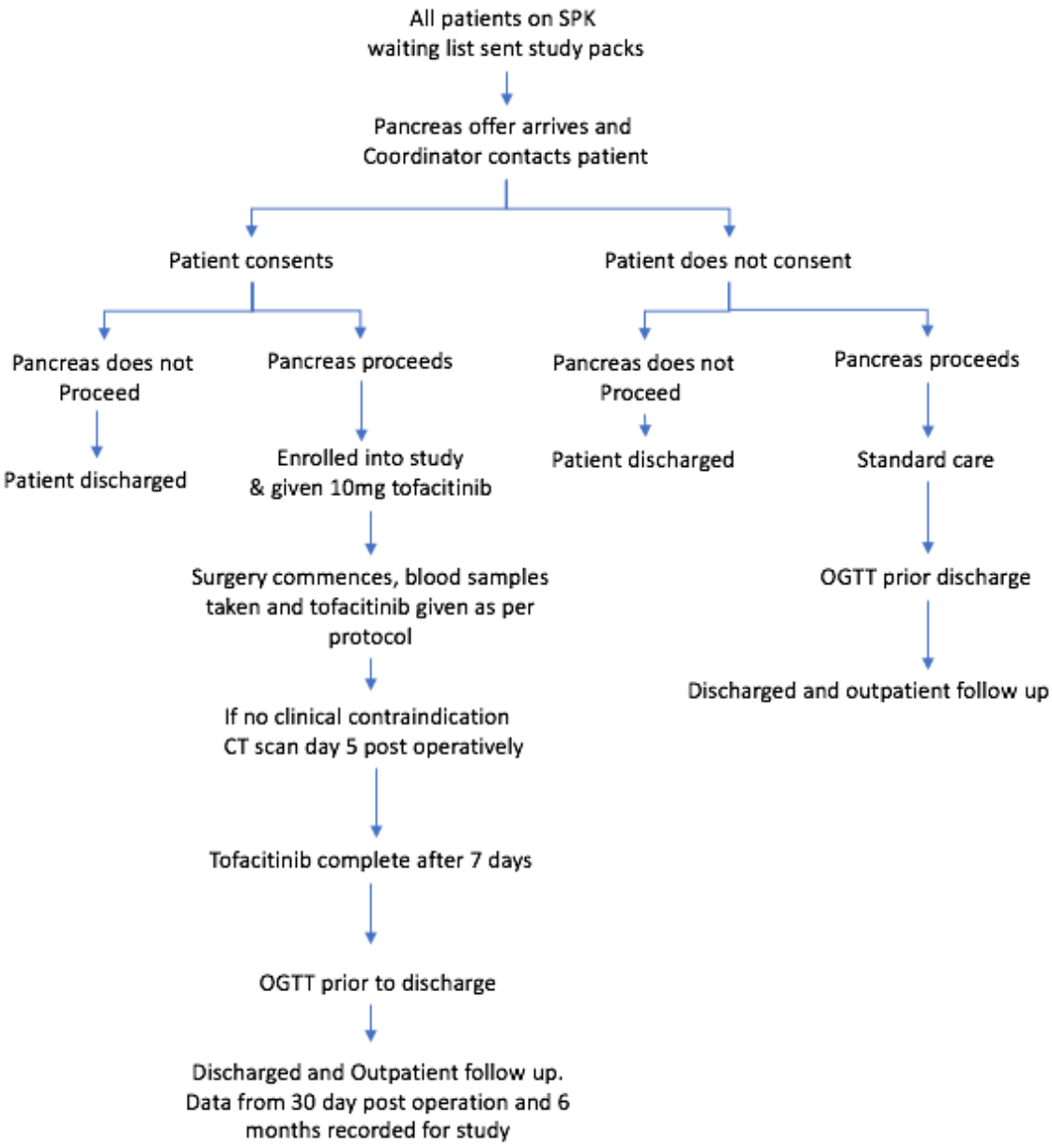
Synopsis

Trial Title	Safety and efficacy of tofacitinib in ameliorating ischaemia reperfusion injury and allograft pancreatitis in solid organ transplantation – a pilot study.
Internal ref. no. (or short title)	JAK inhibition treating allograft pancreatitis & ischaemia reperfusion.
Clinical Phase	Phase 2
Trial Design	Single Arm pilot study.
Trial Participants	Patients undergoing simultaneous pancreas and kidney transplantation with enteric drainage of pancreatic exocrine secretions.
Planned Sample Size	20 patients
Treatment duration	7 days
Follow up duration	6 months
Planned Trial Period	12 months

	Objectives	**Outcome Measures**
Primary	To assess the safety and anti-inflammatory effect of tofacitinib on allograft pancreatitis and ischaemia reperfusion, by measuring inflammatory markers in whole blood and assessing CT imaging using an oral dose of tofacitinib 10mg BD for 7 days.	Whole blood will be tested for the inflammatory marker C-reactive protein (CRP). CRP has been validated as a surrogate marker for allograft pancreatitis and is the primary outcome of the study. WCC will also be monitored as a further inflammatory marker. 5ml blood samples will be taken during reperfusion of the organ at times 0, 0.5, 1, 2, 4, 6, 12 and 24 hours, then once daily for the first 7 days post-surgery. Postoperative CT imaging day 5 post operatively will be performed.

Secondary	To quantify the level of inflammation observed following reperfusion of organs and identify what inflammatory pathways are being affected.	Whole blood samples were taken during reperfusion of the organ at 0, 0.5, 1, 2, 4, 6, 12 and 24 hours, then once daily for the first 7 days post-surgery, tested for full cytokine analysis and cell free circulating DNA. Perfusate samples from pancreas workbench preparation will be analysed for inflammatory markers.
Tertiary	To assess any association between post-operative complications with the administration of the drug. Clinical parameters which affect patient experience and morbidity will also be measured.	Length of hospital stay, number and type of further surgery needed, results of oral glucose tolerance testing and 30-day graft survival will be recorded.
