NOVEL APPROACHES TO PANCREAS ALLOGRAFT SURVEILLANCE

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by

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Abstract

Pancreas transplantation is a successful treatment option for selected patients with advanced complications of diabetes. It offers insulin independence, stabilisation of diabetic complications, and improved survival. However, these are largely conditional on long term pancreas allograft survival. Pancreas graft survival is poorer than that of other transplanted organs and this is mainly due to the absence of accurate and reliable techniques for monitoring the pancreas allograft. Irreversible damage has usually occurred to the pancreas before rejection is diagnosed, such that treatment which would otherwise have been effective, brings no benefit.

This thesis describes a number of novel approaches to pancreas allograft surveillance, aiming to identify graft injury early enough that treatment can be initiated before permanent damage occurs.

- The Homeostatic Model Assessment (HOMA) model has been modified to allow for systemic venous drainage of the endocrine pancreas. HOMAtx has been validated in a cohort of pancreas transplant patients with insulin secretion and sensitivity correlating well with the HOMAtx-derived outputs of %B and %S respectively. A lower threshold for beta cell function (%B) was identified that had a 100% sensitivity and 84.1% specificity for detecting diabetic glucose tolerance during oral glucose tolerance testing.
- Urinary C-peptide creatinine ratio (UCPCR) has been assessed as a marker of pancreas allograft function. Good correlation with serum C-peptide has been confirmed and the use of patient-directed remote monitoring via home UCPCR samples has been shown to be a potential tool for future allograft surveillance.
- The use of a donor-specific vascularised composite allograft or ‘sentinel skin flap’ to detect rejection in pancreas transplantation has been evaluated. Although isolated skin

rejection was observed in 10% of patients, there was no associated rejection of the primary visceral allografts, suggesting that the SSF might be identifying an early alloimmune response and allowing treatment before clinical rejection has occurred.

Together, these studies represent novel approaches to pancreas allograft surveillance that may deliver incremental improvements that are required to advance overall outcomes.

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List of Abbreviations

%B	Beta cell function derived from HOMA/HOMA2/HOMAtx
%S	Insulin sensitivity derived from HOMA/HOMA2/HOMAtx
ACR	Acute cellular rejection
AKI	Acute kidney injury
AMR	Antibody mediated rejection
APC	Antigen presenting cell
AST	Arginine stimulation test
ATG	Anti-thymocyte globulin
AUC	Area under the curve
BCC	Basal cell carcinoma
BKV	BK (polyoma) virus
BMI	Body mass index
BP	Blood pressure
BPAR	Biopsy proven acute rejection
cRF	Calculated reaction frequency
CGM	Continuous glucose monitoring
CIT	Cold ischaemic time
CKD	Chronic kidney disease
CMV	Cytomegalovirus
CNI	Calcineurin inhibitor
CRP	C-reactive protein
CT	Computed tomography
CV	Coefficient of variation
DBD	Donation after brain death
DC	Dendritic cell
DCCT	Diabetes Control and Complications Trial
DCD	Donation after circulatory death
DGT	Diabetic glucose tolerance
DM	Diabetes mellitus

DSA	Donor specific antibodies
dnDSA	De-novo donor specific antibodies
EBV	Epstein-Barr virus
EDIC	Epidemiology of Diabetes Interventions and Complications study
eGFR	Estimated glomerular filtration rate
ELIS	Enzyme-linked immunosorbent assay
ESRF	End stage renal failure
fsOGTT	Frequently sampled oral glucose tolerance test
GAD	Glutamic acid decarboxylase
GLP-1	Glucagon-like peptide 1
GST	Glucagon stimulation test
HbA1c	Glycated haemoglobin
HD	Haemodialysis
HLA	Human leucocyte antigen
HOMA	Homeostatic Model Assessment
HOMA2	Modified Homeostatic Model Assessment
HOMAtx	Homeostatic Model Assessment for pancreas transplantation
HR	Hazard ratio
IFN-γ	Interferon gamma
IGT	Impaired glucose tolerance
INR	International normalised ratio
IU	International units
IVC	Inferior vena cava
IVGTT	Intravenous glucose tolerance test
IVIG	Intravenous immunoglobulin
KDIGO	Kidney Disease: Improving Global Outcomes
KTA	Kidney transplant alone
LADA	Latent autoimmune diabetes in adults
LDK	Living donor kidney
LST	Leucine stimulation test

MDRD	Modification of diet in renal disease
MDT	Multidisciplinary team
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MMTT	Mixed meal tolerance test
MODY	Maturity onset diabetes of the young
MRI	Magnetic resonance imaging
NICE	National Institute for Health and Care Excellence
NHS	National Health Service
NHSBT	NHS Blood and Transplant
OCT	Optimal cutting temperature (OCT compound)
OGTT	Oral glucose tolerance test
OUH	Oxford University Hospitals NHS Foundation Trust
PD	Peritoneal dialysis
PAK	Pancreas after kidney transplant
PASPK	Pancreas after SPK transplant
PBMC	Peripheral blood mononuclear cells
PTA	Pancreas transplant alone
PTLD	Post-transplant lymphoproliferative disorder
QFM	QuantiFERON Monitor
QOL	Quality of life
RI	Resistive index
ROC	Receiver operating characteristic
RRT	Renal replacement therapy
SD	Standard deviation
SEM	Standard error of the mean
SMR	Standardised mortality rate
SNOD	Specialist nurse in organ donation
SNP	Single nucleotide polymorphism
SPK	Simultaneous pancreas kidney transplantation

SSF	Sentinel skin flap
SST	Serum separating tubes
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TEG	Thromboelastogram
TRIG	Transplant research immunology group at the University of Oxford
UCPCR	Urinary C-peptide creatinine ratio
UK	United Kingdom
UKPDS	UK Prospective Diabetes Study
UW	University of Wisconsin
VCA	Vascularised composite allograft
WHO	World Health Organisation

Chapter 1

Introduction

1.1 Diabetes Mellitus

Diabetes mellitus comprises a group of metabolic disorders that are characterised by prolonged hyperglycaemia as a result of defective insulin secretion, insulin action, or both[1]. Diabetes is a major and increasing public health problem due to the rising prevalence worldwide and the associated morbidity, mortality, and economic cost.

The number of people with diabetes is at an all-time high with 4.7 million in the United Kingdom and 425 million worldwide[2,3]. This is expected to rise over the coming decades so that by 2035 more than 10% of the population may have the condition[4]. The World Health Organisation lists diabetes as the 6th commonest cause of death worldwide with 1.6 million deaths attributed to this disease in 2015[5]. However, when focusing on North America and Europe, diabetes is the 3rd biggest killer contributing to between 11.5 and 19.4% of all deaths[6]. This is associated with a huge economic cost - £14 billion per year in the UK which represents 10% of the NHS budget, although the global cost has recently been estimated at $825 billion[7,8].

Glucose is the most important source of energy in human metabolism. All cells in the body can metabolise glucose although some require the presence of other substances, such as insulin, in order to do this. When glucose levels are high, insulin is released from pancreatic beta cells and influences carbohydrate, lipid and protein metabolism in many different tissues within the body. Insulin generally has anabolic effects; for example, it promotes glucose uptake in muscle, adipose and other tissues, whilst stimulating the liver to synthesise glycogen and fatty acids, stimulating the uptake of amino acids, and inhibiting the

breakdown of fat in adipose tissue. Many of these effects cease in the absence of insulin or in the presence of glucagon which is produced in the pancreatic alpha cells in response to low glucose levels.

The principle feature of diabetes mellitus is hyperglycaemia, but the disease is characterised into several types that have very different defining features. It is broadly divided into type 1 (T1DM) and type 2 (T2DM) diabetes, although there are several other forms such as gestational diabetes, maturity onset diabetes of the young (MODY), latent autoimmune diabetes in adults (LADA), type 3 or Alzheimer's related diabetes, secondary diabetes, and many other rare congenital and genetic types. T1DM is typically characterised by an absolute insulin deficiency whereas T2DM is usually due to a combination of insulin resistance and insulin insufficiency. A recent study by Ahlqvist et al. has suggested that the current broad categorisation is too simple, and that diabetes is better defined by 5 subtypes[9]. They argue that treatment targeted to the specific subtypes may lead to improved outcomes.

1.1.1 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus is the most common endocrine condition that occurs in childhood with an annual incidence in the UK of 24.5 per 100,000 children aged 0 to 14 years old[10]; this varies considerably throughout the world with incidence rates ranging from 0.1/100,000 in Venezuela to 57.6/100,000 in Finland. Although T1DM represents only 8% of all patients with diabetes, it accounts for more than 90% of diabetes in children[2]. The incidence rate increases from birth and peaks between the ages of 10 to 14 during puberty[11-14]. There are currently around 400,000 people in UK living with T1DM, with over 30,000 of them children[15]. Most autoimmune diseases disproportionately affect females more than males[11], yet T1DM is generally seen equally in males and females[16]. However, there is evidence that males are more affected in some European and post-pubertal populations[17-19].

T1DM is a chronic autoimmune disease characterised by hyperglycaemia and insulin deficiency caused by the loss of pancreatic islet beta cells. In the majority of patients (70 - 90%[20]) the beta cell loss is associated with the development of autoantibodies and an autoimmune response (i.e. loss of tolerance with migration of self-reactive T-cells to islets[20,21], Figure 1.1). In a small subset of patients, there are no autoantibodies or identifiable immune responses to explain the beta cell destruction.

The aetiology of T1DM remains unclear but is thought to be a combination of genetic and environmental factors. It is a polygenic disease where genetic risk factors are necessary but not alone sufficient for disease. The concordance rate of T1DM among monozygotic twins has been reported in the literature as between 23 - 65% depending on the duration of follow-up[22-24]. First degree relatives of patients with T1DM have at least a 10 to 15 fold increased risk of T1DM relative to the general population[25]. The most strongly linked and studied genetic risk factors for T1DM are the HLA Class II haplotypes. HLA-DR4-DQ8 and HLA-DR3-DQ2 are the two highest risk types for the development of islet autoantibodies and T1DM in the western world, being present in 90% of children with T1DM[20,26]. In addition to the HLA region, more than 50 other regions of the human genome have been identified through genome wide association studies as conferring susceptibility to T1DM[27].

Whilst genetic factors are clearly important for the development of T1DM, multiple environmental factors are also necessary. Gestational events, gestational infections, early diet, and viral infections have all been proposed as potential influences for the development of T1DM[28-30]. The different combinations of exposure to these environmental factors and genetic disposition is likely to explain the variable incidence of the disease both within and between populations.

In the UK, 23% of children with T1DM present with diabetic ketoacidosis[2]. The diagnosis is usually based on a combination of clinical features and confirmatory biochemical investigations. Individuals usually have an array of hyperglycaemic and catabolic symptoms due to insulin deficiency: polyuria, polydipsia, weight loss, myalgia, and fatigue. The American Diabetes Association's criteria for diagnosing diabetes mellitus are based on signs of abnormal glucose metabolism irrespective of diabetes type and age of onset (Table 1.1)[31]. The diagnosis of T1DM can be more challenging in older children and young adults where T2DM is becoming increasingly common. The presence of appropriate symptoms, low insulin/C-peptide and beta cell targeted autoimmunity is usually sufficient for a diagnosis of autoimmune T1DM. In those with a consistent clinical picture for T1DM but no autoantibodies, a diagnosis of idiopathic T1DM is recognised by the American Diabetes Association[20].

Although the development of autoantibodies against beta cells is a predominant feature in T1DM, this often predates by many years the development of glycaemic dysfunction or diagnosis of diabetes (Figure 1.2). Anti-insulin or glutamic acid decarboxylase (GAD) antibodies are usually the first to be seen, followed by autoantibodies that target proteins found in the membrane of beta cell secretory vesicles (e.g. IA-2, IA-2β and ZnT8)[32]. The peak incidence of insulin autoantibody is at the age of 1 - 2 years with it being strongly associated with certain HLA haplotypes. The presence of multiple autoantibodies has a > 50% risk of progression to T1DM over 10 years and even those with single persistent insulin or GAD autoantibodies have a 44% risk of progression[26,33].

The management of T1DM involves a diverse multidisciplinary team with the aim of optimising glycaemic control whilst preventing and treating secondary complications of the disease. This involves significant education to enable self-management given that the average diabetic patient will only spend around 3 hours with a healthcare professional every

year, leaving the remaining 8,757 hours where they must manage their diabetes themselves[2]. The pharmacological management of T1DM usually involves the use of exogenous insulin due to beta cell insufficiency. This can be via different injecting schedules (e.g. basal bolus); in selected patients with difficult to manage diabetes, there is increasingly use of pumps with glucose monitoring devices. Where there is diagnostic ambiguity in older children and adults, particularly those with higher BMI, there is a role for trialling oral hypoglycaemic agents initially.

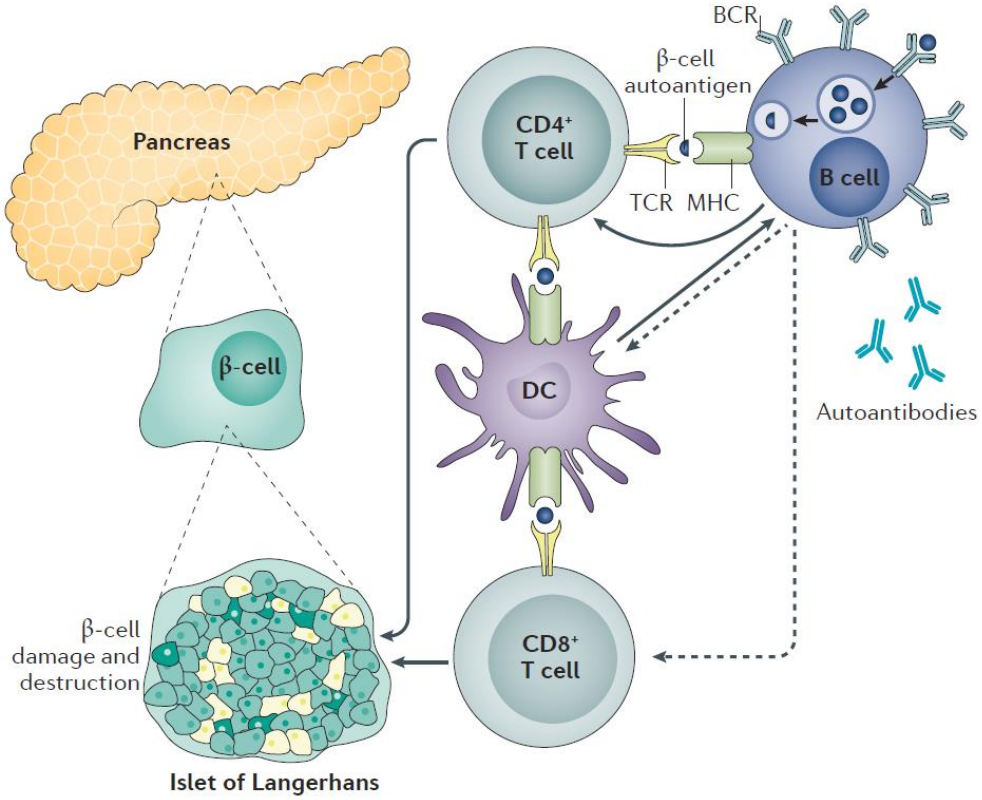


Figure 1.1: Pathophysiology of T1DM. Activated B cells interact with $CD4^+$ and $CD8^+$ T cells, as well as dendritic cells (DC). Antigen presentation by B cells and DCs drives the activation of beta cell specific T cells. The additional exposure of B cells to beta cell autoantigens leads to the production of islet specific autoantibodies. Dashed arrows indicate potential interactions. BCR, B cell receptor; TCR, T cell receptor; MHC, major histocompatibility complex. Image reproduced with permission from Springer Nature[20].

Table 1.1: Criteria for the diagnosis of diabetes mellitus[31].

Fasting plasma glucose of ≥ 7.0 mmol/L
OR
2 hour plasma glucose ≥ 11.1 mmol/L during OGTT
OR
HbA_{1c} ≥ 48 mmol/mol
OR
Random plasma glucose ≥ 11.1 mmol/L in patient with hyperglycaemic symptoms

OGTT – oral glucose tolerance test, performed as per World Health Organisation requirements.

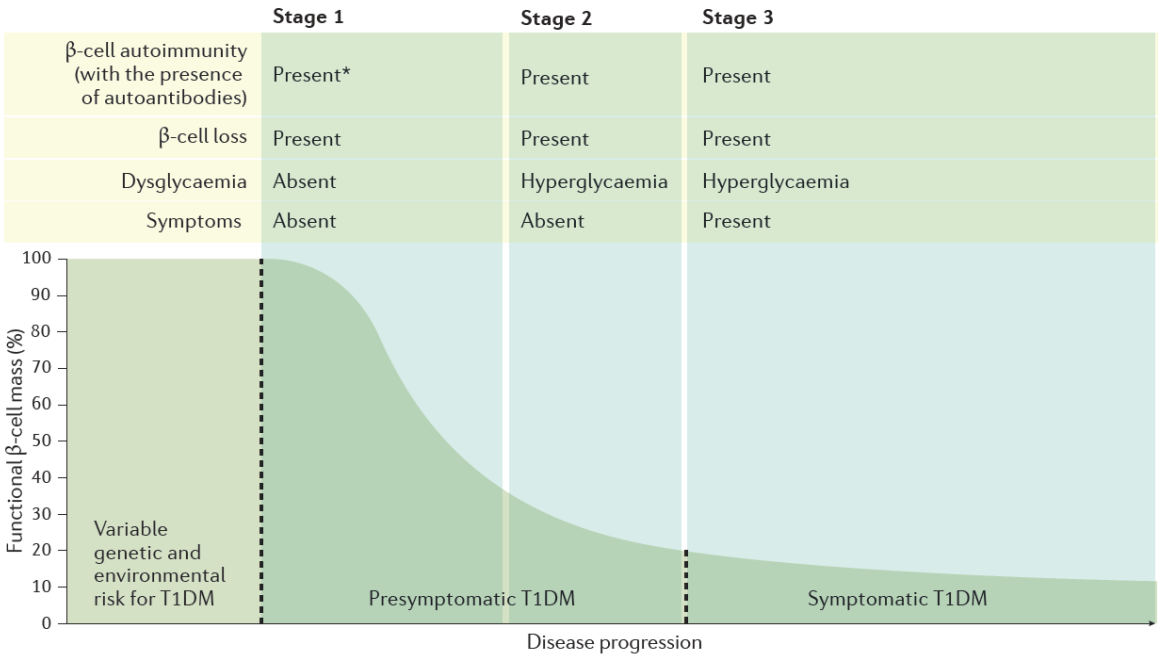


Figure 1.2: Staging of T1DM. It can be divided into 3 stages. Stage 1 is characterised by the presence of autoantibodies and the absence of dysglycaemia. Stage 2 is characterised by the presence of both autoantibodies and dysglycaemia. Symptoms only appear in stage 3 which corresponds with symptomatic T1DM. Reproduced with permission from Springer Nature[20].

1.1.2 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus accounts for more than 90% of diabetes worldwide. The number of people globally with the disease has quadrupled over the past 3 decades and is expected to increase to more than 600 million by 2040 [10]. The incidence and prevalence of T2DM varies according to geographical region but is 2 to 4 times more common in some Asian and Black ethnic groups. Asia has emerged as the epicentre of the global epidemic with China and India accounting for more than 160 million patients with the disease. In comparison, there are approximately 30 million people in the USA and 4 million people in the UK with T2DM[2,34]. T2DM was historically thought of as a disease affecting older adults, however, there has been an increasing incidence in younger adults and children over the last 2 decades. It remains uncommon in children under 10 years old, but in older children and young adults a diagnosis of T2DM is associated with higher morbidity and mortality when compared with older adults[35]. Unfortunately, there are often significant delays in the diagnosis of T2DM; it has been estimated that 49.7% of all cases (or 224 million) of diabetes are undiagnosed[36] - 1 million of which are in the UK[2]. This is important because those with untreated diabetes are at much higher risk of developing complications than those receiving treatment.

T2DM is a heterogeneous disorder with multiple pathophysiological abnormalities and a varied nature. The characteristic features are beta cell dysfunction, insulin resistance, and chronic inflammation – all of which contribute to hyperglycaemia and the development of complications. The amount of insulin produced varies considerably but in contrast to T1DM, most patients do maintain some insulin secretion. There are many distinct pathophysiological abnormalities that contribute to impaired glucose homeostasis and hyperglycaemia (Table 1.2). Risk factors for the development of T2DM are shown in Table 1.3. Similar to T1DM, the aetiology of T2DM is multifactorial with both genetic and environmental factors. First degree relatives of patients with T2DM have a 2 to 3x increased

risk compared with the general population, but this increases to 30x if two siblings have the disease[37]. Delineating the genetic foundations in such a complex polygenic disease has been challenging. Genome wide association studies have identified more than 100 single nucleotide polymorphisms (SNPs) that show a strong association with T2DM[38]. However, obtaining mechanistic insights into these SNPs has been hard and not always helped by animal studies[39]. More than 80% of the heritability seen in T2DM cannot be explained by the SNPs that have been identified so far[40]. This may be because of the heterogeneity of the disease, environmental interactions and epigenetic mechanisms. Despite this, it has been suggested that up to 90% of cases are potentially preventable by maintaining a body mass index (BMI) of less than 25 kg/m^2, having a healthy diet, exercising regularly, and not smoking or drinking alcohol excessively[40-42].

The principles of management of T2DM are similar to that of T1DM, that is, a multidisciplinary approach aiming to minimise complications of the disease. This involves a tailored approach according to the individual's needs and usually involves a combination of lifestyle changes and medication. Many of the risk factors for T2DM that are listed in Table 1.3 are modifiable and can have a profoundly beneficial effect or even reverse the disease[43]. Despite the significant benefits achievable with lifestyle changes, many do not succeed in avoiding the need for pharmacological intervention. Current National Institute for Health and Care Excellence (NICE) guidance recommends that pharmacological intervention is started if HbA_{1c} rises to 48 mmol/mol despite lifestyle interventions[44]. First line therapy is usually metformin monotherapy, and additional agents can be added in as escalation requires. Most patients with T2DM continue to have some beta cell function and many therapeutic agents aim to either increase insulin sensitivity or increase insulin secretion. Nevertheless, many patients will still need exogenous insulin as the disease progresses. This can cause additional complexity due to the fact that it contributes to weight

gain and therefore insulin resistance and thus confounding the problem. Newer therapies such as GLP-1 analogues have had some success in preventing transition to exogenous insulin. There is also an increasing role for bariatric surgery in the treatment of type 2 diabetes with profound weight loss and resolution of T2DM being common after sleeve gastrectomy and gastric bypass surgery[45]. This is reflected in NICE guidance which recommends assessment for bariatric surgery in anyone with recent onset T2DM and a BMI of over 30 kg/m^2 and expedited assessment for anyone with a BMI of over 35 kg/m^2 [46].