Investigational Medicinal Product(s)	Tofacitinib citrate 10mg.	
Formulation, Dose, Route of Administration	Oral formulation XELJANZ 10mg BD.	

Trial design



The trial is a Phase-2 single arm trial consisting of 20 patients undergoing SPK transplant. It is not powered to prove efficacy but designed to proof of concept and escalation to a Phase-3 trial. Participants will be given tofacitinib 10mg twice daily while all other medication will be per standard practice for simultaneous pancreas kidney (SPK) transplant.

Inclusion and Exclusion criteria

Inclusion criteria

- Participant willing and able to give informed consent for participation in the trial.
- Male or Female, aged 18 years to 65 years.
- Participant confirmed surgically fit to proceed with transplantation.
- In the Investigator's opinion, able and willing to comply with all trial requirements.
- Willing to allow his/her General Practitioner and consultant, if appropriate, to be notified of participation in the trial.
- Willing to undertake effective contraception for the duration of the trial.

Exclusion criteria

- Female participant who is pregnant, lactating or planning pregnancy during the course of the trial.
- Bladder drained pancreas transplants.
- Participant with a life expectancy of less than six months.
- Patients with Hepatitis B/C and HIV.
- Any other significant disease or disorder which, in the opinion of the Investigator, may either put the participant at risk because of taking part in the trial, may influence the result of the trial or the participant's ability to participate in the trial.
- Known allergy to tofacitinib.
- Does not speak or understand English.
- Use of contraindicated medication to tofacitinib as per the appendix of inhibitors and inducers.

Trial visits

Baseline assessments/investigations (Day 0)

As part of the routine workup for pancreas transplantation, all preoperative assessments are performed prior to a patient coming in for transplant (before joining the transplant waiting list). These results will be collected from the participants' notes after they consent, and do not need to be repeated as part of this study.

- Demographic data will be obtained: date of birth, gender, ethnicity, smoking status, alcohol consumption.
- Medical history including current medication.
- General physical examination including weight and height.
- Vital signs including blood pressure, heart rate and temperature.

Visit 1 – Day of transplant surgery

Before transplantation, after a patient has been consented for both the study and surgery and as part of routine care, they will receive pre-medication comprising a number of medications both orally and intravenously, as part of the standard immunosuppressive induction regime. At this time, trial patients will be given the first dose of tofacitinib (a 10mg tablet).

During transplantation, blood tests will be taken to measure the level of inflammation in the patients' blood. This will be done through the central venous catheter, which is placed as a normal part of the procedure. 5mls of blood will be taken and aliquoted for analysis, and these blood samples will be taken at regular intervals during surgery (0, 0.5, 1, 2, 4 hours). Hour Zero is defined as the point of reperfusion of the pancreas which occurs part-way

through the surgical procedure hence, samples 0, 0.5, 1, 2, 4 will be done while the patient is still in theatre.

All blood samples taken whilst the participant is having surgery and on the ward, will be taken through the central venous catheter. The volume of samples needed each time is 5mls and the time points are standardised as described above. In total, 70mls of blood will be taken for analysis.

Visit 2 – Following transplantation on ITU (Day 0-1)

Following transplantation, patients are admitted to intensive care for a period of typically 24 hours for close observation prior to repatriation to the ward. The second dose of tofacitinib will be administered by the study team to the patient in the intensive care unit and further blood tests will be taken from the central line as per protocol. If the patient is being ventilated, the medication will be crushed and administered in the same manner as other necessary oral medication, via the NG tube.

After surgery on ITU, blood samples will be taken at 6 and 12 hours in intensive care (the patient may not be awake). Safety checks will be in place for any intolerance to tofacitinib or any adverse effect observed, as outlined by the adverse reaction guidance set out by the European Medicines Authority (EMA), through daily clinical review by the clinical transplant team.

Visit 3 – Following transplantation on the ward (Day 1-7)

Once patients have returned to the ward, administration of tofacitinib will continue in combination with all routinely prescribed medication following the twice-daily regime for

the 7-day course. Further blood samples will be taken once daily (24, 48, 72, 96 hours and once daily for 7 days) from the central line as the patient begins to recover. Safety checks will be in place for any intolerance to tofacitinib or any adverse effect observed through daily clinical review by the transplant team.

Visit 4 – CT scan (Day 5)

As part of the study, all patients following pancreas transplantation will have research CT imaging following surgery during day 5 to assess for any early complications, unless a CT has been requested for clinical reasons any time before this. A peripheral venous cannula will be used to inject a standard contrast medium into the venous circulation and patients will lie flat on the scanner bed. The scan will take 30 minutes and it will be performed in the Churchill hospital radiology department. Safety checks will be in place for any intolerance to tofacitinib or any adverse effect observed through daily clinical review by the transplant team.