Table 1.2: Pathophysiology of hyperglycaemia in T2DM. Adapted from reference [47].

Pancreatic beta cell	Loss of mass and impaired insulin secretion
Pancreatic alpha cell	Dysregulated and increased glucagon secretion
Incretin	Diminished incretin effect
Inflammation	Immune dysregulation
Liver	Increased hepatic glucose output
Muscle	Reduced peripheral glucose uptake and insulin resistance
Adipose tissue	Reduced peripheral glucose uptake and insulin resistance
Kidney	Increased glucose reabsorption
Brain	Increased appetite and lack of satiety
Intestine	Increased rate of glucose absorption
Colon	Abnormal gut microbiome
Vascular	Insulin resistance, impaired insulin mediated vasodilation

Table 1.3: Risk factors for the development of T2DM. Adapted from reference [40,48].

• Older age
• Non-white ancestry
• Family history of type 2 diabetes mellitus
• Genetic factors (e.g. risk alleles of TCL7F2)
• Components of metabolic syndrome (increased waist circumference, increased blood pressure, increased plasma levels of triglycerides, low plasma levels of HDL cholesterol and small, dense LDL cholesterol particles)
• Overweight or obese (BMI $\geq$ 25 kg/m^2)
• Abdominal or central obesity (independent of BMI)
• Unhealthy dietary factors (regular consumption of sugary beverages and red meats and low consumption of whole grains and other fibre-rich foods)
• Cigarette smoking
• Sedentary lifestyle
• History of gestational diabetes mellitus or delivery of neonates > 4 kg in weight
• Some medications, such as statins, thiazides and beta-blockers
• Psychosocial stress and depression
• History of gestational diabetes
• Short and long sleep duration and rotating shift work
• History of atherosclerotic cardiovascular disease
• Polycystic ovary syndrome

1.2 Complications of Diabetes Mellitus

The underlying problem with diabetes is that poor glycaemic control causes microvascular and macrovascular complications. Microvascular complications contribute to significant disabling morbidity and reduce quality of life whilst also contributing to the development of macrovascular disease which itself is the predominant cause of mortality in diabetes. There is a strong correlation between hyperglycaemia, duration of diabetes and the development of complications; it is therefore essential to optimise glycaemic control to improve outcomes in this disease. The development of complications is insidious, but they can usually be detected many years before they cause symptoms or significant organ dysfunction.

Retinopathy is a common microvascular complication and is the leading cause of preventable sight loss in the UK. Worldwide, over one third of patients with diabetes will have signs of diabetic retinopathy with a third of these having vision-threatening disease[49]. After 20 years, nearly all of patients with T1DM and two-thirds of patients with T2DM will have some degree of retinopathy[50]. In the UK, this translates into 1,700 patients each year who develop vision limiting retinopathy[2]. Diabetic retinopathy takes several years to develop and is difficult to reverse once established. Prevention and early diagnosis are therefore crucial to prevent visual loss[51]. For this reason, early retinal screening should form a fundamental part of all diabetic management plans to prevent debilitating and irreversible visual loss.

Nephropathy is a common severe microvascular complication of diabetes. Around 40% of people with diabetes will develop nephropathy and up to half of these will need treatment for end stage renal failure (ESRF) during their lifetime. Like all diabetic complications, the prevalence varies significantly according to country and ethnicity with a higher prevalence

in African Americans, Asians, and Native Americans than in Caucasians[52]. In the UK, more than 30% of patients requiring renal replacement therapy (dialysis or renal transplantation) have diabetes. Progression to ESRF appears to be more common in T1DM, however this can be modified by intense control of risk factors[53]. The incidence of end stage renal failure in T1DM has been reported to be decreasing in many cohorts, presumably due to the widescale adoption of reno-protective regimes and improvements in glycaemic control[54-56]. There are, however, still marked variations in incidence between some groups, such as the 2-fold difference observed between those in Finland and the United States[57]. Renal replacement therapy with dialysis is itself associated with a significant increase in morbidity and mortality (Figure 1.3). 10 year adjusted survival is less than 30% and younger patients with diabetes have a much poorer prognosis than their matched colleagues without diabetes[58].

Neuropathy is another common microvascular complication of diabetes that can cause significant disability and reduced quality of life. When affecting the somatic nervous system, it can cause sensory loss, chronic pain, foot disorders, and unrecognised injuries which combine with hyperglycaemia and tissue hypoperfusion in a cascade towards uncontrollable sepsis and amputation. There are more than 8,500 amputations performed in the UK each year as a result of diabetes. Around half of these people will die within two years and having a diabetic foot ulcer is associated with a 40% chance of death within 5 years; this is also associated with macrovascular disease that contributes to myocardial infarction, heart failure and strokes. Autonomic neuropathy is also common, and like other complications, is associated with poor glycaemic control and the duration of disease, with estimates of prevalence ranging up to 90%[59]. It can lead to cardiovascular instability, gastrointestinal disorders, bladder and sexual dysfunction. These can have a profound effect on quality of life and are often extremely difficult to manage.

Macrovascular complications include coronary artery disease (myocardial infarction, heart failure), cerebrovascular disease (strokes), and peripheral vascular disease (claudication, rest pain, ischaemic ulceration and amputation). The unifying pathology behind all of these is the accelerated development of atherosclerosis although there is increasing evidence of some shared pathogenic features with microvascular disease (e.g. proliferation of vasa vasorum). Each year in the UK, diabetes causes more than 27,000 myocardial infarctions, 100,000 cases of heart failure, and 35,000 strokes[2]. In the developed world, cardiovascular disease is the leading cause of death in people with either T1DM or T2DM[60,61]; however, in some developing countries it lags behind infections and renal failure as the leading cause of death[62].

Since the 1980's there have been several landmark studies investigating the effect of improved glycaemic control and outcomes in diabetes. The Diabetes Control and Complications Trial (DCCT) investigated whether intensive glycaemic control would ameliorate the long term complications of diabetes when compared with conventional therapy at that time[63]. The 1441 patients with T1DM were followed for a mean of 6.5 years and achieved an HbA_{1c} of 7.2% in the intervention group compared with 9.1% in the control group. The study showed that intensive glycaemic control reduced the adjusted mean risk for developing retinopathy by 76% compared with conventional therapy whilst also slowing progression of retinopathy by 54% in those that already had mild retinopathy. The overall risk of microalbuminuria and albuminuria occurring was reduced by 39% and 54% respectively in the intensive treatment group. Clinical neuropathy was reduced by 60%. The small number of events in a relatively young cohort made it difficult for meaningful comparisons to be made about the effect on macrovascular complications. The main adverse event associated with intensive therapy was a nearly threefold increase in severe hypoglycaemia. The trial was stopped 1 year early by its independent oversight committee

and the original conventional therapy control group was taught the intensive therapy and the entire cohort invited to join a new study - the Epidemiology of Diabetes Interventions and Complications study (EDIC)[64]. Numerous EDIC papers have demonstrated that there was a durable effect of the initial intervention therapy despite a convergence of HbA_{1c} between the two groups to 8.0%; the reduction in early-stage microvascular complications seen in DCCT translated into substantial long term reductions in severe complications in T1DM[65-69]. In addition, EDIC 30 year data has shown that intensive glycaemic control in DCCT reduced the risk of any cardiovascular disease event by 30% and the risk of nonfatal myocardial infarction, stroke, or death from cardiovascular disease by 32%[70]. This has translated into an overall mortality in the DCCT intensive therapy cohort similar to that of the general population (standardised mortality rate (SMR) of 0.88), whereas the conventional therapy cohort had a significantly higher mortality rate (SMR 1.31)[71].

The UK Prospective Diabetes Study (UKPDS) was a similar study to DCCT but for people with newly diagnosed T2DM[72]. It assessed the effect of intensive glycaemic control with either sulphonylurea or insulin (and later metformin) compared with a conventional group using primarily dietary changes. Mean HbA_{1c} over 10 years in 3867 patients was 7.0% in the intensive group compared with 7.9% in the conventional group. There was a 12% risk reduction for any diabetes related endpoint and 25% reduction in the development of microvascular complications. When looking specifically at overweight patients on metformin, a reduction in any-cause mortality of 36% was observed. Subsequent post-trial follow-up of these patients demonstrated a similar effect to that seen in DCCT - a convergence of HbA_{1c} yet a persistent protective effect (24% reduction) for microvascular complications in the intensive therapy group[73]. There was a 13% risk reduction for any-cause mortality in the overall intensive therapy group, rising to 27% in the metformin group. Whilst, the benefit of intensive therapy on the development of microvascular complications

has been further substantiated, the effect on macrovascular complications and mortality remains more controversial. A Cochrane review and meta-analysis of 28 trials containing 34,912 patients with T2DM concluded that targeted intensive glycaemic control did not show significant differences in all-cause mortality and cardiovascular mortality[74,75]. The main reason for this is due to the inclusion of studies with a high risk of bias (only 2 of 28 trials were considered to be at low risk of bias). There was a considerable heterogeneity of patients and duration of intervention in the trials included. Furthermore, some trials such as UKPDS recruited patients at the point of diagnosis whereas others like ACCORD recruited patients many years after the diagnosis of T2DM[72,76]. It is probable that a limited intervention in a patient that has had diabetes for more than 10 years (e.g. ACCORD) will produce less benefit than a prolonged intervention initiated in a patient at the time of diagnosis (e.g. UKPDS).

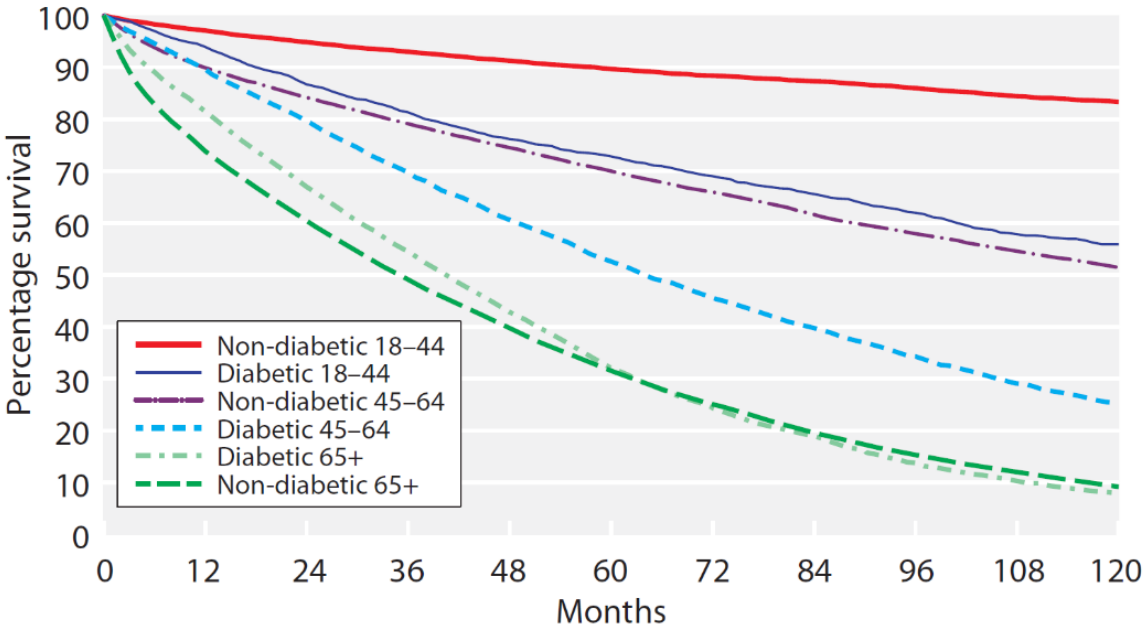


Figure 1.3: Long term survival for renal dialysis patients with and without diabetes by age group. Reproduced with permission from S. Karger AG, Basel[58].

1.3 Insulin and Beta Cell Replacement Therapy

The discovery of insulin by Banting, Best, Colip and Macleod in 1921 laid the foundations for a century of research that has changed T1DM from a usually fatal disease to one that is easily treatable, albeit with significant delayed morbidity and mortality. The advent of human recombinant insulin in 1978 and subsequent refinement to allow for different durations of action, led to the basis of current therapeutic regimes for managing all patients with T1DM and many with T2DM.

Exogenous insulin treatment is not without its problems. The most serious side-effect of insulin is hypoglycaemia. The DCCT, EDIC, and UKPDS trials showed us that the better a person's glycaemic control is, the less likely they are to develop complications of diabetes. However, the price for improved control is increased episodes of hypoglycaemia - nearly 3 times as many episodes in DCCT. Hypoglycaemia is itself associated with significant morbidity and frequent episodes can lead to loss of the normal autonomic responses to neuroglycopaenia - hypoglycaemic unawareness. Hypoglycaemic unawareness has been reported in up to 48% of patients with T1DM and 37% of T2DM patients receiving insulin[77]. Patients with hypoglycaemic unawareness have up to 6 times higher risk of severe hypoglycaemia (requiring third party assistance), as well as fractures, cognitive decline, arrythmias, seizures, coma, and a 3 - 6% annual risk of death[78-81]. Usual practice for the treatment of recurrent hypoglycaemic episodes or hypoglycaemic unawareness is to relax glycaemic control. However, this then offsets the original desired potential benefit in reducing complications derived from tighter glycaemic control.

Patients receiving insulin therapy for both T1DM and T2DM typically suffer from significant weight gain (5.1 kg in DCCT, 6.5 kg in UKPDS)[82]. This compounds existing problems in both groups. In T2DM, patients are usually already obese at the point of

diagnosis and therefore further weight gain is undesired and contributes to insulin resistance, poorer glycaemic control, and escalating insulin requirements. In T1DM, weight gain can contribute to associated psychological morbidity and contribute to 'diabulimia' - an eating disorder where an individual reduces or stops their insulin with the intent of losing weight but exposing themselves to the associated high risk of developing ketoacidosis.

Difficulties with hypo- and hyperglycaemia have led to the development of insulin pumps with the aim of providing more physiological insulin therapy. These infuse a variable basal amount of insulin alongside boluses for meals. Such devices are becoming increasingly sophisticated with algorithms allowing for readings to be input from external glucose monitoring devices as well as inbuilt sensors to detect hypoglycaemia. The benefit of such devices has been recognised by NICE which has supported their use in patients with T1DM who are failing traditional medical therapy[83]. Current NICE guidelines are now more than 10 years old and do not reflect the progress in technology. A closed loop system that incorporates continuous glucose monitoring and autonomous insulin delivery could provide the much coveted 'artificial pancreas'. Two 'hybrid' closed loop devices have been approved so far; the Medtronic 670G in the United States and the Diabeloop DBLG1 in the European Union. However, whilst these represent a significant technological advance in safely administering basal insulin, they are not fully automated and do require the user to input data about carbohydrate intake. Whilst such devices have been shown to reduce episodes of hypoglycaemia[84], their effect on overall glycaemic control, morbidity and mortality is still subject to further work and scrutiny. Ultimately, long term results from DCCT have shown us that current methods of exogenous insulin administration are not good enough and that the threshold level for the risk of developing secondary complications is normal glucose homeostasis. In his Nobel lecture Frederick Banting concluded that *'Insulin is not a cure for diabetes; it is a treatment. It enables the diabetic to burn sufficient*

carbohydrates, so that proteins and fats may be added to the diet in sufficient quantities to provide energy for the economic burdens of life' [85].

Alongside the development of insulin and insulin delivery systems, there has been a parallel quest for a true beta cell replacement that can provide normal physiological glucose homeostasis. Scientists experimented with islet and pancreas transplantation throughout the 19th and 20th Centuries. The first technically successful human pancreas transplant was performed by Kelly and Lillehei at the University of Minnesota in 1966[86]. They transplanted a whole pancreas alongside a kidney from a deceased donor into a 28 year old woman. The recipient was insulin independent but unfortunately died from complications after two months. By 1982 Sutherland et al. reported that 190 pancreas transplants had been performed, but only 9 still had graft survival at 1 year[87]. Outcomes only started to significantly improve with standardisation of surgical techniques and the development of better immunosuppressants - namely cyclosporin A in 1983 followed by mycophenolate and tacrolimus in the 1990s. Further incremental improvements in outcomes can be attributed to the widespread use of lymphocyte depleting antibodies (antithymocyte globulin, muromonab-CD3, alemtuzumab) throughout this period and into the 21st Century. Despite substantial improvements in reducing graft loss from immunological causes, pancreas transplantation has been associated with significant early post-operative complications. The most common and troublesome of these is graft pancreatitis that can result in graft loss, severe morbidity, and death. Improvements in preventing and treating graft pancreatitis have not been forthcoming and, alongside the morbidity of immunosuppression, this is one of the reasons that pancreas transplantation is still reserved for those with the most severe complications of diabetes.

The problems experienced with graft pancreatitis have reinforced the desire to avoid transplantation of the troublesome exocrine pancreatic tissue. Transplantation of only the

pancreatic islets meets the clinical need of a patient with diabetes and avoids the technical complications associated with whole organ pancreas transplantation. However, the islets of Langerhans represent only 2% of the pancreas and effective isolation of the islets has proven to be very difficult. Lacy and Kostianovsky found that infusing collagenase directly into the pancreatic duct allowed isolation of hundreds of islets[88]; this technique has continued to be refined over subsequent decades with successful recovery of up to 50% of islets now achievable[89]. In 1972 Ballinger and Lacy demonstrated successful transplantation of islets in rats[90]. However, it was not until 1980 that Sutherland et al. reported 7 islet allografts and 3 islet autografts[91]; the allografts were not clinically successful but 2 of the 3 autografts remained insulin free for more than a year after pancreatectomy. The main barriers to islet allotransplantation at this time were a lack of consistent and efficient methods of islet isolation, effective and non-toxic immunosuppressive regimes, and availability of human pancreases[92]. Although islet isolation has continued to improve, poor islet yield remains the main reason for unsuccessful transplantation after pancreas retrieval and it is not uncommon for multiple donors to be required to achieve a suitable islet mass or for patients to receive further islet transplants in the future. Shapiro et al. subsequently reported a steroid-free immunosuppression regime that became known as the 'Edmonton protocol'; outcomes with this regime were much better, with insulin independence observed in 7 recipients with T1DM and this was subsequently confirmed in a multicentre trial[93,94]. More than 1000 islet transplants have now been performed worldwide and the UK was the first country to set up a government funded islet transplant programme[95]. Outcomes have continued to improve with insulin independence rates reported in a phase 3 trial of 52% and 42% at 1 and 2 years respectively. More pertinent, however, is that 87.5% of patients had an HbA_{1c} level $\leq$ 7% and freedom from severe hypoglycaemic events at 1 year (71% at 2 years)[96].

Vascularised pancreas and islet cell transplantation are currently the only beta cell replacement techniques available. Both have the requirement for chronic immunosuppression which carries its own considerable morbidity, and both have procedural risks although these are much less significant in islet cell transplantation. There are several techniques that may offer revolutionary advances in the future. These include islet encapsulation, stem cells, xenotransplants, and prevention of T1DM by treatment during its earliest phases. The 'holy grail' of beta cell replacement would achieve the longstanding restoration of normoglycaemia without the punitive morbidity of immunosuppression (possibly by means of immunological tolerance). Whilst it is exciting that there are so many areas of research addressing diabetes, progress in the translation of these techniques has often been overestimated[97].

1.4 Pancreas Transplantation

Pancreas transplantation is now an established treatment option for some patients with advanced complications of diabetes. It offers insulin independence and significant improvements in both mortality and quality of life. So far 48,000 pancreas transplants have been performed worldwide with more than 29,000 of these performed in the US and over 2,500 performed in the UK[98-100]. Although pancreas transplantation has been performed in the UK since the 1970's, the majority of transplants have been performed over the last 20 years. The number of pancreas transplants performed annually in the UK has remained fairly static over the last 10 years whereas there has been a decreasing trend in the US and in some other parts of Europe (Figure 1.4)[98]. This is surprising given the increasing prevalence of T1DM and improving outcomes seen in pancreas transplantation. The reasons for the decline seen in other countries are multifactorial but may include improved medical management of diabetes, poorer quality donor organs, and reduced frequency of referrals from diabetologists, endocrinologists and nephrologists.

Pancreas transplantation is most commonly performed alongside kidney transplantation for patients with diabetic end-stage renal disease. Simultaneous pancreas kidney transplantation (SPK) accounts for approximately 80% of all pancreas and islet transplants in the UK with 168 being performed in 2017/18 (Figure 1.5)[101]. Whole organ pancreas transplantation alone (PTA) is performed in diabetic patients with severe metabolic complications, particularly hypoglycaemic unawareness, or after previous living donor kidney transplantation (PAK). Solitary pancreas transplantation accounts for less than 10% of all pancreas and islet cell transplants; indeed, numbers have been reducing over the last 10 years with only 17 performed in the UK in 2017/18 compared with 53 in 2008/09. Islet transplants are performed in similar patients to PTA, that is those with life-threatening hypoglycaemic unawareness. Annual numbers of islet transplants in the UK significantly increased after the

UK Islet Transplant Consortium was commissioned in 2008/09 and have fluctuated between 22 to 33 since 2011 (Figure 1.5).