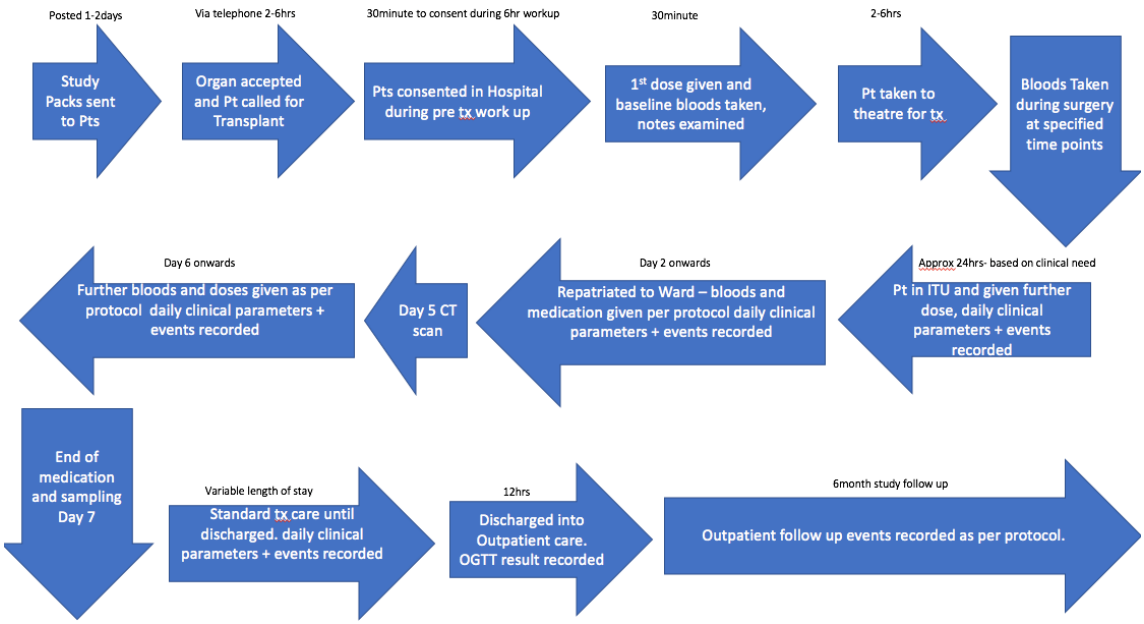
Visit 5 – 2 weeks' post-transplant

On the day of discharge, typically around 2 weeks' post-transplant, all patients have an Oral Glucose Tolerance Test (OGTT). This is to stimulate the transplanted pancreas and assess its ability to secrete insulin. This test is part of normal clinical practice and not an additional research procedure. The results of this test will be recorded in the study. Study participants will also be offered the chance to be reviewed by the study team during any outpatient visit, in order to address any concerns they may have. Safety checks will be in place for any intolerance to tofacitinib or any adverse effect observed through daily clinical review by the transplant team during the inpatient stay and any clinical outpatient appointment.

Visit 6 – Outpatient follow up

Following discharge from the hospital, all patients will be followed up as outpatients. Results of routine clinical testing and recording of all events will be measured for the study for a period of 6 months. Study time points will be linked to clinical time points and so, the 6-month assessment will coincide with the patient's 6 month clinical follow up. No additional appointments are necessary and the data from these clinic follow-ups will be used for the study analysis.

Study Schedule – Summarising timeline and safety monitoring:



Discontinuation / Withdrawal of participants from trial treatment

Each participant has the right to withdraw from the trial at any time. This is expected to be a rare event as tofacitinib is only given for 7 days. The most likely reasons for non-completion would be an adverse medical event such as CVA or MI, which would trigger the stopping of further tofacitinib administration, or if there is acute allograft thrombosis prompting pancreatectomy.

Other reasons a patient may be withdrawn include:

- Significant protocol deviation or inability to comply with study procedures
- Clinical decision
- Patient decision

In addition, the Investigator may discontinue a participant from the trial treatment at any time if the he/she considers it necessary for any reason including:

- Pregnancy – although highly unlikely
- Ineligibility (either arising during the trial or retrospectively having been overlooked at screening)
- Significant non-compliance with the treatment regimen, protocol deviation or trial requirements such as inability to administer tofacitinib for clinical reasons
- An adverse event which requires discontinuation of the trial medication, or results in an inability to continue to comply with trial procedures
- Disease progression which requires discontinuation of the trial medication, or results in an inability to continue to comply with trial procedures
- Withdrawal of consent
- Loss to follow up

Significant drug interactions occur with known inhibitors and inducers of CYP3A4 pathway such as with ketoconazole, fluconazole, clarithromycin, rifampicin. None of these drugs are routinely used for transplant patients and if indicated due to clinical need, further administration of tofacitinib to the patient would terminated. Patients who have received the study drug but had to stop because of other medication which is known to interact with tofacitinib, will be followed by the clinical team to ensure patients receive all necessary treatment and avoid any complication. If the participant is withdrawn due to an adverse event, the Investigator will arrange for follow-up visits or telephone calls until the adverse

event has resolved. The reason for withdrawal will be recorded in the CRF. A patient may also withdraw consent to provide samples for research at any time without giving a reason. In this case, the Investigator must ensure that their wishes are recorded in the medical record. If a patient withdraws, research samples and study data already collected will be retained for use in the study analysis with the patient's permission. Patients who withdraw consent will be replaced and the study will continue until we have reached 20 patients who have completed the study (7-day intervention with all tests). The total number of patients enrolled may increase to allow flexibility in order to replace withdrawn patients, as per the trial design.