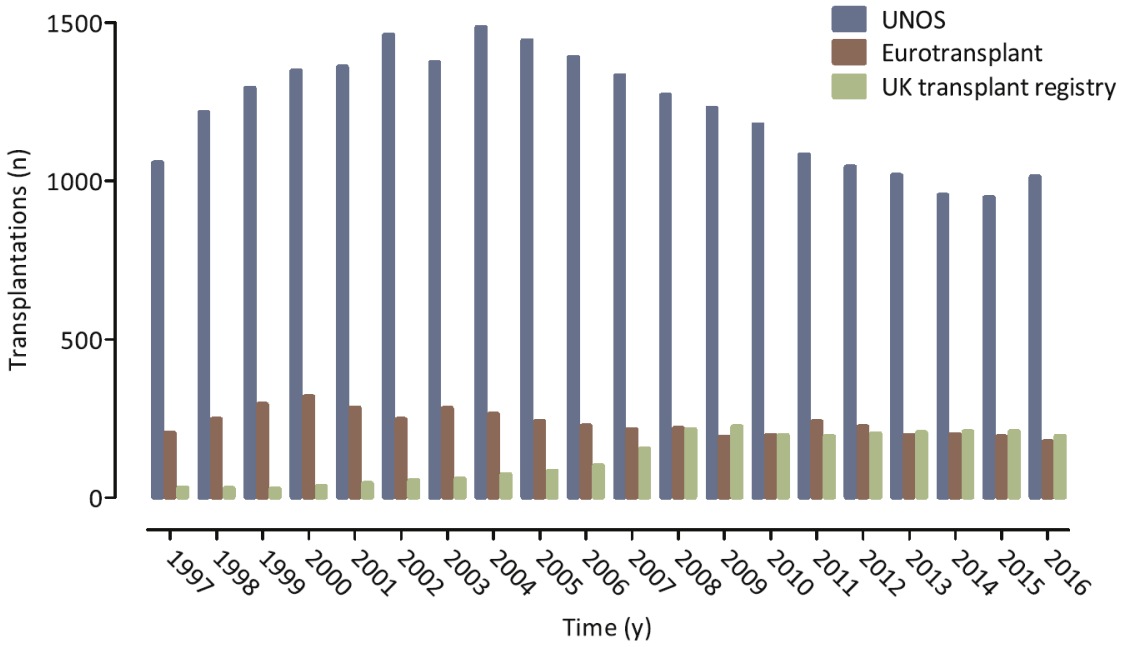


Figure 1.4: Yearly number of pancreas transplants for the past 20 years, for UNOS, Eurotransplant, and the UK transplant registry. Reproduced with permission from Elsevier[98].

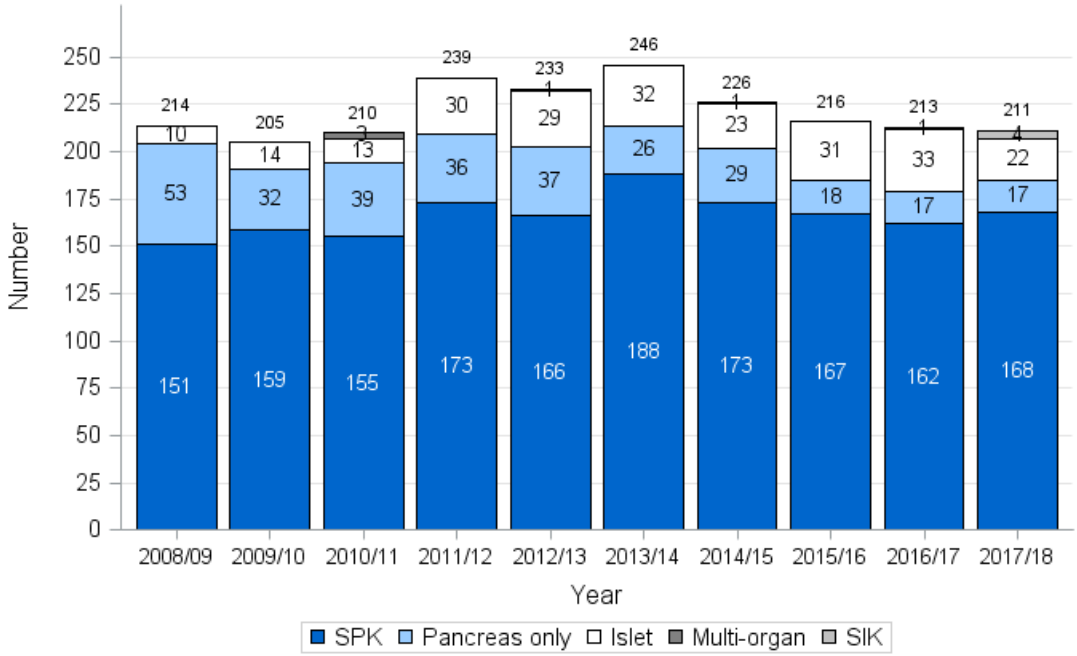


Figure 1.5: Pancreas and islet transplants performed in the UK, 1 April 2008 to 31 March 2018 by financial year and transplant type. Reproduced from NHSBT Annual Report on Pancreas and Islet Transplantation 2017/18[101].

1.4.1 Technical aspects of pancreas transplantation

Since 1966 there have been numerous refinements to the surgical techniques used in pancreas transplantation in the effort to improve outcomes. With the exception of a few small cohorts[102], nearly all pancreas transplants have been from deceased donors. The main issues are defined by the anatomy, that is arterial inflow, venous drainage, and exocrine drainage. These are illustrated in Figure 1.6. Arterial supply has generally been standardised with units utilising a 'Y' graft of the donor's iliac arteries to reconstruct onto the superior mesenteric and splenic arteries to form a common conduit for anastomosis onto the recipient's artery. This is usually the right common iliac artery in units that position the pancreas intraperitoneally; a small number of units use an extraperitoneal approach and therefore use the external iliac vessels in a similar manner to renal transplantation. It is considered good practice to test for communication between pancreaticoduodenal arterial arcades in the head of the pancreas by assessing backflow of perfusion fluid through the gastroduodenal artery, so that where this is absent then further reconstruction can be performed[103,104].

Venous drainage of the pancreas has provided a source of controversy. There have been two main techniques proposed - systemic venous drainage by anastomosis of the donor portal vein to the recipient's inferior vena cava or iliac vein, and portal drainage by anastomosis of the donor portal vein to the recipient's superior mesenteric vein. Anastomosis to the IVC produces direct systemic drainage of the pancreatic endocrine hormones; the main effect of this is systemic hyperinsulinaemia, although regulatory measures ensure that normoglycaemia ensues (discussed further in Chapter 3). There has been concern that hyperinsulinaemia itself can contribute to accelerated atherosclerosis and cardiovascular disease[105,106], however this has not been corroborated in pancreas transplantation[107]. In addition to a more physiological hormone delivery, it was proposed that portal venous

drainage may improve immunological outcomes due to development of 'portal tolerance'[108]. However, this remains unproven, and it has not translated into any difference in graft outcomes[107,109,110]. More than 90% of pancreas transplants are now performed with systemic venous drainage[111].

Exocrine drainage of the pancreas allograft has also been contentious. Although many pancreas transplant pioneers used segmental pancreas grafts (comprising the body and tail of the pancreas vascularised via the splenic vessels), it is now almost universal practice to transplant the whole pancreas together with a short segment of donor duodenum, as a conduit for drainage. Drainage of the duodenum to the bladder has largely given way to direct anastomosis to the small intestine of the recipient. Enteral drainage includes duodenal, jejunal, and ileal locations with or without the use of a Roux limb. Bladder drainage was used to avoid the risks of enteric anastomotic leaks, but also provides a potential advantage in terms of urinary amylase monitoring and trans-cystoscopic access for biopsy. However, bladder drainage is associated with unique metabolic and urinary complications that contribute to significant morbidity. Enteric drainage is currently practised in more than 80% of all pancreas transplants performed[111].

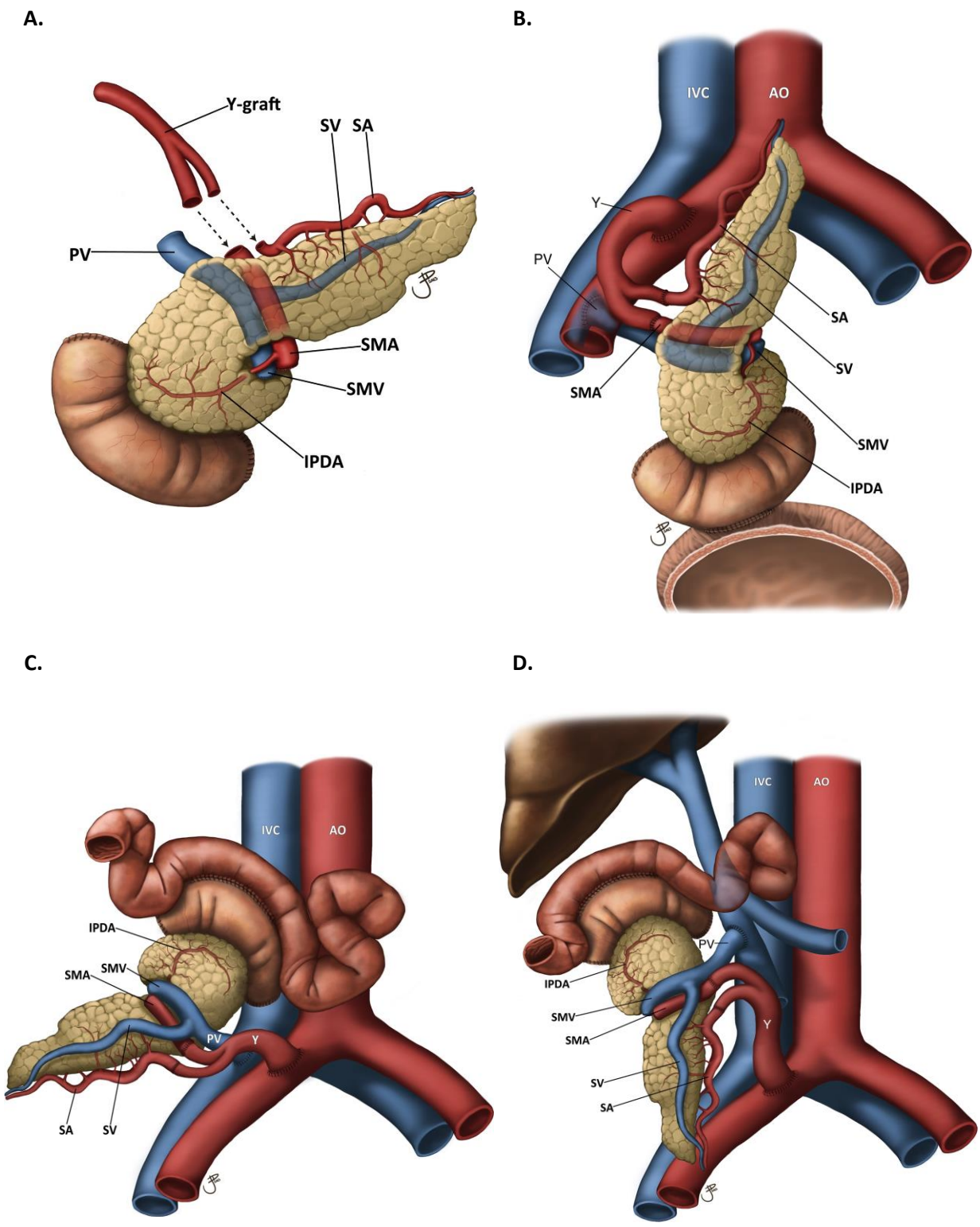
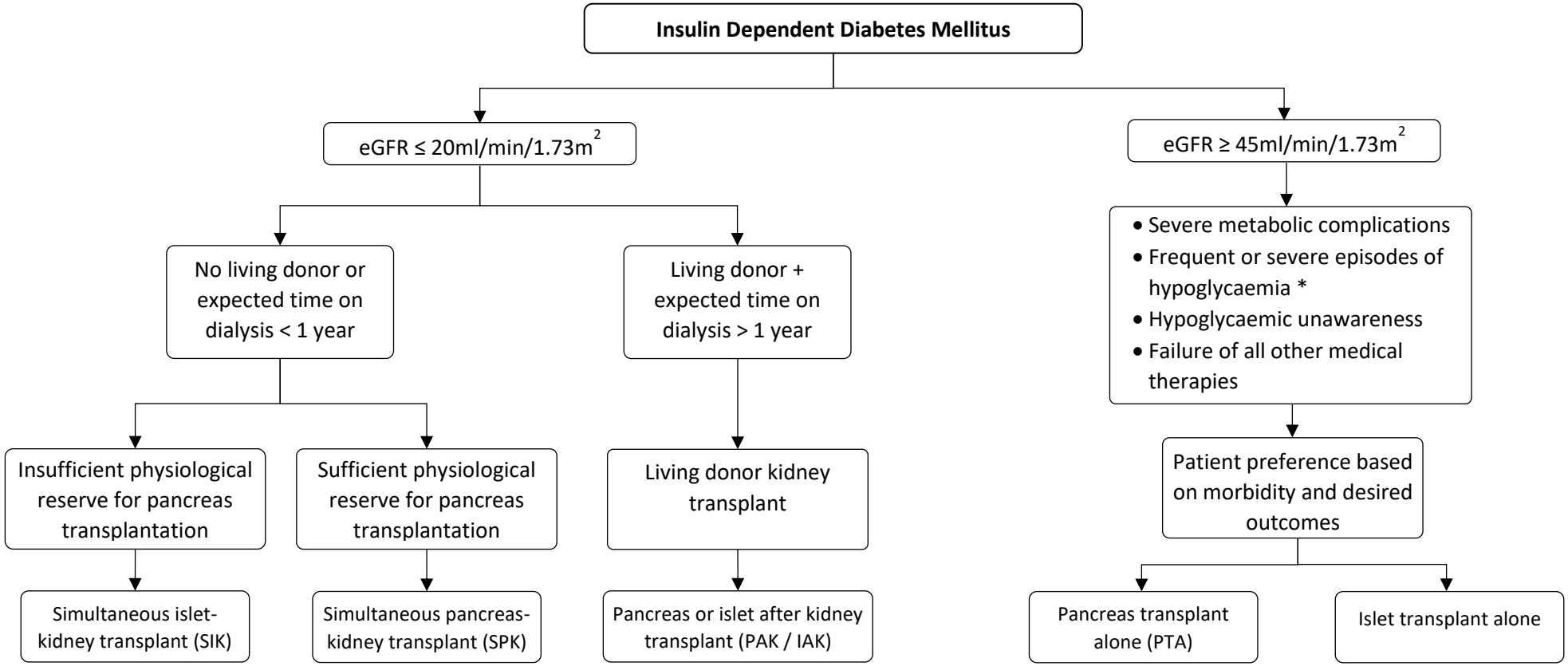


Figure 1.6: Schematics of pancreas transplantation. (A) explanted pancreas with donor Y-graft; (B) systemic vascular connections with duodenocystostomy; (C) systemic venous drainage; (D) portal venous drainage transplanted pancreas. Ao = native aorta, IPDA = inferior pancreaticoduodenal artery, IVC = native inferior vena cava, PV = donor portal vein, SA = donor splenic artery, SMA = donor superior mesenteric artery, SMV = donor superior mesenteric vein, SV = donor splenic vein, Y = donor Y graft. Images courtesy of Medical College of Wisconsin and Dr Jennifer Davies[112].

1.4.2 Indications for pancreas transplantation

The decision regarding the type of beta cell replacement can be difficult (Figure 1.7). Pancreas and islet transplantation are alternative procedures chosen on the basis of surgical risk. Pancreas transplantation requires a major abdominal operation with prolonged general anaesthetic and patients need to have adequate physiological reserve, not only to survive this and any subsequent complications, but also to benefit from the procedure. The main indication for SPK transplantation is end-stage diabetic nephropathy in a patient without any absolute contraindications to the procedure (Figure 1.7). The main indication for PTA in the UK is that of recurrent episodes of hypoglycaemic unawareness; however, internationally this has been diversified to include those with other severe metabolic complications of diabetes and those with 'brittle diabetes'. In the UK, the NHSBT Pancreas Advisory Group is empowered to authorise exemptions from the standard listing criteria in specific individual circumstances.



Contraindications to pancreas transplantation:

Relative	Absolute
BMI > 30 kg/m^2	BMI > 35 kg/m^2
Hepatitis B, C or HIV	Poor compliance (e.g. ongoing substance abuse, psychiatric illness)
Significant history of non-compliance	Active malignancy (excluding localised skin malignancy)
Significant cardiovascular disease	Immunosuppression likely to lead to life-threatening complications

Figure 1.7: Types of beta cell replacement therapy and an algorithm for clinical decision making for patients with T1DM. * In the UK, frequent episodes of severe hypoglycaemia is the main indication for pancreas alone or islet transplant. Adapted with permission from BMJ Publishing[99].

1.4.3 Outcomes after pancreas transplantation

The success rates of pancreas transplantation have continued to improve. Patient overall survival rates are > 96% at 1 year and > 88% at 5 years[101,111]. Graft survival rates (defined by insulin independence) are dependent on the type of pancreas transplant performed. Historically, SPK graft outcomes have been noticeably better than solitary pancreas transplants (both PTA and PKA), although there is some evidence that the gap may be narrowing[113]. The latest registry data from the UK show that risk-adjusted 1- and 5-year pancreas graft survivals for first SPK transplants are 89% and 79% respectively[101]. These compare with 89% and 73% in the US[111,114]. For PTA grafts the 1- and 5-year pancreas allograft survival rates in the UK are 88% and 52% respectively compared with 82% and 53% in the US. Where registry data exists, 10-year graft survival is nearly 60% for SPK and 40% for PTA and the estimated half-life of a pancreas allograft has been estimated at 10 to 15 years[111].

It is important to note that the definition of graft survival in pancreas transplantation is itself controversial. Graft failure has customarily been defined as a return to exogenous insulin. However, it has become increasingly clear that many patients who return to insulin still have some residual allograft beta cell function which reduces the amount of insulin therapy required and maintains some metabolic control that continues to stabilise baseline symptoms such as hypoglycaemic unawareness. This has been recognised for some time in the setting of islet transplantation where graft failure is not defined by insulin requirement but by stimulated C-peptide and clinical scoring tools (especially the absence of episodes of hypoglycaemia unawareness). A group of expert clinicians in the field has recently proposed 'the Igls criteria' for defining outcomes in beta cell replacement therapy, including pancreas transplantation[115]. This defines graft function as 'Optimal, Good, Marginal, Failure' depending on a combination of functional and clinical outcomes; it currently includes

measures of glycaemic control, C-peptide production, hypoglycaemic events, and insulin requirements. The authors recognise that this is still a work-in-progress and that the definition requires validation; however, it is progress towards a mechanism for meaningful comparisons across the range of beta cell replacement therapies. For the purpose of this thesis, graft failure in the context of pancreas transplantation is defined as a return to exogenous insulin unless otherwise specified.

1.4.4 Survival benefit with pancreas transplantation

Kidney, pancreas and islet transplants are performed to increase quality of life, with a longer-term benefit in terms of life expectancy, unlike liver and heart transplants which are more immediately lifesaving. A patient with end-stage diabetic nephropathy has the choice between dialysis, kidney transplantation alone, simultaneous pancreas-kidney transplantation, pancreas after living donor kidney transplantation or simultaneous islet-kidney transplantation. The advantages of renal transplantation compared with dialysis are well established, but the benefits in diabetic renal disease are less than those seen in other cohorts[116]. There is evidence that having an SPK transplant confers a significant survival advantage over a deceased donor kidney transplant alone[117-119]. Living donor kidney transplantation was thought to confer a similar survival advantage to that of SPK[120], however there is increasing evidence that the improved metabolic control of a pancreas transplant confers additional survival benefit over that of purely restoring renal function[113,121-123]. Survival after PTA and islet transplantation in patients with preserved renal function remains controversial. A paper by Venstrom et al. suggested that PTA patients had a higher mortality rate compared to patients remaining on the waiting list receiving conventional therapy[124]. However, there were significant methodological criticisms of the way in which this paper used registry data. Several further analyses have

since shown that mortality rates after PTA transplantation are in fact no worse than those receiving conventional therapy[113,125]. Data on islet transplants is more limited due to smaller numbers that do not allow robust conclusions about survival. It is important to bear in mind the very high mortality rate of diabetic patients on dialysis, and also the substantial mortality associated with hypoglycaemia-unawareness.

1.4.5 Quality of life and diabetic complications after pancreas transplantation

In addition to potential improved survival, there is evidence that pancreas transplantation may improve quality of life (QOL)[126-128]. The quality of life gained from the freedom from insulin, glucose monitoring, dietary restrictions and hypoglycaemia should not be underestimated and are the main reasons why many patients choose retransplantation after pancreas graft failure despite the operative risks. In the case of SPK transplantation, these benefits must be offset against the risk of additional operative morbidity over kidney transplantation alone. However, for PTA recipients, the stakes are much higher - high operative risks, poorer graft outcomes, and the morbidity of long-term immunosuppression and monitoring. Good quality data for patient reported outcomes after pancreas transplantation is limited - many studies are small and limited by generic tools for measuring QOL[129].

The DCCT/EDIC studies clearly demonstrated that improved glycaemic control could reduce microvascular complications of diabetes. Given that pancreas transplantation usually affords tighter glycaemic control than can be achieved with insulin, it is reasonable to extrapolate that it would also improve diabetic complications. Indeed there is evidence to support this, although much of it is from small single centre studies[130].

Diabetic renal changes have been reported within 2 years of kidney transplantation in patients with T1DM[131]; however, in the presence of a functioning pancreas transplant, the kidney allograft seems to be protected[132-134]. Furthermore, in PTAs there are reports of stabilisation and even reversal of diabetic lesions in the native kidneys[135-137]. This is encouraging given the nephrotoxicity of calcineurin inhibitors (CNI) that form an important part of all modern maintenance immunosuppression protocols.

There is also evidence for pancreas transplantation having positive effects on diabetic retinopathy. Giannarelli et al. investigated a group of PTA patients and compared them with a similar control group of patients with T1DM[138]. The follow-up was relatively short but most of the PTA patients had stabilisation or improvement of their retinopathy compared with 70% of the control group who had deterioration. This was supported by another study by the same group who investigated retinal findings in SPK patients[139]. A later study by Boggi et al. had larger numbers (n = 71) and longer follow-up of PTA patients. They also described stabilisation of retinal disease in most patients and improvement of non-proliferative retinopathy in 75% of patients[135]. A more recent study by Kim et al. with 153 patients confirmed these findings[140]; 80% of patients had stabilisation of diabetic retinopathy, although they also highlighted that 20% of patients can have significant deterioration within the first year of transplantation.

The evidence for amelioration of neuropathy after pancreas transplantation is mixed. There is evidence to support improvement in nerve conduction although this may occur more in somatic than autonomic nerves[135,141]. On the other hand, Havrdova et al. found no improvement in electrophysiological parameters after 8 years (only 12 patients) whereas Tavakoli et al. described significant improvements in corneal nerve fibre characteristics after just 12 months[142,143]. Similar to calcineurin inhibitor nephrotoxicity and renal outcomes, it is also important to acknowledge that immunosuppressants, particularly

tacrolimus, can cause significant neurotoxicity that may impair assessment of potential transplant outcomes. It would appear that the effect of pancreas transplantation on diabetic neuropathy is related to the severity at the time of transplantation and the length of follow-up.

Studies investigating the effect of pancreas transplantation on macrovascular complications are limited. Biesenbach et al. described significantly lower incidences of myocardial infarction, stroke and amputations in SPK versus kidney transplant alone (KTA) recipients[144]. Whilst they did achieve a median follow-up of 10 years, there were only 12 SPK patients being compared with 10 KTA patients. Lindahl et al. compared several cardiac parameters in 25 SPK and 17 LDK transplant patients[145]. They did not see any difference in coronary calcification or left ventricular systolic function. However, Fiorina et al. did see a significantly higher left ventricular ejection fraction in SPK patients compared with KTA and T1DM control patients[146].

There is a lack of high quality prospective studies investigating the effect of transplantation on diabetic complications. Many of the reported studies are small single centre studies with low numbers and short follow-up. It is, however, highly likely that any significant stabilising or reversing effect obtainable from pancreas transplantation requires a functioning graft for a long period of time. This makes it even more important that graft loss is minimised in the long-term as well as the short-term.

1.5 Causes of Pancreas Allograft Failure

Despite significant improvements in pancreas allograft survival over the past 3 decades[147], there is still a high rate of graft loss. The rate of graft loss differs between SPK and PTA, but there is still a common pattern across all pancreas transplant cohorts (Figure 1.8). There is a rapid rate of graft loss observed in the first 4 weeks after transplantation, followed by a moderate rate of failure over the next 12 to 18 months and then a more consistent lower rate of graft failure thereafter.

A common cause of early graft loss is acute vascular thrombosis of the graft[148-151]. This usually occurs within the first 2 weeks and often within 72 hours of transplantation. It is mainly secondary to acute venous thrombosis and is frequently described as a 'technical' loss, inferring surgical error. Whilst there are technical factors such as graft positioning and the use of venous extension grafts that may contribute, in reality the aetiology is diverse involving multiple donor and recipient factors. Many recipients undergo a period of hypotension necessitating vasopressors, potentially exacerbated by a hypercoagulable state[152,153]. In addition, there is low blood velocity in the high capacitance splenic and portal veins (following removal of the spleen), adding a further risk for thrombosis. Several techniques to ameliorate this have been attempted such as creating an iatrogenic A-V fistula between the splenic artery and vein[154]. Most centres currently use prophylactic anticoagulants to reduce the risk of acute thrombosis[155,156], at the expense of increased haemorrhagic complications. Some centres advocate early exploration if there are any untoward findings and have reported limited success with graft salvage if thrombosis is identified early enough[157]. This has not translated into improved graft survival rates though. Isolated early arterial thrombosis is rare and is usually related to iatrogenic arterial injury during retrieval, back-table preparation or implantation[158]. Most centres use a Y graft reconstruction technique which is associated with lower rates of thrombosis[159].

A very common, and often related, cause of early pancreas loss is graft pancreatitis. All pancreas transplants develop some degree of pancreatitis from ischaemia reperfusion injury, although in most cases this is mild and self-limiting[160,161]. However, in a substantial minority of cases this develops into severe pancreatitis and triggers a systemic cytokine storm and associated inflammatory response syndrome with progressive organ dysfunction. Graft necrosis can occur, complicated by sepsis in a highly immunocompromised patient. Relook laparotomies with washouts, debridement and laparostomy can achieve graft salvage at the expense of increased morbidity but not infrequently graft pancreatectomy is the only treatment option. There are several factors that are associated with a lower risk of graft pancreatitis such as donor quality, organ perfusion with University of Wisconsin solution, and a cold ischaemia time of less than 12 hours[162-164]. However, there is no strong evidence to support any interventions in the donor or recipient that could reduce the incidence or severity of graft pancreatitis.

There are other rarer causes of early pancreas graft loss such as infection, enteric anastomotic leak and haemorrhage, but these can usually be salvaged. What is less frequently discussed however is the role of immunological causes of early graft failure. Drachenberg et al. analysed 74 graft pancreatectomies and of those lost in the first 4 weeks, 33% had histological evidence of acute rejection[165]. These grafts showed immune mediated vascular injury that had subsequently caused acute thrombosis and graft loss. Given that rejection can also cause acute graft pancreatitis, it is possible that some of the early losses attributable to pancreatitis may also have an immunological component[166]. Therefore, some of the early graft losses that are described as ‘technical losses’ may actually be related to alloimmune damage. This is loosely supported by data from Humar et al. who investigated 123 cases of graft loss[150]. They describe early graft loss rates of 24.4% between 1985 to 1989, 18.3% between 1990 and 1994, and 13.1% between 1994 and 2003. There was no

substantial change in their surgical techniques during this period and so it is highly probable that much of this reduction in graft loss is due to improvements in immunosuppression protocols. In their series of 94 pancreas allograft biopsies in 162 pancreas transplants, Niederhaus et al. described acute rejection in 5% of samples taken within 1 month of transplantation[167]. Thus, although markedly reduced, early rejection does still occur in the current era of lymphocyte depleting induction agents and we should be mindful not to overlook this as a potential contributing factor to early pancreatitis and thrombosis.

After 6 weeks, rejection is by far the most common cause of graft failure accounting for more than 50% of graft losses[168]. The rate of pancreas rejection at 1 year has been reported as between 11.5 and 22%[167,169-172] for SPK transplant recipients, and in a study by Dong et al., this increased to 26.6% by 5 years[169]. The figure for PTA recipients is thought to be higher given the poorer graft outcomes. The true long-term rate of rejection is difficult to calculate but has even been reported as high as 91% at 5 years[173]. Nevertheless, most rejection is successfully treated and this translates into a 1 year graft loss rate of up to 6% in recent literature, but depends greatly on transplant type and series reported[174,175]. The types of rejection reported vary with Niederhaus et al. describing a fairly even incidence of cellular mediated, antibody mediated, and mixed rejection episodes whereas Uva et al. described cellular rejection in more than 80% of biopsies and antibody mediated rejection in the remainder[167,172]. Antibody mediated rejection is known to contribute to microvascular injury, intimal hyperplasia and graft fibrosis which accounts for most long term graft loss[176]. Indeed, a study by Mittal et al. demonstrated that the development of donor specific antibodies was associated with a much higher rate of graft loss[177]. The type of rejection clearly has implications for treatment options and their success (Section 1.8).

Graft thrombosis and pancreatitis can contribute to graft failure in the medium and long term, but they are much less common than in the early post-operative period and are usually

reflective of another underlying pathology such as immunological or infectious assault. Late haemorrhage has also been attributed to causing late graft loss. This is usually due to the development of arterio-enteric fistulae or pseudoaneurysms which are typically mycotic in nature[178].

A return of islet autoantibodies may also contribute to graft loss after pancreas transplantation despite immunosuppression. Several studies have shown histological features of recurrent T1DM in the absence of any features of rejection and this has been associated with rising autoantibody titres in some cases[179-181]. There is a general paucity of data, but the most compelling evidence comes from Vendrame et al. who described a T1DM recurrence rate of 7% in their cohort of 233 pancreas transplant recipients[182]. The diagnosis was confirmed by hyperglycaemia, biopsies demonstrating insulitis and beta cell loss, and positive autoantibody serological testing. Patients that were autoantibody negative at the time of transplantation and subsequently converted to autoantibody positivity had the highest risk of developing recurrent T1DM.

In T2DM there are several characteristic histopathological features seen in the pancreas such as reduced beta cell mass, increased beta cell apoptosis and deposition of islet amyloid[183-185]. Interestingly, some of these features may also play a role in long-term pancreas allograft failure[176]. Fradejas et al. described 2 patients with graft failure at 5 and 8 years who had prominent accumulation of islet amyloid[186]. This has been corroborated in Oxford where 3 functioning pancreas grafts removed for other reasons had evidence of amyloid deposition and increased alpha cell populations (Clarke et al., unpublished). Whilst chronic rejection and allograft fibrosis is thought to account for the majority of long term graft loss, the role of alternative pathological processes like amyloid deposition is unknown.

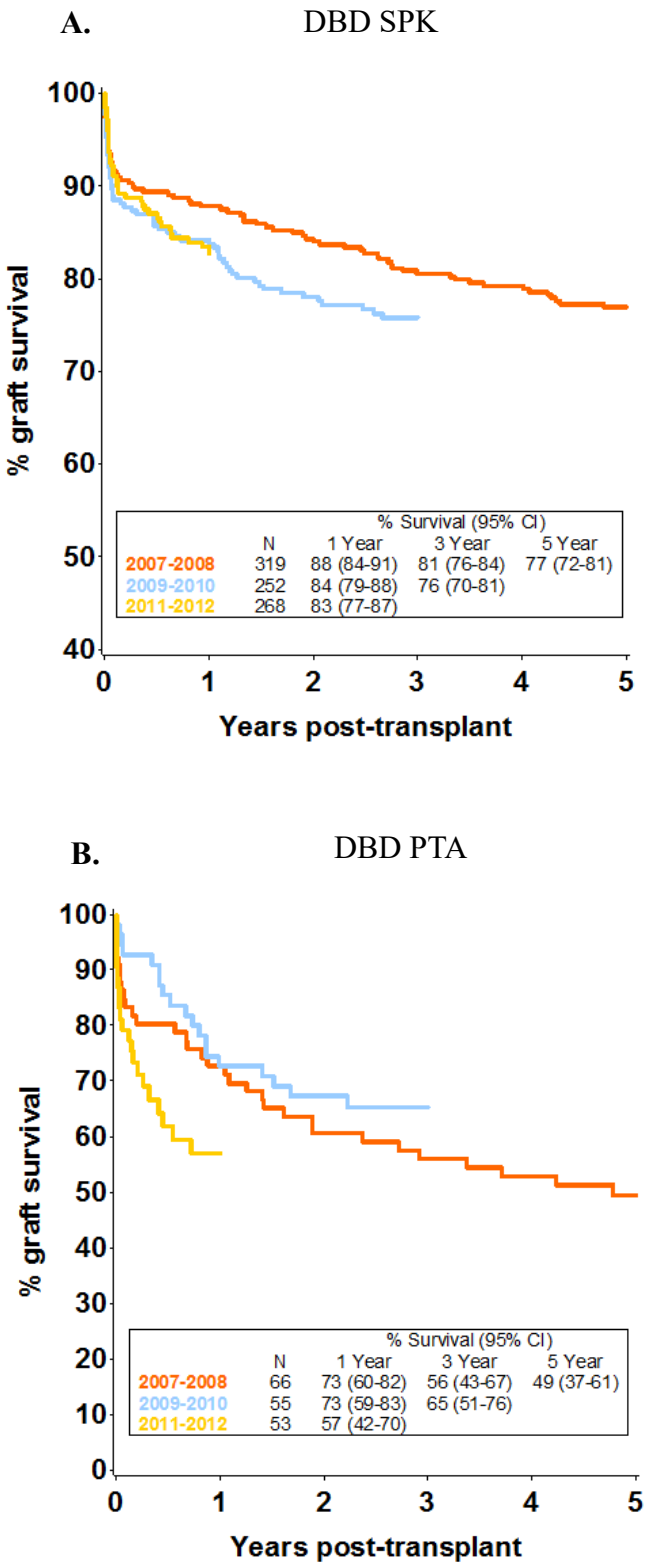


Figure 1.8: Pancreas allograft survival in the UK. (A) DBD SPK graft survival 2010 – 2014 (B) PTA graft survival by donor type 2010 - 2014. NHSBT pancreas activity data[187].

1.6 Pancreas Allograft Surveillance

Pancreas allograft survival remains poor in comparison to outcomes seen in liver and kidney transplantation. This is particularly pronounced for PTA and PAK transplantation although even in SPK transplantation the pancreas outcomes are notably worse than that of the co-transplanted kidney. This is generally believed to be due to the fact that we do not have a robust and reliable method of monitoring the transplanted pancreas. Clinical signs and symptoms of graft rejection are often absent and by the time hyperglycaemia is present, the damage is usually irreversible. Most pancreas transplant units employ a range of biochemical, pathological, immunological, and radiological investigations to monitor the transplanted pancreas. However, none have an equivalent level of utility to those used in kidney and liver transplantation.

1.6.1 Biochemical Surveillance

Biochemical measures of pancreas function in diabetes are commonly used in pancreas transplantation but have not been validated. The physiology of the pancreas and glucose metabolism is substantially altered due to transplantation of a denervated pancreas in a heterotopic position. The effect of this on islet function, liver metabolism, the incretin effect and other neurohormonal feedback loops are not known.

The exocrine tissue of the pancreas makes up 98% of the pancreas gland and is often used as a surrogate for endocrine tissue. Serum amylase and lipase are frequently elevated in episodes of pancreas rejection with a study by Klassen et al. describing a mean increase in amylase and lipase of 3.6 and 8.3 respectively[188]. Unfortunately, neither is sensitive nor specific enough to be wholly reliable. Sensitivity has been reported as 50% to 79% and specificity of 54% to 80% [188-190]. There are many other causes of raised pancreatic enzymes

depending on timepoint after transplantation, whilst the origin can also be from the native pancreas (Figure 1.9).

One of the main advantages of using bladder drainage for the exocrine pancreas is the ability to monitor exocrine function. Urinary amylase can be easily measured either in a 24-hour collection or as a spot sample with a creatinine ratio[191]. A reduction of more than 50% has been associated with acute rejection, and it has been suggested that rejection of the exocrine tissue can precede rejection of the islets, thereby enabling early treatment[192,193]. Because of a false positive rate of up to 53% [194], in some units a reduction in urinary amylase triggers further investigations, possibly including trans-cystoscopic biopsy of the donor duodenum or pancreas (discussed further in Section 1.6.2). Nevertheless, bladder drainage of the pancreas is associated with significant additional morbidity. Cystitis, reflux graft pancreatitis, dehydration, acidosis, reflux nephropathy and severe electrolyte disturbance can occur requiring often recurrent additional inpatient treatment. Some centres have reported that up to 50% of patients require conversion to enteric drainage necessitating further laparotomy. Despite this additional morbidity, Adler et al. have recently reported that this has no detrimental effect on allograft survival[195]. However, in the current era of immunosuppression there is limited evidence to suggest that the ability to monitor urinary amylase has any significant beneficial effect on pancreas graft survival; indeed the complications of bladder drainage may contribute to a decline in renal function[164,196]. This is the main reason that most pancreas transplants are now carried out with enteric drainage.

The endocrine function of the pancreas allograft can be assessed using similar methods to those used in diabetes mellitus. The simplest method is self-monitoring with a finger prick capillary glucose measurement, with which most diabetic patients are familiar. Patients are advised to contact their clinical team if they observe high values which then prompt further investigation. Compliance in the medium to long-term is frequently poor and

hyperglycaemia is usually a sign of irreversible allograft damage. Continuous glucose monitoring can be used but this still requires capillary glucose calibration and active patient engagement. In pancreas transplantation this is more frequently used in a research setting rather than routine clinical practice to identify graft dysfunction.

Serum insulin and C-peptide are routinely measured in clinical follow-up of pancreas allografts in many centres. Random non-fasting or starved samples are unlikely to be helpful given the significant endocrine reserve of the pancreas. Stimulated samples are likely to be the most useful and indeed form a core part of islet transplant assessments such as the Beta score[197]. However, in order to detect a deterioration in endocrine function at a time that allows early intervention, the test would have to be performed very frequently. Many patients have to travel considerable distances to their pancreas transplant centre and are poorly compliant with requests to attend starved and then stay for several hours for a stimulated test to be performed. C-peptide has traditionally been used as a surrogate for insulin secretion due to loss of insulin by first pass liver metabolism. However, in pancreas transplantation with systemic venous drainage, serum insulin can be reliably measured. However, the effects of systemic endocrine drainage, variable renal allograft function, and immunosuppression on glycaemic control are unknown. It is therefore difficult to interpret the results with any meaningful conclusions about graft function at each timepoint or longitudinally, or indeed make comparisons between patients. In diabetes, several indices have been developed that attempt to help with this. One example is the Homeostatic Model Assessment (HOMA)[198], that combines known formulas for insulin and glucose metabolism in a variety of tissues involved with glucose metabolism. A serum glucose and either insulin or C-peptide value is entered, and the model calculates a beta cell function and insulin sensitivity output as a percentage of a standardised population. It is useful for intra- and inter-individual comparisons and has utility in both clinical practice and research. It is not

currently used in pancreas transplantation due to transplant and model specific problems that will be discussed further in Chapter 3.

Stimulated tests such as the oral glucose tolerance test (OGTT) and mixed meal tolerance test (MMTT) are frequently used in diabetes and several studies have shown that worsening glucose tolerance is associated with the subsequent development of T1DM[199]. Both of these tests are used in pancreas transplantation but have not been validated as reliable measures of pancreas allograft function. Mittal et al. demonstrated that early impaired glucose tolerance during OGTT was associated with a much higher rate of graft failure at 1 year[200]. The problem remains though of how we translate such a prediction into improved graft surveillance that can actually influence timely intervention and graft outcomes.

Glycated haemoglobin (HbA_{1c}) has been used for many decades as a marker of glycaemic control over the preceding 2 to 3 months. In the DCCT trial HbA_{1c} reliably predicted the development of secondary diabetic complications and it now forms a routine part of diabetic monitoring. It has been shown to correlate with the development of diabetes and, although controversial, forms part of the World Health Organisation's diagnostic criteria for diabetes[201]. It has also been shown to correlate with subsequent graft failure in pancreas transplantation[202]. However, it does not track short-term variability in glycaemic control nor current allograft dysfunction. Therefore, it is less likely to be useful for graft monitoring due to the infrequency of sampling and delay before HbA_{1c} rises.

There are several other biomarkers that have been investigated for the purpose of pancreas allograft surveillance. Whilst tests such as serum CRP and procalcitonin can indicate an acute inflammatory response that may correlate with graft pancreatitis or rejection, their low sensitivity and specificity mean they are unreliable for longer term graft surveillance[203,204].

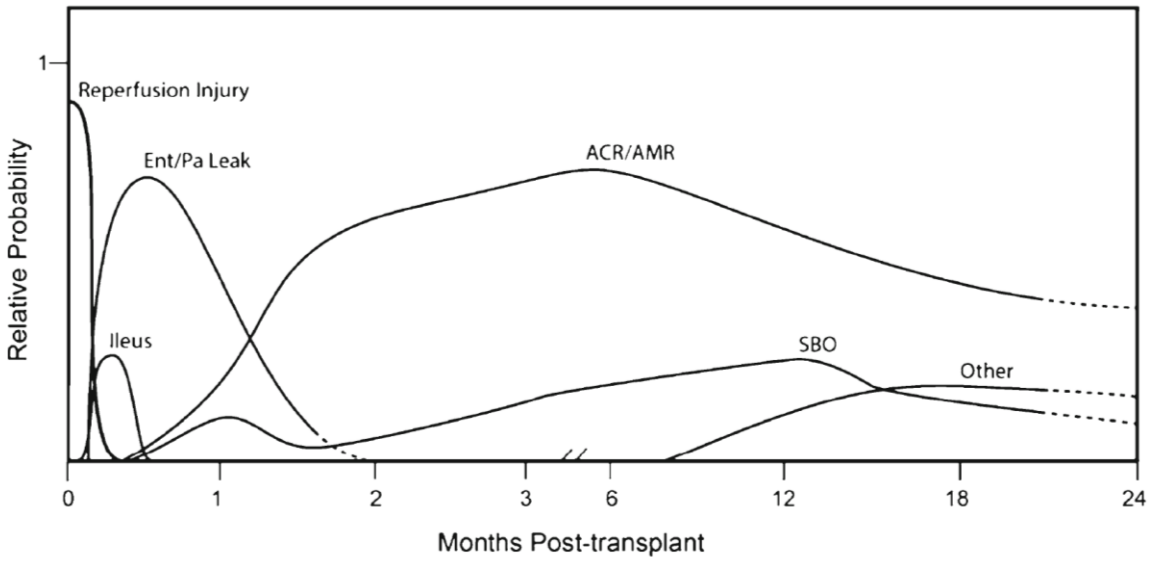


Figure 1.9: Temporal relationship between raised amylase/lipase to aetiology. Note Y axis does not represent overall probability of incidence but conveys relative probability that these diagnoses are associated with elevated pancreatic enzymes. Complete graft thrombosis rarely presents with increased pancreatic enzymes. 'Other' causes include transplant duct stricture, native pancreatitis, IPMN. Reproduced with permission from Springer Nature[170].

1.6.2 Allograft Biopsies

Pancreas parenchymal biopsy with immunohistochemical assessment remains the gold standard for diagnosis of pancreas allograft rejection[170,205]. However, whilst some centres have a large series of ultrasound or CT guided percutaneous biopsies of the pancreas with complication rates lower than 2%, others have described more frequent and serious complications from this technique[170,206,207]. Laparoscopic and open pancreas biopsies have therefore been utilised by other groups[208]. The risk of biopsy failure has been reported as high as 10 to 20% and therefore the morbidity associated with post-procedural haemorrhage, exocrine leak, injury to other viscera, and further surgery must be balanced with the need for a definitive diagnosis[175,209,210]. For this reason, many units do not routinely practice direct biopsy of the pancreas allograft.