Description of statistical methods

Due to the small size of the trial, both intention to treat and per-protocol analysis will be performed for this trial as both approaches should support non-inferiority. All tests will be completed at a 5% two-sided significance level and all comparative outcomes will be presented as summary statistics with 95% confidence intervals and reported appropriately.

This pilot study has not been powered to prove efficacy. Data from the Oxford Transplant Centre has shown that 44.3% of patients develop allograft pancreatitis, as shown by CT. With typically 70 transplants being performed annually, an expected 31 patients each year are estimated to develop allograft pancreatitis. In the context of this trial, we therefore expect to see evidence of reperfusion pancreatitis in up to 9 patients, with an anticipated lower incidence depending on the effect of the intervention. The results from this trial may be used for the purpose of the power calculation for a future larger study in which efficacy would be the primary endpoint.

APPENDIX 4:

Protocol approvals and associated documents

NHS
Health Research Authority

London - City & East Research Ethics Committee
Bristol Research Ethics Committee Centre
Whitefriars
Level 3, Block B
Lewins Mead
Bristol
BS1 2NT

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

10 May 2018

Mr Sham Dholakia
Oxford Transplant Centre
Churchill Hospital
Oxford
OX3 7LE

Dear Mr Dholakia

Study title:	**Safety and efficacy of Tofacitinib in ameliorating ischaemia reperfusion injury and allograft pancreatitis in solid organ transplantation – a pilot study**
REC reference:	**18/LO/0560**
Protocol number:	**13005**
EudraCT number:	**2017-002784-18**
IRAS project ID:	**221443**

Thank you for your letter of 8 May 2018, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a Sub-Committee of the REC. A list of the Sub-Committee members is attached.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact hra.studyregistration@nhs.net outlining the reasons for your request.

NHS
Health Research Authority

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA and HCRW Approval (England and Wales)/ NHS permission for research is available in the Integrated Research Application System, at www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

Clinical trial authorisation must be obtained from the Medicines and Healthcare products Regulatory Agency (MHRA).

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites listed in the application, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet completed any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as an SSA application(s) has been reviewed. In the meantime no study procedures should be initiated at non-NHS sites.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Oxford Insurance]		01 August 2017
GP/consultant information sheets or letters [GP letter]	1.0	25 February 2018
Investigator's brochure / IMP Dossier [EMA investigator brochure]		
IRAS Application Form [IRAS_Form_01052018]		01 May 2018
Letter from funder [funding letter]	1.0	21 June 2017
Letter from sponsor [Letter from sponsor]		13 March 2018
Letters of invitation to participant [study reply slip]	Track Changes	
Other [Peer Review Letter]	1.0	13 March 2018
Other [NHS feasibility R&D template]	1.0	
Other [Reply to REC revisions]	1.0	20 April 2018
Participant consent form [Patient Consent Form]	Track Changes	
Participant information sheet (PIS) [PIS]	1.0	25 February 2018
Participant information sheet (PIS) [PIS]	1.0	25 February 2018
Participant information sheet (PIS) [PIS]	Track	20 April 2018

	Changes	
Research protocol or project proposal [Study Protocol]	REC track changes	20 April 2018
Summary CV for Chief Investigator (CI) [Peter Friend CV]		01 January 2018
Summary CV for Chief Investigator (CI) [CI CV]	1.0	
Summary CV for student [Sham CV]		01 January 2018
Summary of product characteristics (SmPC) [SmPC]	1.0	