In the case of SPK transplantation, it is sometimes assumed that any alloimmune response will be present in both kidney and pancreas grafts. The kidney can therefore be used as a surrogate and is much easier to monitor with serum markers of renal function, ultrasound and percutaneous biopsy. However, there is considerable discrepancy in the literature regarding concordance of rejection between the pancreas and kidney. Papers have been confined to small numbers of retrospective studies with considerable bias. It was recognised in the 1980s that discordant rejection could occur between the pancreas and kidney with total loss of one organ and preservation of the other[211]. A study by Troxell et al. in 2010 looked at 21 paired biopsies in SPK recipients[190]; discordant rejection occurred in 38% of cases and 29% had rejection of the pancreas without kidney rejection. The University of Wisconsin group is a vocal proponent of percutaneous pancreas biopsy and has performed more than 400 to date[170]. On retrospective review of their data, they identified 40 pairs of pancreas and kidney biopsies in SPK patients[212]. Discordance between the organs occurred in 38% of cases with 75% of these having isolated rejection of the pancreas. Furthermore,

in those that did have concordant rejection, 36% had different types and grade of rejection in the pancreas and kidney. However, the discordant findings in both of these studies have been questioned[213]. Timings of the paired biopsies differed by up to 30 days which is a long time in the development and treatment of an alloimmune response. Additionally, the fact that a difference in type of rejection was observed between organs has highlighted the challenges associated with the diagnosis of antibody mediated rejection. A recent study by Uva et al. provides significant additional insight into this conundrum given their routine practice of taking simultaneous biopsies of both the kidney and pancreas when dysfunction occurs in either organ[172]. Laparoscopic biopsies of both organs were taken with very low rates of non-diagnostic samples[208]. They describe 101 kidney and pancreas biopsies in 70 patients. Concordance was noted in 66% of patients, although when looking at just those with rejection only 40% of cases were concordant with 27% showing isolated pancreas rejection. They also confirmed a difference in type and grade of rejection between the organs. However, the authors concluded that, in the context of pancreas dysfunction, a positive kidney biopsy predicts pancreas rejection in 86% of cases. When considering the 'sentinel' aspect of the kidney biopsy in these 3 studies, it appears that the chance of having isolated, and therefore untreated, rejection of the pancreas is in the region of 14 - 29%.

In units that do not routinely perform direct biopsy of the pancreas, some have advocated the use of endoscopic biopsy of the donor duodenum. In the case of bladder drained grafts, this is straightforward with a transcystoscopic approach. With enterally-drained grafts the position of the anastomosis determines the ease of the procedure; an enteroscope is required to access the jejunum whereas Hummel et al. described an anastomosis to the 3rd part of the recipient duodenum that facilitates access with standard upper gastrointestinal endoscopy[214]. However, in a similar manner to SPK transplants, the question of whether there is concordant rejection between the pancreas and duodenum arises. Nakleh et al. first

made the observation that in pigs a normal duodenum did not rule out rejection of the pancreas[215]. The same group further corroborated these findings in humans with 25 concurrent duodenum and pancreas biopsies that demonstrated discordant rejection in 64% of cases although there was isolated pancreas rejection in 28% where the duodenum was normal[216]. Brockman et al. have recently described their experience with duodenal biopsies performed as part of routine surveillance protocols[175]. Although not paired with pancreatic biopsies to enable direct comparison of concordance, they report diagnosing rejection in 18 of 27 consecutive patients that underwent SPK and PAK transplantation. They were able to show histological improvement after treatment of rejection and also diagnose other complications of transplantation such as venous thrombosis and cytomegalovirus (CMV) infection.

1.6.3 Immunological surveillance

Routine clinical immunological surveillance is limited to assessment of donor specific anti-HLA antibodies (DSA) in most transplant units. There is a clear association between preformed DSA or *de novo* DSA (dnDSA) and graft survival in several transplanted organs[217-219]. Data is more limited in pancreas transplantation, but two large studies suggest a similar negative relationship between DSA and pancreas allograft outcomes. Mittal et al. analysed a cohort of 354 pancreas transplant recipients that had post-transplant DSA assessment[177]. 37% developed dnDSA and this was associated with significantly poorer graft survival at 1- and 3-years (hazard ratio of 4.66 for graft failure). However, they were not able to comment on associated episodes of rejection due to the lack of pancreas biopsies at this centre. Parajuli et al. described a cohort of 541 pancreas transplant recipients of which 22% developed dnDSA[220]. In the group with dnDSA, 42% of patients had biopsy proven acute rejection and 36% developed graft failure.

Other markers such as non-donor HLA and anti-endothelial antibodies, membrane glycoprotein sCD30, chemokines CXCL9 and CXCL10, as well as T cell reactivity have been used to try and identify patients at higher risk of rejection but have not been shown to correlate reliably with acute rejection[221]. Cashion et al. described serial analysis of granzyme B, perforin and HLA-DR in 13 pancreas transplant recipients[222]. Patients with rejection were found to have a significant increase in levels of these biomarkers up to 5 weeks before the clinical diagnosis of rejection and the granzyme B and perforin subsequently decreased following treatment. This was a small study with limited and varied follow-up, but the results are encouraging and warrant further investigation; the fact that there has been nothing else reported in the literature in the 9 years since this study is however ominous.

1.6.4 Radiological surveillance

Imaging is now an essential component of investigation in most aspects of modern medicine. Its value in the surveillance of pancreas allografts is however varied and depends specifically on the question being asked of it. Duplex ultrasonography and CT are routinely used in the early post-operative period to assess graft perfusion and look for complications such as collections, anastomotic leaks, and thrombosis[112,223]. In the longer term, duplex ultrasonography, CT, and MR angiography are useful for assessing pancreatitis, arterial stenoses and venous thromboses.

The role of imaging in aiding the diagnosis and monitoring of allograft rejection is less clear. Wong et al. used duplex ultrasonography to assess pancreas allograft and correlate findings with biopsy results[224]. They demonstrated a 58% sensitivity and 100% specificity for rejection. Gimenez et al. retrospectively analysed 182 sonographic pancreas allograft examinations in 51 patients[225]. A resistive index (RI) in the splenic artery of greater than 0.65 suggested ‘a problem’ with 86% sensitivity and 47% specificity. The level of elevated

RI could provide further clues as to the likely nature of the problem; an RI of greater than 0.75 may indicate splenic vein thrombosis, pancreatitis, or rejection, whereas an RI of greater than 0.9 is highly suggestive of rejection. There were small numbers with pathology in this paper and there have been no further prospective studies to validate this work. A study by Krebs et al. investigated the use of MRI in 25 patients and correlated results with a pancreas allograft biopsy performed within 3 days[226]. Rejection was reported in 18 patients and the MRI technique used had an impressive 96% sensitivity and 67% specificity. MRI technology has progressed significantly in the 20 years since this study, yet there have been no further reports of MRI being used in pancreas allograft surveillance.

1.7 Pancreas Allograft Salvage

Allograft surveillance is only worthwhile if there are interventions available that can modify the course of the identified pathology. Whilst many complications of pancreas transplantation will ultimately result in graft failure if left untreated, most can be reversed if identified in time.

As described in Section 1.5, venous thrombosis is the commonest cause of early pancreas graft loss. Complete occlusion of the major vessels of the pancreas allograft are usually associated with sudden onset graft dysfunction with rapid progression towards permanent tissue damage and necrosis if not diagnosed within hours. In most cases this results in graft failure and pancreatectomy although there are reports of successful surgical and radiological thrombectomy[227,228]. Some centres advocate intensive early surveillance whilst others have a low threshold for exploratory laparotomy[229,230]. The Indiana group reported that 10% of their patients had a return to theatre because of suspected thrombosis[157]; 57% of these had a compromised blood flow and 45% were salvaged by repositioning or thrombectomy. If this protocol led to more patients being salvaged than in other units, it should be reflected in graft outcome figures, yet this does not seem to be the case. Partial thrombosis of the splenic and portal veins and superior mesenteric artery are a common incidental finding on imaging of the pancreas allograft. They often reflect stasis of blood in the blind stumps of the splenic vein, superior mesenteric vein and superior mesenteric artery but there is concern they could propagate and render the organ ischaemic or produce systemic venous emboli. Such incidental thromboses are usually treated empirically with anticoagulation to prevent clot propagation but there is limited evidence to support or refute this practice[158,231]. Vascular thrombosis in the late post-operative period is usually associated with chronic alloimmune graft injury and salvage is rarely attempted[155]. Nevertheless, focal arterial stenoses of the Y graft, superior mesenteric or splenic artery can occur and if identified can

be successfully targeted with angioplasty[232]. Interventional radiology can also play a key role in the salvage of grafts with pseudoaneurysms and arterio-venous fistulas. Several centres have reported successful outcomes with placing covered stents to avoid laparotomy and probable pancreatectomy[233-236]. This is despite the fact that many late presenting pseudoaneurysms are mycotic in nature.

Acute rejection of the pancreas allograft is the main cause of pancreas graft failure from 6 weeks onwards. With modern immunosuppression it occurs in up to 22% of SPK recipients by 1 year but may be much higher over the long term, particularly if including features of chronic rejection[176]. Unlike kidney transplantation, acute rejection of the pancreas allograft is associated with poorer graft outcomes[167]. This is likely to reflect the lack of early diagnosis rather than a failure of treatment. Acute T cell mediated rejection (ACR) is highly treatable if identified before significant allograft damage occurs (i.e. when some beta cell function remains). Some centres use steroids as first line therapy whilst others maintain strict steroid avoidance strategies and will opt for muromonab-CD3, antithymocyte globulin (ATG) or alemtuzumab. Antibody mediated rejection is harder to treat successfully and there is a lack of consensus as to the best strategy to employ. In the presence of DSA some advocate the use of intravenous immunoglobulin (IVIG) and plasmapheresis with escalation to anti-B cell therapies such as rituximab, bortezomib, or eculizumab if appropriate[170]. This is seemingly based on the 2009 KDIGO guidelines for treatment of AMR in renal transplantation where it accounts for up to 72% of death-censored graft failure beyond the first year[237]. A recent systematic review and metanalysis of the treatment of AMR in renal transplantation concluded that there is a notable absence of evidence for the efficacy of these treatments despite plasmapheresis and IVIG in particular becoming standard of care[238]. The treatment of AMR in pancreas transplantation is even less well defined.

For pancreas allografts that have suboptimal glycaemic control but have not failed there may be options available for graft salvage. Glucagon-like peptide 1 (GLP-1) is an incretin hormone produced in the ileum that leads to a glucose dependent increase in insulin release. It also has other non-insulinotropic effects including glucose-dependent suppression of glucagon secretion and an improvement in hepatic insulin resistance and glycaemic control. In-vitro animal and human islet cell studies have shown that treatment with incretin hormones can promote insulin secretion and increase beta cell mass[239-241]. This is thought to be by stimulation of beta cell growth and differentiation but there is also evidence to support an anti-apoptotic and cytoprotective effect of GLP-1 on beta cells[242-247]. These are clearly desirable qualities for any potential therapeutic candidate. The role of GLP-1 therapy in pancreas transplantation is unknown and there is a paucity of clinical research in the field. Rickels et al. demonstrated that use of the GLP-1 agonist exenatide can enhance glucose-dependent insulin secretion in human islet and solid organ transplantation whilst Fadaji et al. showed that exenatide can help restore graft function in patients with islet graft failure[248,249]. However, benefit could not be attributed to exenatide alone and this is not transferable to clinical practice for solid organ pancreas transplants. Bellin et al. investigated the use of sitagliptin in islet autotransplantation after pancreatectomy and did not see any improvement in metabolic outcomes with the intervention[250]. However, sitagliptin has a different mechanism of action (DPP-4 inhibitor) than direct GLP-1 receptor agonists that had been previously used in most studies of GLP-1 therapies in islet and pancreas transplantation. A study by Cariou et al. investigated the use of liraglutide in 6 pancreas transplant recipients who had signs of a failing grafts many years after transplantation[251]. Whilst they observed a significant improvement in glycaemic control in this cohort, there has not been any further prospective studies of GLP-1 agonists in human pancreas transplantation.

1.8 Research Rationale

Pancreas transplantation has become an established treatment option for some patients with advanced complications of diabetes. Unlike other forms of beta cell replacement therapy, its overall success depends on graft survival, maintenance of insulin independence, and preservation of relative normoglycaemia. However, there is a need to improve graft outcomes to levels at least comparable to those experienced by other transplanted organs. Improved outcomes could then justify an expansion of indications and enable more people to benefit from this treatment. This would also produce significant social and economic rewards by avoiding advanced complications of diabetes and reducing healthcare costs.

The main factor limiting further advances in graft outcomes is the lack of sensitive and specific techniques for identifying graft injury before permanent damage occurs. The development of sensitive and specific methods that allow reliable allograft surveillance and timely intervention is essential to improving outcomes in pancreas transplantation.

1.9 Aims

This thesis describes a number of clinical studies performed in pancreas transplant recipients. The overall aim was to explore whether several novel approaches to pancreas allograft surveillance could provide advanced warning of imminent graft injury and thereby give the opportunity for early intervention and graft salvage.

The specific aims of this thesis are:

- To validate the HOMAtx model in pancreas transplant recipients. This is a modified version of HOMA model that allows for altered anatomy in pancreas transplantation (Chapter 3).
- To assess the use of urinary C-peptide creatinine ratio in pancreas transplant recipients and whether home samples could be used for remote monitoring of pancreas graft function (Chapter 4).
- To explore the use of vascularised donor derived composite allografts (sentinel skin flaps) in clinical pancreas transplantation (Chapter 5).
- To investigate immunological characteristics of using sentinel skin flaps in pancreas transplantation (Chapter 6).

Chapter 2

Common Methods

The methods described in this chapter are commonly used throughout this thesis. Specific methods for each chapter are found within the methods section of those chapters.

All participants that were involved with research in this thesis underwent pancreas transplantation at the Oxford Transplant Centre. Clinical procedures and protocols relevant to this thesis are summarised here.

2.1 Preoperative Work-up

All patients referred for pancreas transplantation undergo a vigorous assessment process prior to listing for transplantation. Once it is confirmed that they meet the indications for pancreas transplantation, and have no contra-indications to transplantation or immunosuppression, patients undergo extensive physiological, metabolic, and psychological assessment by the multidisciplinary team. Investigations include stress echocardiography, myocardial perfusion scintigraphy, spirometry, cardiopulmonary exercise testing and invasive angiography if indicated. Patients who are deemed too high risk by the MDT or who are unlikely to gain significant benefit from transplantation are returned to the care of their referring centre.

All patients undergo ABO blood group assessment, HLA genotyping and assessment of alloantibodies. Alloantibody assessment is repeated several times a year whilst on the transplant waiting list. Patients are reviewed annually whilst waiting for transplantation.

2.2 Organ Allocation and Retrieval

In the UK, pancreases are usually allocated for either solid organ or islet transplantation to named recipients according to a defined points-based scoring system (Appendix A)[252]. Organ donors are defined as either donor after brain death (DBD) or donor after circulatory death (DCD). All abdominal organs are retrieved by the NHSBT National Organ Retrieval Service (NORS) in accord with NHSBT published standards[253]. The general principles of retrieval are to safely remove organs without any damage, to minimise warm ischaemic time, and to ensure adequate perfusion of the organs with cold preservation solution. When the pancreas is being retrieved, all abdominal organs should be perfused with University of Wisconsin (UW) preservation solution. Dual perfusion via both the aorta and portal vein is used to allow adequate perfusion of both the liver and pancreas. During pancreas retrieval, the spleen is left attached to the pancreas and the duodenum is typically stapled through D1 and D4. The portal vein should be divided at least 1 cm from the confluence of the splenic and superior mesenteric veins. Organs are packed in sterile UW solution and stored in boxes of ice for transplantation to the receiving centres (static cold storage).

2.3 Pancreas Transplantation in Oxford

All transplantation at the Oxford Transplant Centre occurs at the Churchill Hospital in Oxford. Once a pancreas offer has been accepted, the recipient is asked to attend the Hospital where they are assessed and final suitability for transplantation is confirmed (e.g. no new illness or deterioration in cardiorespiratory function). Immunological crossmatch with the donor is checked with a combination of complement-dependent cytotoxicity, flow cytometry, and Luminex antibody testing.

The pancreas is unpacked and assessed for final suitability for transplantation, specifically looking for steatosis, fibrosis, and retrieval damage. It then undergoes back table preparation for transplantation. This involves removing the spleen, redundant duodenum, and additional non-pancreatic tissue. The arterial blood supply is reconstructed with a Y-graft using the donor iliac bifurcation (as in Figure 1.6A). The pancreas is flushed again with UW solution and collateral flow through the gastroduodenal artery is checked before it is ligated.

Once the pancreas has been checked and confirmed to be suitable for transplantation, the recipient is anaesthetised, and surgery commences simultaneously with the back table preparation of the pancreas. This reduces the cold ischaemic time of the pancreas. A partial Cattel-Braasch right sided visceral mobilisation is performed to expose the IVC and right iliac vessels. In the case of SPK transplantation, the pancreas is transplanted first and is placed on the retroperitoneum adjacent to the IVC with the head facing cranially. The portal vein is usually anastomosed to the IVC and the arterial Y-graft is anastomosed onto the right common iliac artery. The donor duodenum is passed through a window in the right mesocolon and anastomosed to a segment of recipient jejunum with a 2-layered side to side anastomosis. The kidney is usually transplanted onto the left external iliac vessels with either an intra- or extra-peritoneal position. In the case of PTA or PAK transplantation, the

procedure is very similar except that the pancreas is placed with the head facing caudally and the donor duodenum is anastomosed to the dome of the bladder.

2.4 Immunosuppression and Prophylactic Medications

All pancreas recipients received induction immunosuppression with a single dose of 30mg alemtuzumab subcutaneously and 500mg methylprednisolone intravenously before reperfusion of the pancreas. Prior to 2015, all patients received two 30mg doses of intravenous alemtuzumab - one during surgery and another on day two. Additional medication given during surgery included meropenem, micafungin, mannitol, and unfractionated heparin.

Standard maintenance immunosuppression was with 750mg mycophenolate mofetil and tacrolimus twice a day. Tacrolimus dose was varied to maintain a trough level of 8 - 12 ng/ml for the first 6 months after surgery and then 6 - 10 ng/ml thereafter. Steroids were not routinely used in maintenance immunosuppression.

All patients received aspirin, ranitidine, and atorvastatin post-operatively. Cytomegalovirus prophylaxis with valganciclovir was given based on donor and recipient seropositivity. Tuberculosis prophylaxis was given to anyone with risk factors for latent disease. Cotrimoxazole was given to all patients for 1 year to protect against pneumocystis jirovecii infection.

2.5 Post-operative Care and Follow-up

All pancreas transplant patients were cared for in the intensive care unit post-operatively before being transferred to the Wytham transplant ward when clinically appropriate. A duplex ultrasound scan was performed within 24 hours of surgery to assess kidney and pancreas graft perfusion. Any concern about graft perfusion in this early post-operative period would lead to a prompt return to theatre.

Once recovering back on the ward, an unexplained rise in serum glucose would trigger an urgent CT angiogram to assess graft perfusion before returning to theatre if appropriate. All patients had repeated assessment of coagulability with thromboelastography (TEG). Prior to 2017, all patients would receive a variable rate Dextran 40 infusion that was titrated according to TEG results. Supply problems with Dextran led to cessation of this practice; after this, patients identified as being hypercoagulable on TEG would receive additional heparin. All patients received daily anticoagulation with heparin and aspirin. Prior to discharge, all patients underwent an oral glucose tolerance test (according to WHO criteria - fasted overnight, 75g oral glucose consumed over 5 minutes, 0- and 120-minute serum glucose tested).

After discharge, patients would be seen regularly in a post-transplant clinic (according to local practice for some patients discharged back to their referring regional team). Patients would self-monitor capillary glucose and blood pressure. At the Oxford Transplant Centre, the standard frequency of follow-up included weekly visits for 3 months before being tapered down to monthly visits after 6 months. Standard review in the clinic would include clinical assessment of graft function and management of any problems or complications identified. Routine blood tests at each visit included: full blood count, renal function tests, liver function tests, amylase, lipase, C reactive protein, glucose, insulin, C-peptide, and

tacrolimus levels. A urine sample would be tested for infection and urinary amylase (if PTA) at each visit. Less frequently, patients would undergo oral glucose tolerance testing, HbA_{1c}, DSA surveillance, cytomegalovirus and BK virus surveillance. At approximately 4 to 6 weeks after surgery, patients were admitted for removal of the ureteric stent under local anaesthetic as a day case procedure.

Pancreas biopsies are not performed at the Oxford Transplant Centre. Where acute rejection of the pancreas was felt to be likely (based on clinical, biochemical, radiological parameters, or biopsy proven rejection of the kidney in case of SPK transplants), patients were treated with three daily doses of 500 mg methylprednisolone intravenously and escalation of maintenance immunosuppression if appropriate. In rare cases of steroid resistant rejection, escalation of immunosuppression was given according to the multidisciplinary transplant team - usually ATG or alemtuzumab.

2.6 Statistical Analyses

Statistical analyses were performed using Graphpad Prism (Version 8, GraphPad Software, La Jolla, California, USA) and SPSS 25 (Version 25, IBM, Armonk, New York, USA) for Windows software.

For continuous variables, distribution of data was assessed, and normality tested using D'Agostino-Pearson normality test. Descriptive statistics were used to summarise group characteristics. Mean values with standard deviation, or median values with range were reported. Parametric continuous variables were compared using unpaired t-test or one way analysis of variance (ANOVA). Non-parametric continuous variables were compared using Mann-Whitney U tests. Categorical variables were compared using chi-squared test. Where multiple comparisons were performed, correction was performed using the Bonferroni-Sidak method. Correlation was assessed using Pearson or Spearman correlation analyses. Survival analyses was performed using Kaplan-Meier curves and log-rank tests. A p value of < 0.05 was considered significant.

Chapter 3

Homeostatic Model Assessment in Pancreas Transplantation

3.1 Introduction

During the last century there have been significant advances in our understanding of the pathophysiology of diabetes mellitus and its management. Whilst overall this has translated into increasing life expectancy and reduced morbidity, one of the main problems that challenges patients and clinicians is knowing how the disease will progress and when the appropriate time is to optimise management.

The onset of clinical signs of type 1 diabetes mellitus (i.e. hyperglycaemia) is often stated to only occur once there has been a 90 - 95% reduction in beta cell mass[254], although it has also been suggested that this can be as low as a 40% reduction in different age groups[255]. The rate and duration of beta cell loss is variable, but it is generally thought to be months to years before overt diabetes develops. There is a clear window for immunological intervention here and a recent study by Herold et al. did show that treatment of high risk individuals with a short course of teplizumab delayed progression of type 1 diabetes by nearly 2 years[256]. In type 2 diabetes, the onset of symptoms is more indolent and there is often a more prolonged period of flux in management options from simple lifestyle interventions to the requirement for exogenous insulin, with many permutations of hypoglycaemic agents in between. In islet and pancreas transplantation, beta cell loss can also occur secondary to immunological assault in the form of rejection, however the timescale is usually days to weeks.