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

18/LO/0560	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

NHS
Health Research Authority

Yours sincerely

Rebecca Hawkins

Pp

Dr John Keen
Chair

Email:nrescommittee.london-cityandeast@nhs.net

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers"

Copy to: *Mr Clinical Trials and Research Governance*
Mr Research and Development Department, Oxford University Hospitals NHS Foundation Trust

NHS
Health Research Authority

London - City & East Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 14 May 2018

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr John Keen	GP (REC Chairman)	Yes	
Ms Anna (Renqian) Song	Pharmacist	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Mr Rajat Khullar	REC Manager

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Mr Sham Dholakia
Oxford Transplant Centre
Churchill Hospital
Oxford
OX3 7LE

Email: hra.approval@nhs.net
Research-permissions@wales.nhs.uk

25 July 2018

Dear Mr Dholakia

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**Safety and efficacy of Tofacitinib in ameliorating ischaemia reperfusion injury and allograft pancreatitis in solid organ transplantation – a pilot study**
IRAS project ID:	**221443**
EudraCT number:	**2017-002784-18**
Protocol number:	**13005**
REC reference:	**18/LO/0560**
Sponsor	**Clinical Trials and Research Governance**

I am pleased to confirm that **HRA and Health and Care Research Wales (HCRW) Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

How should I continue to work with participating NHS organisations in England and Wales?
You should now provide a copy of this letter to all participating NHS organisations in England and Wales, as well as any documentation that has been updated as a result of the assessment.

Following the arranging of capacity and capability, participating NHS organisations should **formally confirm** their capacity and capability to undertake the study. How this will be confirmed is detailed in the "*summary of assessment*" section towards the end of this letter.

You should provide, if you have not already done so, detailed instructions to each organisation as to how you will notify them that research activities may commence at site following their confirmation of capacity and capability (e.g. provision by you of a 'green light' email, formal notification following a site initiation visit, activities may commence immediately following confirmation by participating organisation, etc.).

It is important that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details of the research management function for each organisation can be accessed here.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within the devolved administrations of Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) has been sent to the coordinating centre of each participating nation. You should work with the relevant national coordinating functions to ensure any nation specific checks are complete, and with each site so that they are able to give management permission for the study to begin.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?
The document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

I am a participating NHS organisation in England or Wales. What should I do once I receive this letter?
You should work with the applicant and sponsor to complete any outstanding arrangements so you are able to confirm capacity and capability in line with the information provided in this letter.

The sponsor contact for this application is as follows:

Name: Clinical Trials and Research Governance
Email: ctrg@admin.ox.ac.uk

Who should I contact for further information?
Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **221443**. Please quote this on all correspondence.

Yours sincerely

IRAS project ID	221443

Miss Lauren Allen
Senior Assessor

Email: hra.approval@nhs.net

Copy to: *Mr Clinical Trials and Research Governance*
Mr Research and Development Department, Oxford University Hospitals NHS Foundation Trust

List of Documents

The final document set assessed and approved by HRA and HCRW Approval is listed below.

Document	*Version*	*Date*
Clinical Trial Authorisation		24 July 2018
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Oxford Insurance]		01 August 2017
GP/consultant information sheets or letters [GP letter]	1.0	25 February 2018
HRA Schedule of Events	1	22 March 2018
HRA Statement of Activities	1	22 March 2018
Investigator's brochure / IMP Dossier [EMA investigator brochure]		
IRAS Application Form [IRAS_Form_01052018]		01 May 2018
IRAS Checklist XML [Checklist_23032018]		23 March 2018
Letter from funder [funding letter]	1.0	21 June 2017
Letter from sponsor [Letter from sponsor]		13 March 2018
Letters of invitation to participant [study reply slip]	Track Changes	
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Research protocol or project proposal [Study Protocol]	REC track changes	20 April 2018
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Summary CV for Chief Investigator (CI) [CI CV]	1.0	
Summary CV for student [Sham CV]		01 January 2018
Summary of product characteristics (SmPC) [SmPC]	1.0	

IRAS project ID	221443

Summary of assessment

The following information provides assurance to you, the sponsor and the NHS in England and Wales that the study, as assessed for HRA and HCRW Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England and Wales to assist in assessing, arranging and confirming capacity and capability.