The common denominator for the requirement of exogenous insulin in T1DM, T2DM, islet, and pancreas transplantation is the loss of a critical mass of beta cells. Furthermore,

diabetologists and transplant physicians alike are limited by their inability to accurately assess or predict when patients are nearing this critical beta cell mass in the clinical setting. The gold standard method for measuring beta cell function through insulin secretion capacity is the hyperglycaemic clamp technique whereas the hyperinsulinaemic-euglycaemic clamp technique is used to measure insulin sensitivity[257,258]. The hyperglycaemic clamp involves raising the serum glucose concentration acutely (e.g. to 12 mmol/L) and it is maintained at this level by varying a glucose infusion. The insulin secretion capacity is assessed by the rate of glucose infusion required to maintain the hyperglycaemic plateau as well as by the direct measurement of serum insulin and C-peptide levels. During a hyperinsulinaemic-euglycaemic clamp, the serum insulin concentration is acutely raised by a continuous insulin infusion whilst the serum glucose concentration is held constant by a variable rate glucose infusion. When a steady state is achieved, the glucose infusion rate equals the glucose uptake rate in all the tissues in the body which is therefore a measure of insulin sensitivity. These clamps, however, are invasive, complicated, and expensive meaning that they are best suited to small research studies and not easily applied to larger studies or clinical practice.

Several methods, from which surrogate indices have been derived and validated, have been developed that calculate insulin sensitivity and insulin secretion rate. These typically use either fasting samples or are combined with other samples following administration of a glucose load and essentially estimate insulin sensitivity and insulin secretion from historic validation studies. Some of these indices are applicable in a clinical setting due to being quick, minimally invasive, and cheap. A comprehensive review of all techniques is beyond the scope of this work; however, the more commonly used methods and models are summarised in Table 3.1.

Diabetologists have used such models for decades to assess both individual disease progression longitudinally and also the response to therapeutic interventions. This is particularly attractive in the context of islet and solid organ pancreas transplantation where there are currently no validated biomarkers of graft function. The ‘holy grail’ would be a biomarker or model that can be used clinically to provide an objective assessment of pancreas graft function and therefore earlier recognition of failing grafts at a time when intervention is possible. One of the most useful metabolic predictors of pancreas allograft failure is impaired glucose tolerance during an oral glucose tolerance test[200]. This is currently part of clinical protocols in many units with either routine or adhoc use to identify graft dysfunction although it has only been shown to be sensitive to predict overall graft failure at 1-year post transplant (rather than diagnose and monitor dysfunction). The OGTT is time-consuming for patients who are frequently not prepared to spend several hours in a transplant unit every time they visit.

Table 3.1: A comparison of different techniques and indices for assessing beta cell function and insulin sensitivity.

Method / Model	Advantages	Disadvantages
Hyperglycaemic clamp	Gold standard for measuring β-cell function	Invasive, laborious, expensive, not practical for clinical assessment
Hyperinsulinaemic-euglycaemic clamp	Gold standard for measuring insulin sensitivity	Invasive, laborious, expensive, not practical for clinical assessment
OGTT, MMTT	Measure of stimulated secretory function Includes neurohormonal/incretin effect Less technically demanding than clamps	Inconvenient, time consuming, unpopular with patients in clinical environment
IVGTT	Measure of stimulated secretory function	Inconvenient, time consuming, unpopular with patients in clinical environment
AST, LST, GST	Measure of stimulated secretory function Less technically demanding than clamps Removes neurohormonal/incretin effect	Supraphysiological insulin secretory response Inconvenient, time consuming, unpopular with patients in clinical environment
Short insulin tolerance test	Simple test for measuring insulin sensitivity	Doesn't estimate β-cell function
Homeostatic model assessment (HOMA)[259]	Validated measure of % β-cell function and insulin sensitivity Simple and quick	Steady state only
Continuous infusion of glucose with model assessment (CIGMA)[260]	Validated measure of % β-cell function and insulin sensitivity in the non-steady state	Time-consuming, unpopular with patients in clinical environment
Minimal Model[261,262]	Uses fsIVGTT – easier than clamp studies	Still requires multiple sampling over 2-3 hours. Inaccurate if IGT or T2DM (needs insulin or tolbutamide)

Matsuda Index[263]	Uses OGTT – easier than clamp studies	Poor correlation in subjects with IGT or T2DM
Quantitative insulin sensitivity check index (QUICKI)[264]	Similar to HOMA	Steady state only
Belfiore index[265], Cederholm index[266], Avignon index[267], Gutt index[268], McAuley index[269], Stumvoll index[270]		Only suitable for epidemiological/research purposes Absence of reference values for normal and impaired insulin sensitivity

OGTT – oral glucose tolerance test

MMTT – mixed meal tolerance test

IVGTT – intravenous glucose tolerance test

AST – arginine stimulation test

LST – leucine stimulation test

GST – glucagon stimulation test

The Homeostatic Model Assessment (HOMA) is a sophisticated model which was developed in 1985 to provide a simple objective measure of beta cell function and insulin sensitivity[259]. HOMA was developed into a computerised assessment of insulin sensitivity and pancreatic beta cell function based on a structural mathematic model of the normal physiology of glucose-insulin interaction, where organ specific responses to these two inputs are described by equations based on published empirical physiological data[259]. Figure 3.1 shows the HOMA model in several ways - a cartoon of tissues involved, the dose-response curves of these tissues, and finally the equations that govern glucose-insulin metabolism in these tissues and which are combined to form the HOMA model[198]. It provides a calculation for beta cell function and insulin sensitivity using only fasting glucose and insulin values. It has been validated against both hyperglycaemic and hyperinsulinaemic-euglycaemic clamps and has been used clinically for a variety of diabetic and non-diabetic patients for several decades[271-273]. In practice the model is simple to use; it requires only two inputs, fasting serum glucose with either a serum C-peptide or insulin value. The model then produces two outputs - %B which represents beta cell secretory capacity and %S which represents systemic insulin sensitivity. These values are provided as a percentage, with 100% being representative of the control populations that were used in the development of the original model. HOMA2 is a development of the original model that accounts for variations in hepatic and peripheral glucose resistance, increases in the insulin secretion curve for plasma glucose concentrations above 10 mmol/L (180 mg/dL), and the contribution of circulating proinsulin[274]. The model was also recalibrated to give %B and %S values of 100% in normal young adults when using currently available assays for insulin, specific insulin or C-peptide. The HOMA models (and all models in Table 3.1) assume normal pancreatic anatomy with insulin delivery to the portal circulation; this is not the case after a pancreatic transplantation.

Pancreas transplantation results in highly altered physiology. The donor pancreas is denervated and implanted heterotopically, typically with venous drainage of endocrine secretions directly into the systemic circulation via the inferior vena cava. Therefore, the normal delivery of islet-derived hormones directly to the liver via the portal vein does not occur. There is a loss of first-pass metabolism of insulin through the liver with reversal of the normal portal to peripheral insulin and glucagon gradients. As a result, the physiology of glucose homeostasis is altered, and the HOMA2 calculation cannot be applied meaningfully. The HOMA model has several clear advantages over other models and techniques that would make it superior for assessing graft function in pancreas transplant recipients (if it were modified to allow for systemic venous drainage of the pancreas). Its main advantage is its ease of use and low cost. Only a single fasting blood sample is required for a pre-calibrated model rather than several stimulated samples that need modelling to each individual.

The 'HOMAtx' model was therefore developed as a modification of the HOMA2 model incorporating a change from portal to peripheral delivery of insulin from the pancreas allograft. This involved changing the formulas within the model to allow for systemic delivery of insulin and therefore avoid first pass metabolism in the liver. HOMAtx represents a group of complex mathematical calculations and computations to modify HOMA2 for use in pancreas transplantation and was developed by Dr Jonathan Levy who has been a part of the HOMA group in Oxford since its inception in the 1980s. It has not been validated in pancreatic transplant recipients or related to persons with normal physiology.

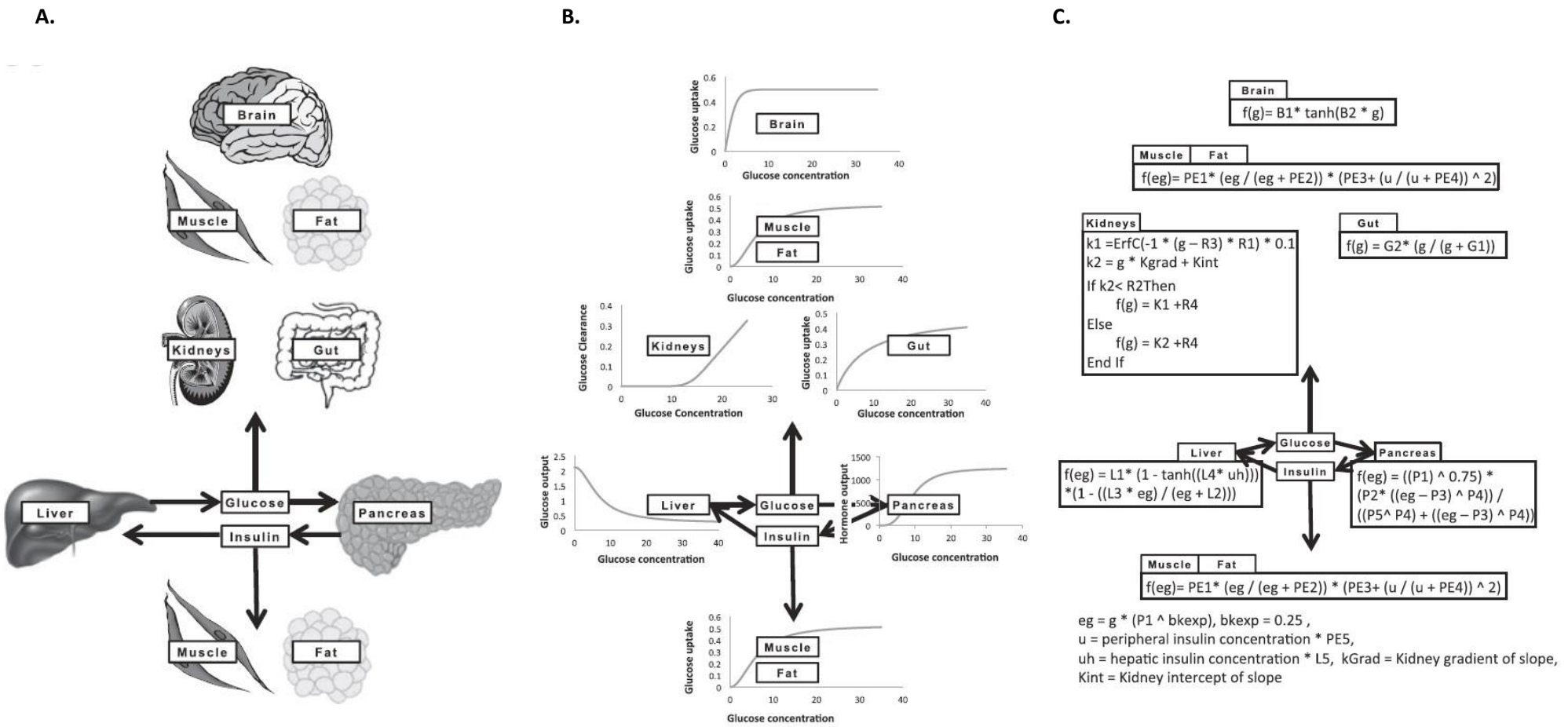


Figure 3.1: The Homeostatic Model Assessment (HOMA) incorporates all tissues in the body that are involved in glucose metabolism. The model is shown in 3 forms: A) cartoon form B) dose-response curves modelled for each tissue C) equations that govern how each tissue performs in the model. Reproduced with permission from American Diabetes Association[198].

3.2 Aims and Objectives of Chapter 3

The aim of this chapter was to develop and validate the new HOMAtx model in pancreas transplant recipients.

The objectives of this chapter were:

Primary:

- To validate HOMAtx as a measure of beta cell function in pancreas transplant recipients.
- To validate HOMAtx as a measure of insulin sensitivity in pancreas transplant recipients.

Secondary:

- To compare HOMAtx in pancreas transplant recipients with HOMA2 in a matched population of non-diabetic controls.
- To assess glycaemic control in pancreas transplant recipients and in the healthy comparison population.

3.3 Methods

3.3.1 Study design

This was an observational study to validate the HOMAtx model for assessing beta cell insulin secretion and insulin sensitivity in the context of pancreas transplantation.

12 pancreas transplant recipients and 12 healthy controls were invited to participate in the study. Participants attended for two research visits. At the first research visit, participants underwent an oral glucose tolerance test with 75g glucose solution. At the second visit, participants underwent a 2-stepped hyperglycaemic clamp study. Research ethics and Trust approvals were gained for this study prior to recruitment (Appendix F).

3.3.2 Outcome measures

Primary:

i) Correlation of HOMAtx derived beta cell function (%B) and insulin secretion measured during hyperglycaemic clamps.

ii) Correlation of HOMAtx derived insulin sensitivity (%S) and insulin sensitivity measured during hyperglycaemic clamps.

Secondary:

i) Comparison of correlations of beta cell function and insulin sensitivity from HOMAtx and HOMA2.

ii) Frequently sampled oral glucose tolerance tests (fsOGTT) with emphasis placed on the 0 and 120 minute values categorised according to WHO criteria.

3.3.3 Study participants

Pancreas transplant patients (SPK only): subjects were identified by the clinical team from a database of pancreas transplants performed at the Oxford Transplant Centre.

Healthy adults: subjects were identified from the Oxford Biobank (a cohort of healthy adults aged 18-60 years who had previously given consent to be re-contacted for future research). Healthy adults were matched to pancreas transplant participants by age, gender, and BMI.

3.3.3.1 Inclusion criteria

Pancreas transplant group:

- Age 18-60 years
- Long-standing functioning pancreas graft (as part of SPK)
- No acute illness
- No hypoglycaemic agents
- Able to give consent

Healthy control group:

- Age 18 - 60 years
- Normal fasting glucose
- No acute illness
- No hypoglycaemic agents
- Able to give consent

3.3.3.2 Exclusion criteria

Both groups:

- Inability to give informed consent
- Diabetic glucose tolerance
- Diabetes or failed pancreas graft (return to exogenous insulin)
- Abnormal renal function (eGFR < 60)
- Likely failure to cannulate
- Pregnancy
- Severely visually impaired

3.3.4 Study procedures

Clinical data:

For each participant, clinical data was collected against a unique study identifier. Demographic and observational data was recorded for date of birth, gender, BMI, blood pressure, heart rate, comorbidities, details of transplant and medications.

Frequently sampled oral glucose tolerance test (fsOGTT):

Participants were asked to starve from midnight the night before the research visit and to avoid strenuous exercise or alcohol the preceding evening and morning of the research visit. At the research visit, a peripheral intravenous cannula was sited through which blood samples were taken according to the sampling protocol (Figure 3.2).

75g of anhydrous glucose was dissolved in 250 ml of water and this was drunk by the research participant over 4 - 5 minutes. A timer was started when drinking commenced, and participants were asked to remain rested in bed whilst blood samples were taken over 120

minutes after the glucose drink was consumed. The oral glucose tolerance test was complete after 120 minutes and participants were provided with something to eat and drink and were observed for another 30 minutes until they achieved a stable glucose level without exogenous support. The peripheral cannula was removed, and participants were free to leave.

Hyperglycaemic clamp:

Participants were asked to starve from midnight the night before the research visit and to avoid strenuous exercise and alcohol the preceding evening and morning of the research visit. At the research visit, two peripheral intravenous cannulas were sited in different arms. One cannula was used to take blood samples according to the sampling protocol (Figure 3.3). The other cannula was used for a variable rate 20% glucose infusion. The dextrose was infused quickly to achieve and maintain hyperglycaemia at 8 mmol/l for 90 minutes (first stage), and then 12 mmol/l for a further 90 minutes (second stage). The infusion was adjusted every 2.5 minutes over 180 minutes to maintain the required level of hyperglycaemia according to Haemacue values. Haemacue samples for glucose and blood samples for future assays were taken over 180 minutes according to the sampling protocol (Figure 3.3). The hyperglycaemic clamp test was complete after 180 minutes and participants were provided with something to eat and drink and were observed for another 30 minutes until they achieved a stable glucose level without exogenous support. The peripheral cannulas were removed, and participants were free to leave.

Sampling protocol:

Blood samples were taken at each time-point as shown in Figures 3.2 and 3.3. 1 ml of blood was discarded at the beginning of each sample and the cannula was flushed with 1 ml saline at the end of each sample. At all timepoints 8 ml of blood was taken into a heparinised

plasma tube, for testing of glucose, C-peptide and insulin. All samples were placed on ice and centrifuged at 4 °C and 3000 rpm for 10 minutes. Plasma was then separated into aliquots which were stored at -80 °C, pending assaying in batches in order to minimise batch and kit variability. Samples were analysed with commercial chemiluminescent (glucose) or immunoassay (specific insulin and C-peptide) kits (Architect/Abbott Laboratories, Illinois, USA). Samples were analysed by me at the Oxford University Hospitals NHS Foundation Trust biochemistry laboratories.

HOMAtx and HOMA2 calculations:

The pancreas transplant participants had HOMAtx calculations for %B and %S derived from mean fasting glucose and insulin or C-peptide data using a modification of HOMA2 to incorporate systemic endogenous insulin delivery from the pancreas graft. The non-diabetic control participants had HOMA calculations using HOMA2[273]. Both versions were made available to me as an independent computer program (Figure 3.4). HOMA2 has been openly available to download from the University of Oxford for many years via the internet at: https://www.dtu.ox.ac.uk/homacalculator/

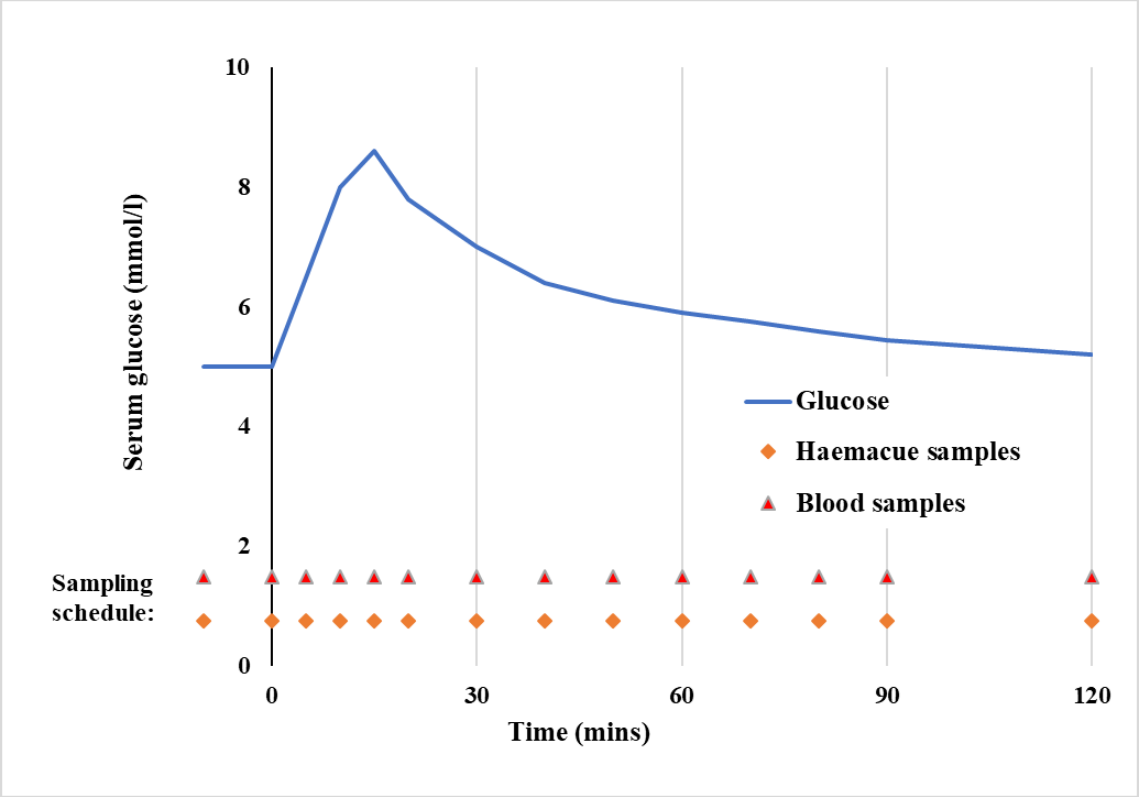


Figure 3.2: Schematic of frequently sampled oral glucose tolerance tests with sampling timepoints.

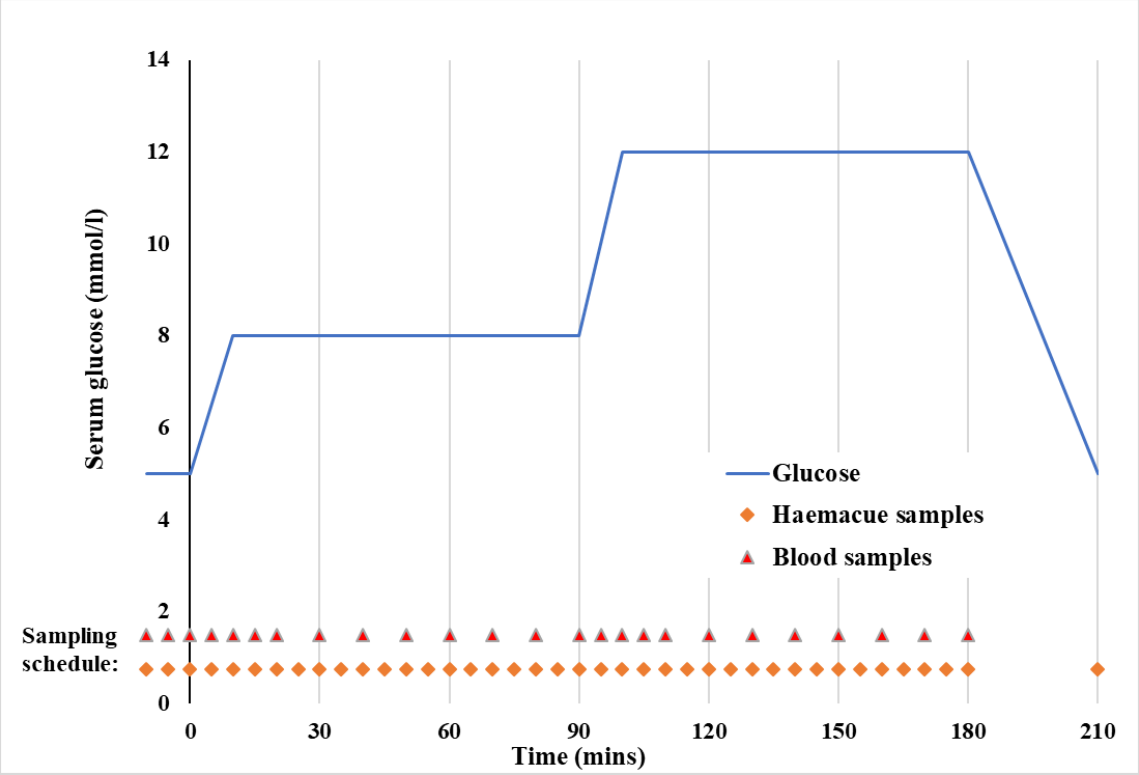


Figure 3.3: Schematic of hyperglycaemic clamps with sampling timepoints.

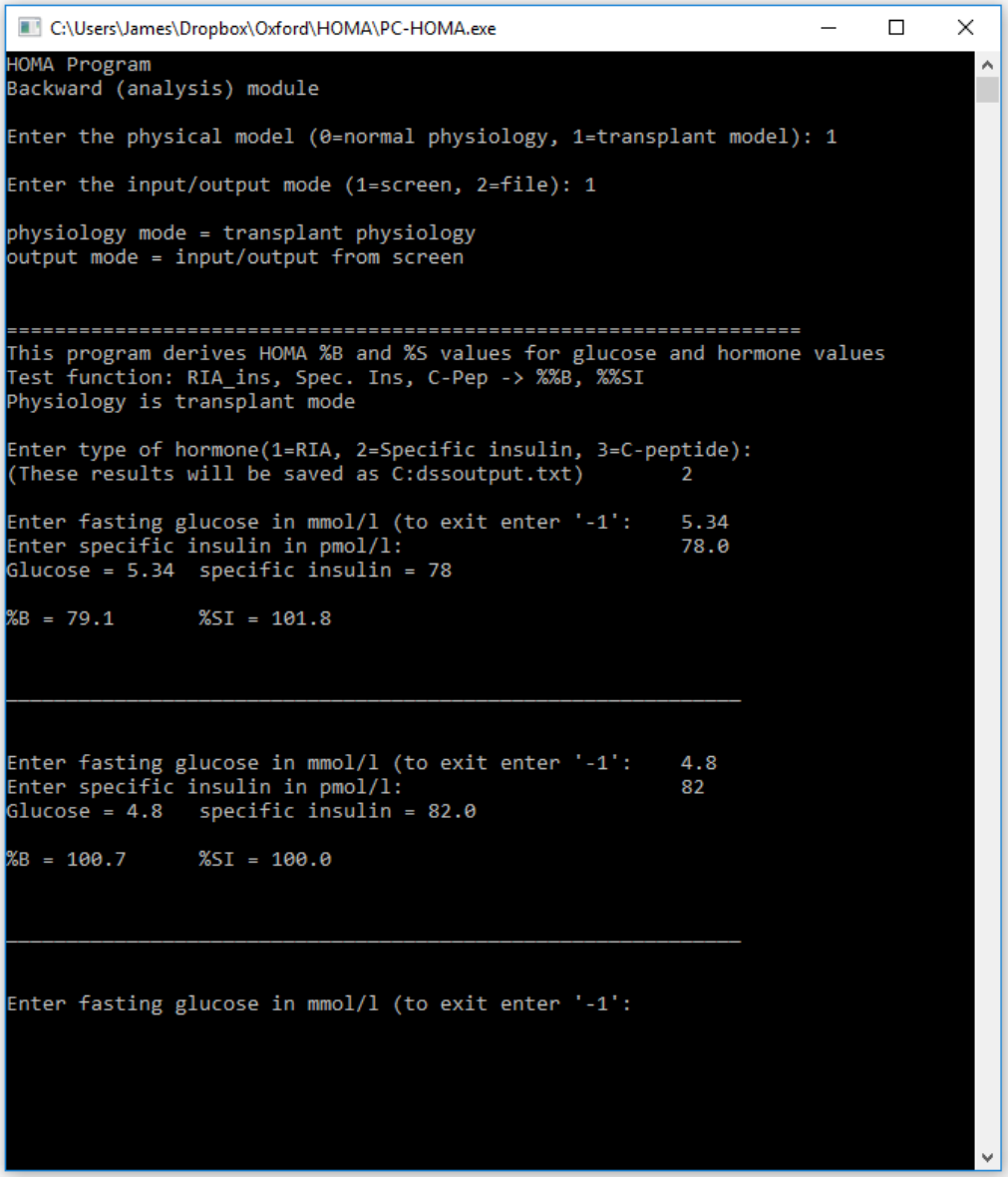


Figure 3.4: Screenshot of the computer program used for this study that incorporated HOMAtx and HOMA2.

3.3.5 Statistical analyses

The pancreas transplant group and healthy control group were compared for demographic and baseline factors using the student's t-test for continuous variables and the Fisher's Exact test for categorical variables.

The hyperglycaemic clamp was tested for glucose variability at each clamp stage by assessing mean glucose using four samples over the last 30 minutes of each step i.e. at 60, 70, 80 and 90 minutes for 8 mmol/l and 150, 160, 170 and 180 minutes for 12 mmol/l. A coefficient of variation of < 5% was considered acceptable.

Hyperglycaemic clamp measure of beta cell function was calculated from insulin secretion rates over the last 30 min of each clamp stage. Hyperglycaemic clamp measure of peripheral insulin sensitivity was calculated from glucose infusion rates divided by the peripheral insulin concentration and corrected for participant weight.

Beta cell function and insulin sensitivity measures derived from HOMA and hyperglycaemic clamp were compared using Pearson's or Spearman's rank correlation co-efficient between individual values.

Statistical analyses were performed using SPSS version 25 for Windows (IBM SPSS Statistics, Armonk, New York, USA) and Graphpad Prism version 8 for Windows (GraphPad Software, La Jolla, California, USA). A p value of < 0.05 was considered to be statistically significant.

3.4 Results

3.4.1 Demographics

12 participants with pancreas transplants and 12 healthy controls were recruited to the study (Table 3.2). There were 10 men and 2 women in each group. The mean age of the pancreas transplant group was 50.1 years (SD = 8.9) and 47.2 years (SD = 2.1) in the control group. The mean BMI of the pancreas transplant group was 27.2 kg/m^2 (SD = 3.33) and 26.5 kg/m^2 (SD = 1.80) in the control group. There was no significant difference between groups for mean age (p = 0.28) or mean BMI (p = 0.52). The transplant cohort consisted of 12 simultaneous pancreas kidney transplant recipients. The median time after transplant that the clamp studies were performed was 40.5 months (range 17 - 121).

3.4.2 Baseline graft function

The glomerular filtration rate, calculated using the MDRD equation (eGFR), was > 90 ml/min/1.73m^2 in all healthy controls whereas in the pancreas (and kidney) transplant group the mean was 72.3 ml/min/1.73m^2 (SD = 13.85). Mean HbA_{1c} was 36.2 mmol/mol (SD = 2.55) in the pancreas transplant group and was not measured in the healthy controls.

All 24 patients successfully completed a 120-minute OGTT (Figure 3.5). Fasting glucose was higher in the transplant group at 5.9 mmol/l (SD = 0.74) versus 5.4 mmol/l (SD = 0.36) in the control group (p = 0.047). This was also reflected in the OGTT where total AUC glucose in the transplant group was significantly higher the control group (p = 0.002). All patients in the control group had normal OGTT (according to WHO criteria i.e. 120-minute glucose < 7.8 mmol/l). Two patients in the transplant group were found to have impaired glucose tolerance at OGTT (serum glucose at 120 mins of 9.9 mmol/l and 10.1 mmol/l) and

one had a diabetic glucose tolerance (serum glucose at 120 mins of 11.9); all had normal HbA_{1c} fasting glucose levels.

Fasting hormone profiles were significantly different between transplant and control groups. Mean serum insulin was significantly higher in the transplant group than in the control group ($p < 0.001$). Mean serum C-peptide was higher in the transplant group than in the control group ($p = 0.035$).

3.4.3 Hyperglycaemic clamp studies

All 24 participants underwent successful 2-stage hyperglycaemic clamp studies (Figure 3.6). The variability in the 60 - 90 minute and 150 - 180 minute periods was very low as measured by the coefficient of variation: 1.85% in the transplant group and 2.21% in the control group at the 8 mmol clamp and 1.35% in the transplant group and 2.30% in the control group at the 12 mmol clamp.

Table 3.2: Demographic and baseline data for the pancreas transplant and control groups.

	Pancreas Tx	Controls	P value
Participants	12	12	-
Age (years) †	50.1 (8.9)	47.2 (2.1)	0.28
Gender (male)	10	10	-
BMI (kg/m^2) †	27.2 (3.33)	26.5 (1.80)	0.52
eGFR (ml/min/1.73m^2) †	72.3 (13.85)	>90	-
HbA$_{1c}$ (mmol/mol) †	36.2 (2.55)	-	-
AUC Glucose (units)	1115	818.5	0.002
Fasting Glucose (mmol/l) †	5.9 (0.74)	5.4 (0.36)	0.047
Fasting Insulin (pmol/l) †	76.9 (30.11)	40.2 (15.79)	0.001
Fasting C-peptide (pmol/l) †	577.5 (141.61)	444.2 (148.40)	0.035

† Values are mean (SD)

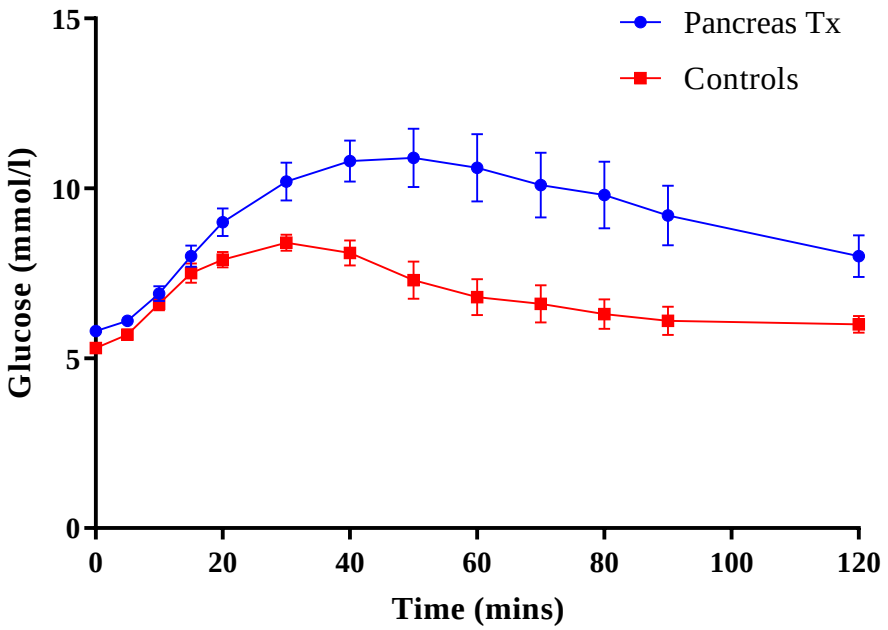


Figure 3.5: Oral glucose tolerance tests in pancreas transplant and control groups. Bars represent SEM.

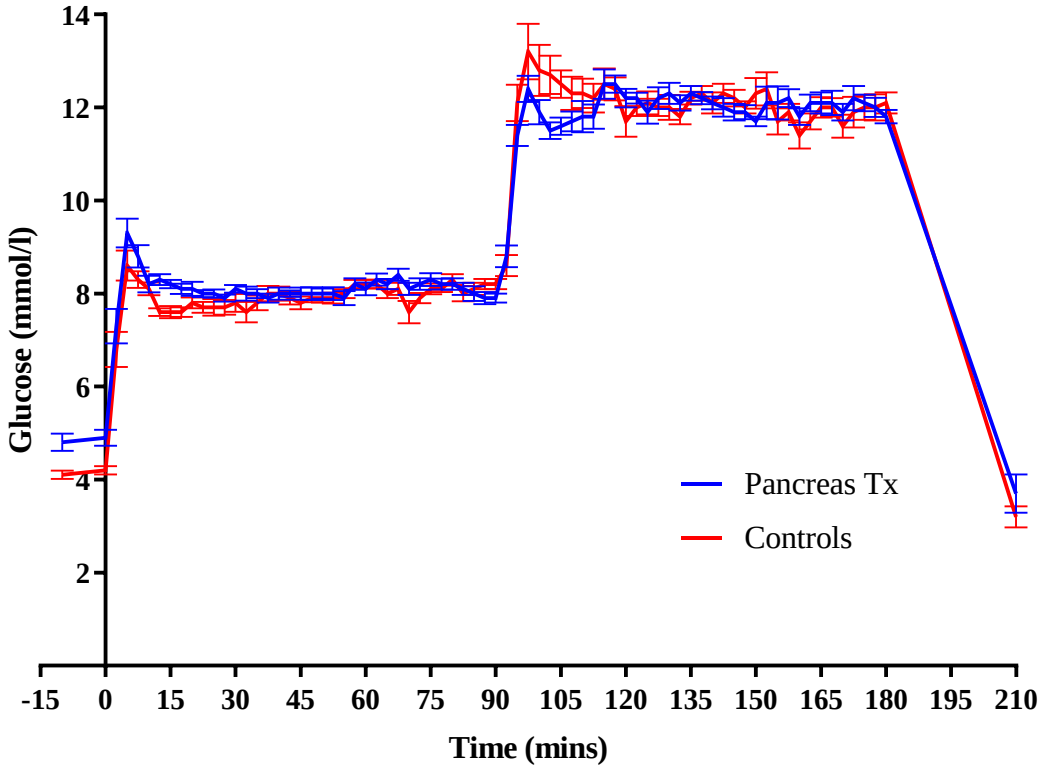


Figure 3.6: Hyperglycaemic clamps in pancreas transplant and control groups. Bars represent SEM.

3.4.4 The HOMAtx model in pancreas transplant participants

Beta cell function (%B) and insulin sensitivity (%S) were calculated for pancreas transplant participants using the HOMAtx model using fasting serum insulin (Table 3.3 and Figure 3.7). Mean %B was 64.4% (SD = 14.73) and mean %S was 114.2% (SD = 34.70).

3.4.4.1 Beta cell function in pancreas transplant participants

The %B was plotted against mean serum insulin values for each participant at the 8 mmol and 12 mmol glucose clamp stages (Figure 3.8). Pearson correlation analysis confirmed an r coefficient of 0.84 at the 8 mmol clamp level ($p < 0.001$) and 0.75 at the 12 mmol clamp level ($p = 0.008$). The %B was also plotted against mean serum C-peptide values at both clamp stages (Figure 3.9). This demonstrated Pearson coefficients of 0.70 ($p = 0.010$) and 0.63 ($p = 0.027$) at the 8 and 12 mmol clamp stages respectively. Log transformation did strengthen some of the correlation coefficients, particularly those derived from serum C-peptide (Figures 3.10 and 3.11).

3.4.4.2 Insulin sensitivity in pancreas transplant participants

The %S was plotted against insulin sensitivity calculated from the 2 clamp stages (M/I/kg; where M = mean steady state glucose infusion and I = mean steady state insulin, both calculated over the last 30 mins of each clamp stage and corrected for weight) (Figure 3.12). Pearson correlation analysis confirmed an r coefficient of 0.60 ($p = 0.039$) at the 8 mmol clamp level and 0.47 ($p = 0.143$) at the 12 mmol clamp level. There was no improvement after log transformation (Figure 3.13).

Table 3.3: Hyperglycaemic clamp outcomes in pancreas transplant recipients and controls.

		Pancreas Tx	Control	P value
	%B	64.4 (14.73)	71.5 (17.51)	0.29
	%S	114.2 (34.70)	138.2 (64.28)	0.27
Clamp 8	**Insulin**	281.4 (130.07)	162.8 (60.32)	0.009
	M/I/kg	0.0039 (0.0024)	0.0097 (0.0011)	<0.001
Clamp 12	**Insulin**	598.5 (314.66)	486.4 (223.47)	0.33
	M/I/kg	0.0064 (0.0038)	0.0110 (0.0046)	0.014

All values are mean (SD)

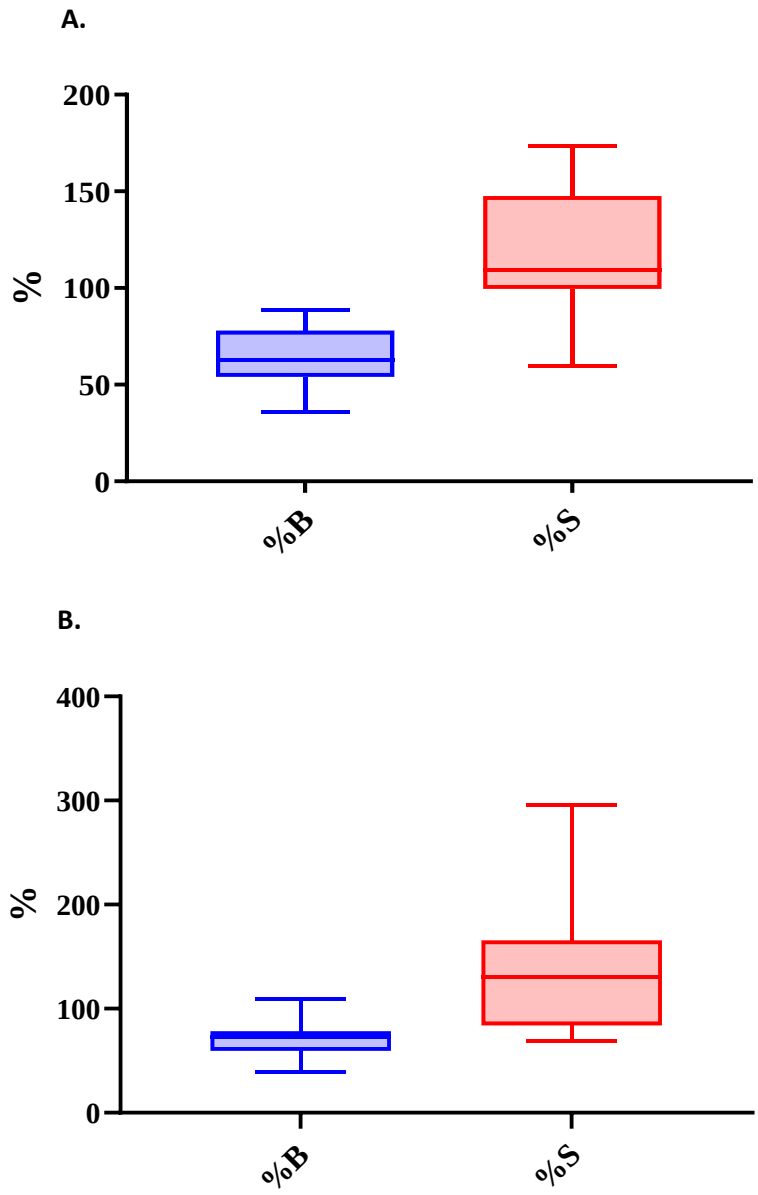


Figure 3.7: Boxplots. (A) %B and %S in the pancreas transplant group derived from HOMAtx using serum insulin; (B) %B and %S in the control group derived from HOMA2 using serum insulin.

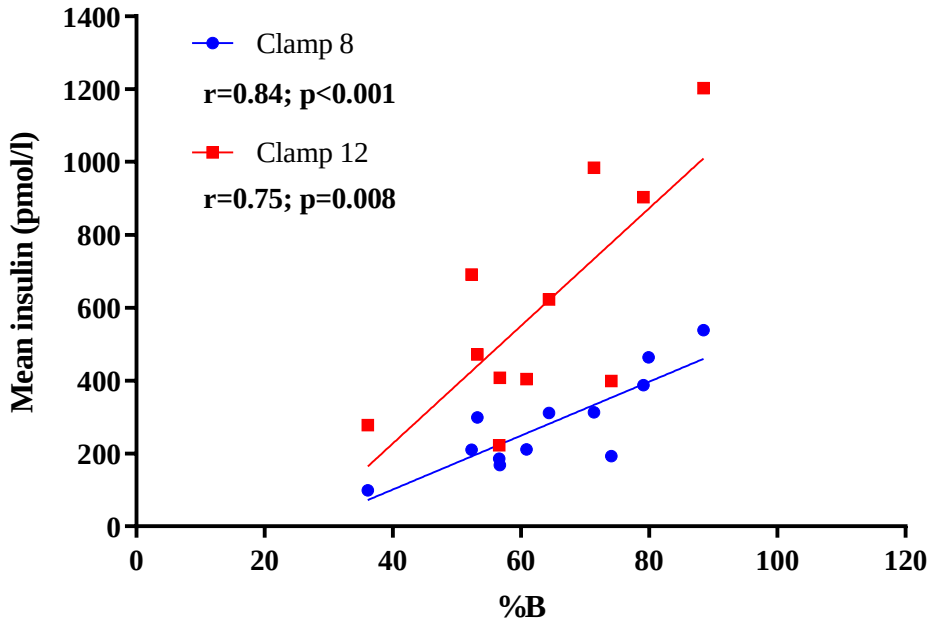


Figure 3.8: Scatterplot demonstrating the relationship between HOMAtx %B and mean serum insulin in the pancreas transplant group during hyperglycaemic clamps.