Assessment criteria

Section	HRA Assessment Criteria	Compliant with Standards?	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	The sponsor has confirmed that the ECG listed in the protocol table of events is part of routine care.
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	The sponsor intends to use the Statement of Activities and Schedule of Events as the agreement with the site.
4.2	Insurance/indemnity arrangements assessed	Yes	Where applicable, independent contractors (e.g. General Practitioners) should ensure that the professional indemnity provided by their medical defence organisation covers the activities expected of them for this research study
4.3	Financial arrangements assessed	Yes	The sponsor has confirmed in the Statement of Activities finance appendix that no funding will be provided to the site.
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments

Section	HRA Assessment Criteria	Compliant with Standards?	Comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Yes	No comments
5.3	Compliance with any applicable laws or regulations	Yes	The sponsor has confirmed samples will be stored at University of Oxford.
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	No comments
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Yes	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Participating NHS Organisations in England and Wales

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
There is one participating site. All study activity will be conducted at the site as detailed in the Protocol and supporting documents. The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England and Wales in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. Where applicable, the local LCRN contact should also be copied into this correspondence. If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England and Wales which are not provided in IRAS, the HRA or HCRW websites, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net or HCRW at Research-permissions@wales.nhs.uk. We will work with these organisations to achieve a consistent approach to information provision.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and Wales, and the minimum expectations for education, training and experience that PIs should meet (where applicable).
A Principal Investigator should be in place at the site. The sponsor has confirmed there are no specific training requirements for Principal Investigators. GCP training is not a generic training expectation, in line with the HRA/HCRW/MHRA statement on training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
Access arrangements will not be applicable as all study activity will be conducted by local staff at the site.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England and Wales to aid study set-up.
The applicant has indicated that they intend to apply for inclusion on the NIHR CRN Portfolio.

Medicines & Healthcare products Regulatory Agency

MHRA

MHRA
10 South Colonnade
Canary Wharf
London
E14 4PU
United Kingdom

gov.uk/mhra

Prof P J Friend
UNIVERSITY OF OXFORD
CHURCHILL HOSPITAL
OLD ROAD, HEADINGTON
OXFORD
OX3 7LE
UNITED KINGDOM

24/07/2018

Dear Prof P J Friend

THE MEDICINES FOR HUMAN USE (CLINICAL TRIALS) REGULATIONS 2004 S.I. 2004/1031

Our Reference:	21584/0397/001-0001
Eudract Number:	2017-002784-18
Product:	XELJANZ® (tofacitinib citrate)
Protocol number:	13005

NOTICE OF ACCEPTANCE OF AMENDED REQUEST

I am writing to inform you that the Licensing Authority accepts your amended request for a clinical trial authorisation (CTA), received on 23/07/2018.

REMARKS
For information – It is assumed that contraceptive use for the duration of the trial (as stated in the protocol), refers to 6 months use. This is acceptable as it covers the 4 weeks of effective contraception use that is advised in the SmPC.

For additional clarification please email Dr Lisa Campbell on lisa.campbell@mhra.gov.uk

The authorisation is effective from the date of this letter although your trial may be suspended or terminated at any time by the Licensing Authority in accordance with regulation 31. You must notify the Licensing Authority within 90 days of the trial ending.

Finally, you are reminded that a favourable opinion from the Ethics Committee is also required before this trial can proceed; changes made as part of your amended request may need to be notified to the Ethics Committee.

APPENDIX 5:

References

1. https://www.nih.gov/news-events/news-releases/rates-new-diagnosed-cases-type-1-type-2-diabetes-rise-among-children-teens [Checked on the 20th November 2018].
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