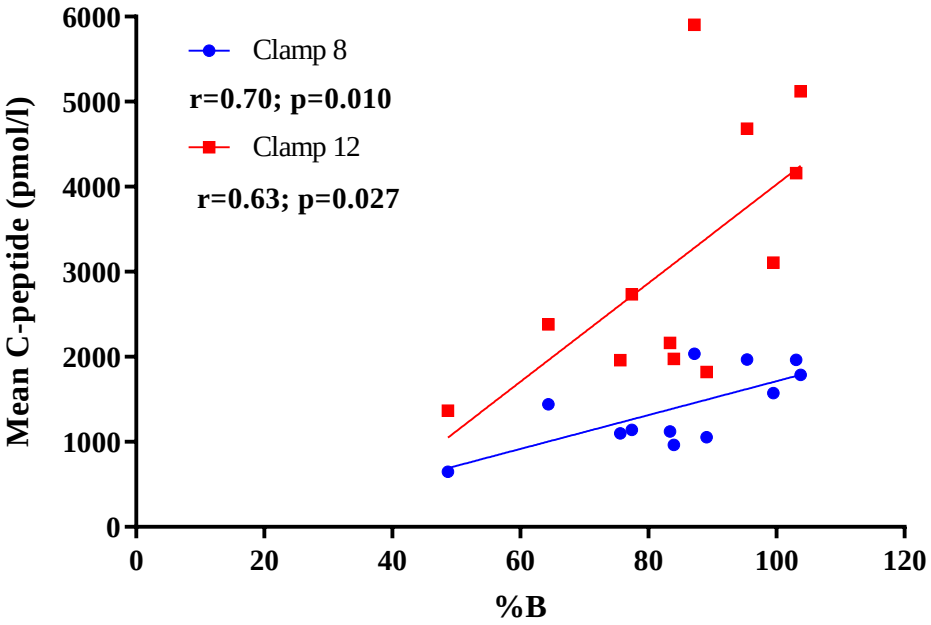


Figure 3.9: Scatterplot demonstrating the relationship between HOMAtx %B with mean serum C-peptide in the pancreas transplant group during hyperglycaemic clamps.

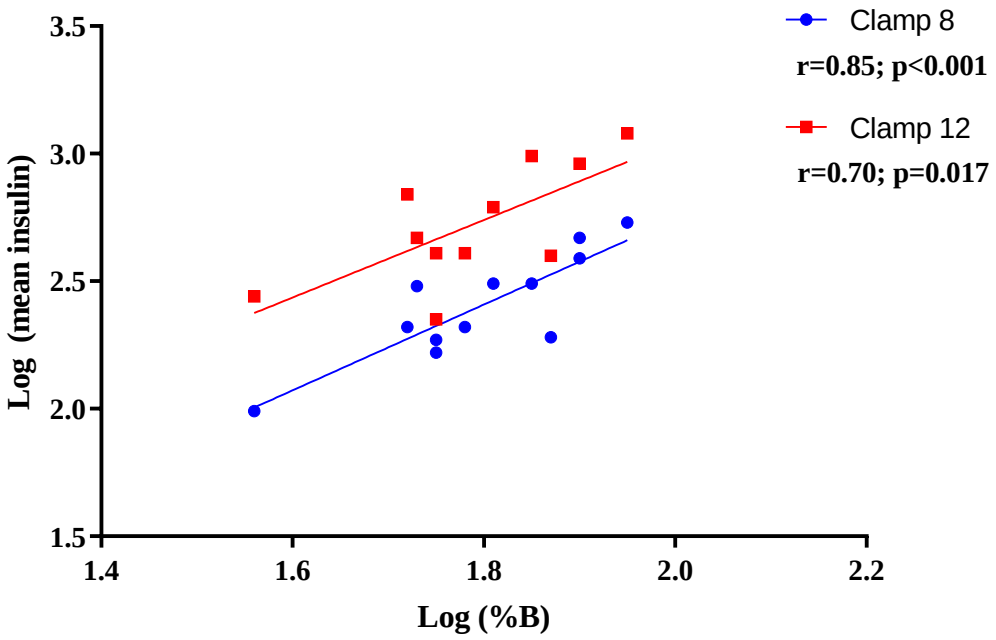


Figure 3.10: Scatterplot demonstrating the relationship between HOMAtx %B with mean serum insulin in the pancreas transplant group during hyperglycaemic clamps (following log transformation).

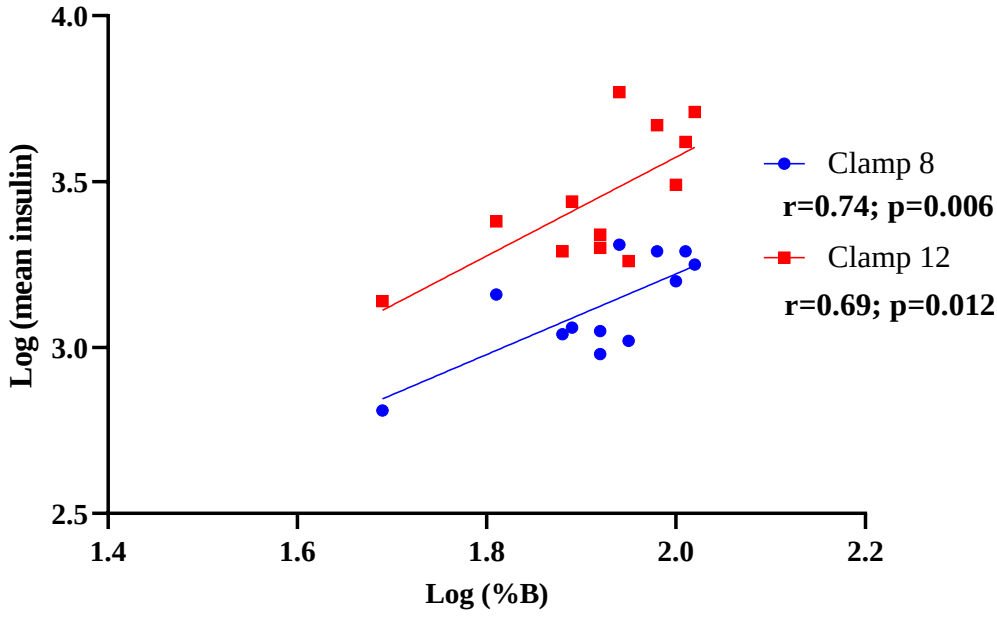


Figure 3.11: Scatterplot demonstrating the relationship between HOMAtx %B with mean serum C-peptide in the pancreas transplant group during hyperglycaemic clamps (following log transformation).

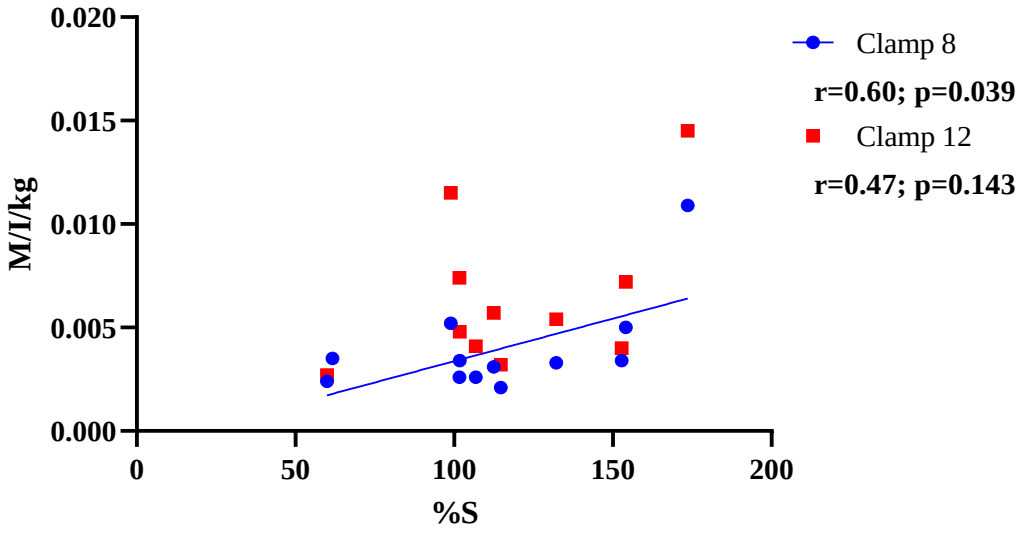


Figure 3.12: Scatterplot demonstrating the relationship between HOMAtx %S with insulin sensitivity (M/I/kg) in the pancreas transplant group during hyperglycaemic clamps.

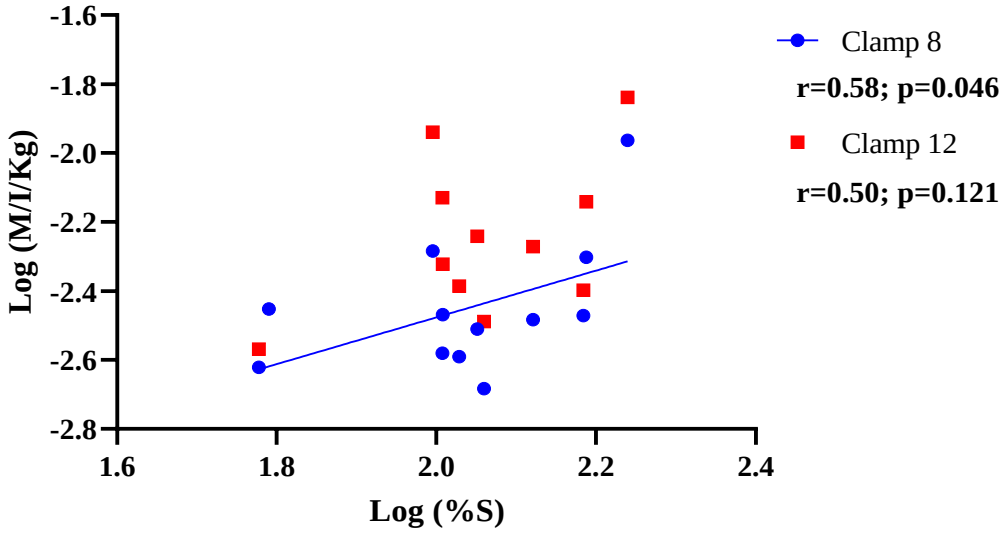


Figure 3.13: Scatterplot demonstrating the relationship between HOMAtx %S with insulin sensitivity (M/I/kg) in the pancreas transplant group during hyperglycaemic clamps (following log transformation).

3.4.5 The HOMA2 model in control participants

Beta cell function (%B) and insulin sensitivity (%S) were calculated for control participants using the HOMA2 model using fasting serum insulin (Table 3.3 and Figure 3.7). Mean %B was 71.5% (SD = 17.51) and mean %S was 138.2% (SD = 64.23).

3.4.5.1 Beta cell function in control participants

The %B was plotted against mean serum insulin values for each participant at the 8 mmol and 12 mmol glucose clamp stages (Figure 3.14). Pearson correlation analysis confirmed an r coefficient of 0.60 at the 8 mmol clamp level ($p = 0.041$) and 0.54 at the 12 mmol clamp level ($p = 0.071$). As in the transplant group, the %B was also plotted against mean serum C-peptide values at both clamp stages, however there was no significant correlation with this method (Figure 3.15). Log transformation did strengthen the correlation between %B and mean C-peptide (Figure 3.16), with $r = 0.64$ ($p = 0.025$) and $r = 0.61$ ($p = 0.034$) at the 8 mmol and 12 mmol clamp stages respectively.

3.4.5.2 Insulin sensitivity in control participants

The %S was plotted against insulin sensitivity calculated from the 2 clamp stages as previously described (Figure 3.17). Pearson correlation analysis confirmed an r coefficient of 0.79 ($p = 0.002$) at the 8 mmol clamp level and 0.78 ($p = 0.008$) at the 12 mmol clamp level. There was no improvement after log transformation (Figure 3.18).

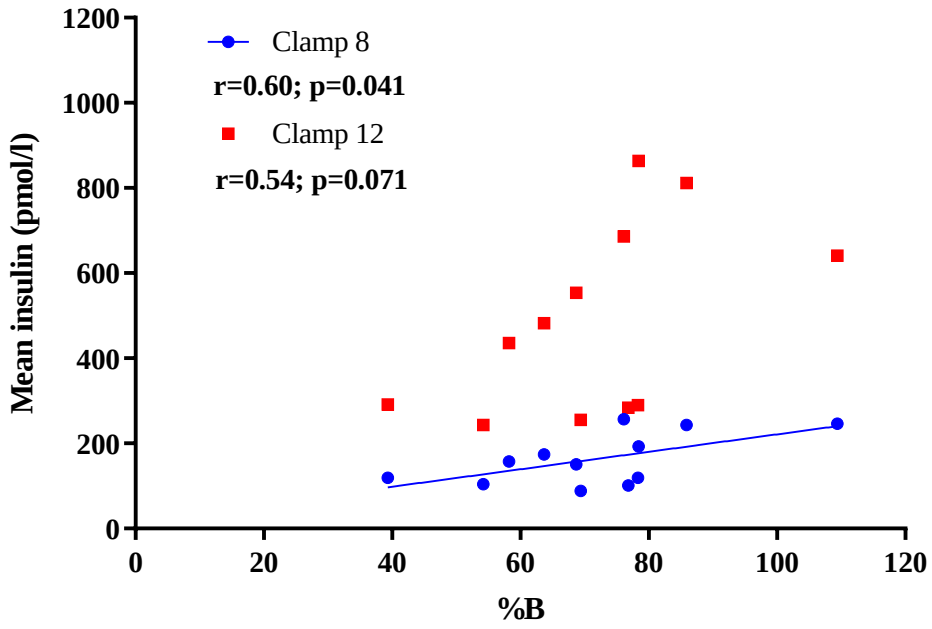


Figure 3.14: Scatterplot demonstrating the relationship between HOMA2 %B with mean serum insulin in the control group during hyperglycaemic clamps.

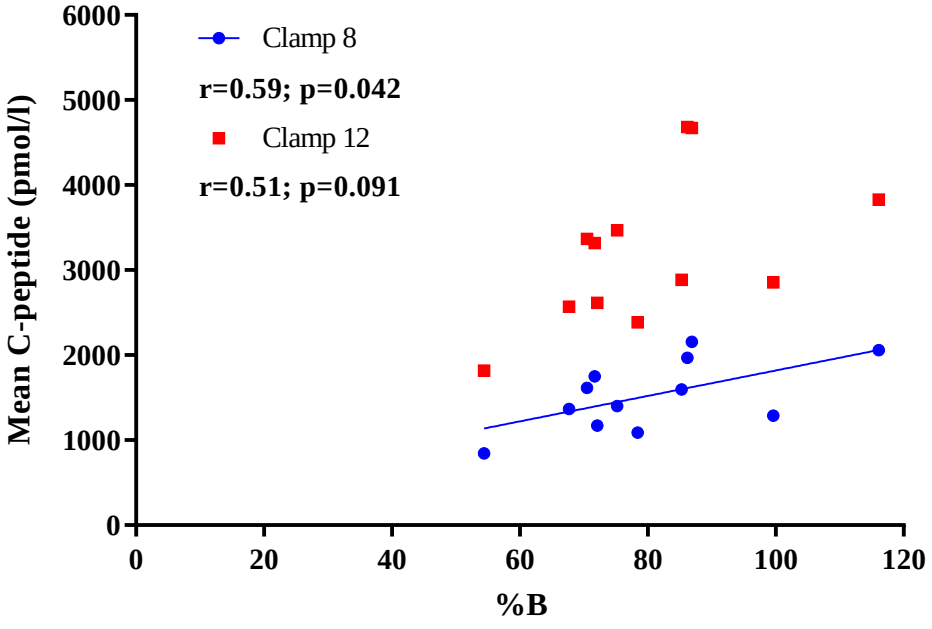


Figure 3.15: Scatterplot demonstrating the relationship between HOMA2 %B with mean serum C-peptide in the control group during hyperglycaemic clamps.

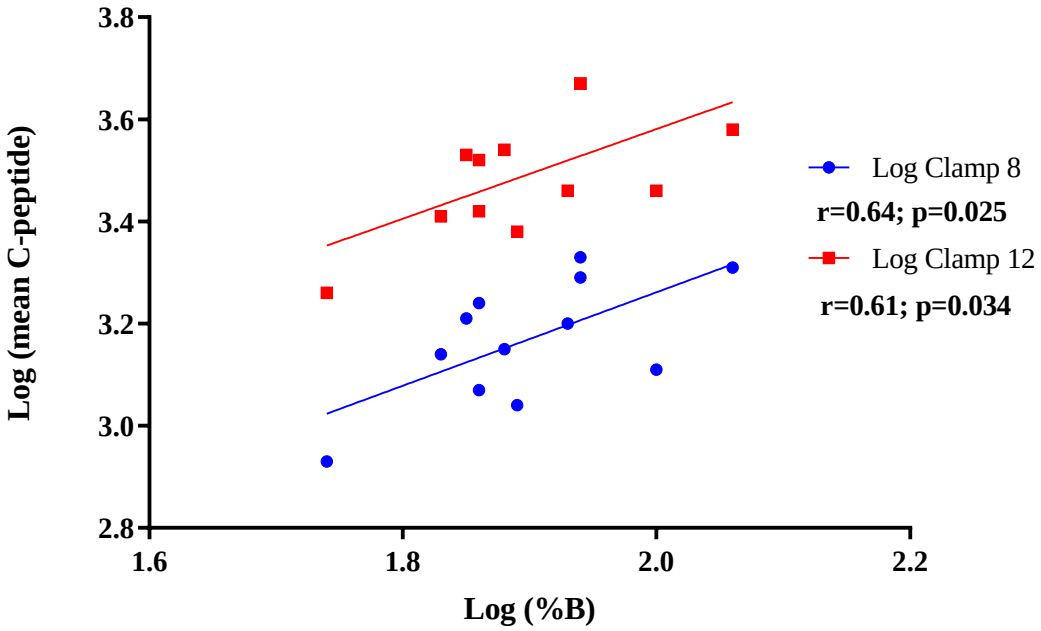


Figure 3.16: Scatterplot demonstrating the relationship between HOMA2 %B with mean serum C-peptide in the control group during hyperglycaemic clamps (following log transformation).

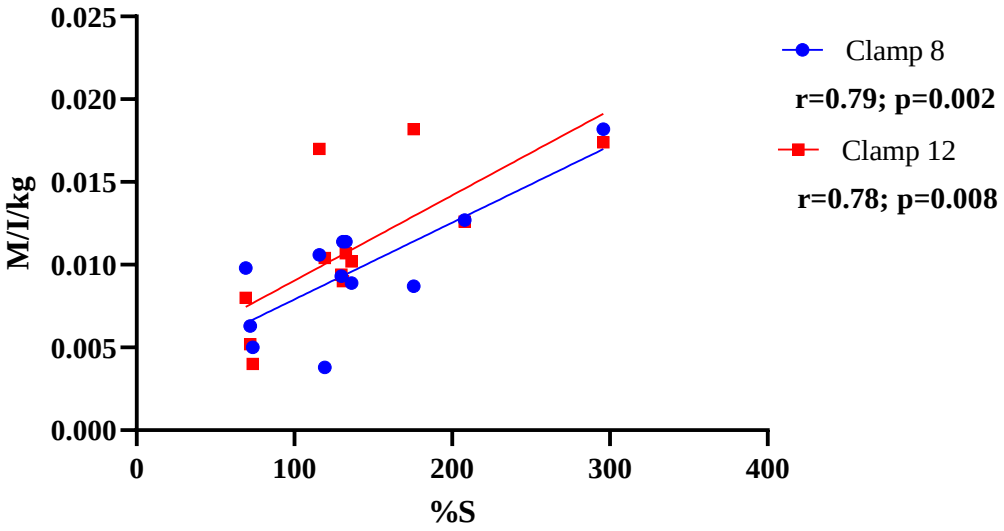


Figure 3.17: Scatterplot demonstrating the relationship between HOMA2 %S with insulin sensitivity (M/I/kg) in the control group during hyperglycaemic clamps.

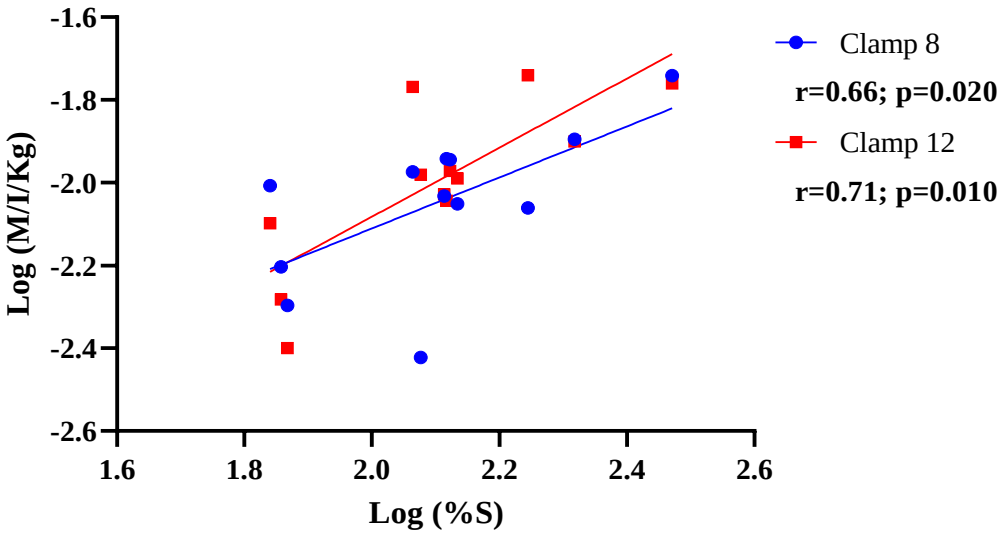


Figure 3.18: Scatterplot demonstrating the relationship between HOMA2 %S with insulin sensitivity (M/I/kg) in the control group during hyperglycaemic clamps (following log transformation).

3.4.6 HOMAtx using insulin or C-peptide

The HOMAtx model should produce similar results for %B and %S when derived from either serum insulin or C-peptide. The relationship between these was assessed and good correlation was observed for both (Figure 3.19 and Figure 3.20).

3.4.7 HOMAtx and glycaemic control

Only 3 participants in this study had impaired or diabetic glucose tolerance during the 12 OGTTs (according to WHO criteria). This is not enough to make accurate or meaningful assessments regarding the use of HOMAtx for evaluating glycaemic control in pancreas transplant recipients. The 12 OGTTs in this study were therefore combined with the 88 OGTTs performed as part of the UCPCR study in Chapter 4 to produce a cohort of 100 OGTTs that had been performed in pancreas transplant recipients. Table 3.4 summarises the characteristics of this combined cohort.

HOMAtx %B and %S were analysed against OGTT outcome for this group (Figure 3.21). There was a significant difference in HOMAtx %B between groups ($p = 0.009$) and %B was significantly higher for patients with a normal glucose tolerance test than it was for those with a diabetic glucose tolerance test result ($p = 0.006$). ROC curve analysis demonstrated an AUC of 0.94 ($p < 0.001$; Figure 3.22). A %B of 64.25% had a 100% sensitivity and 84.1 % specificity for the detection of diabetic glucose tolerance during OGTT.

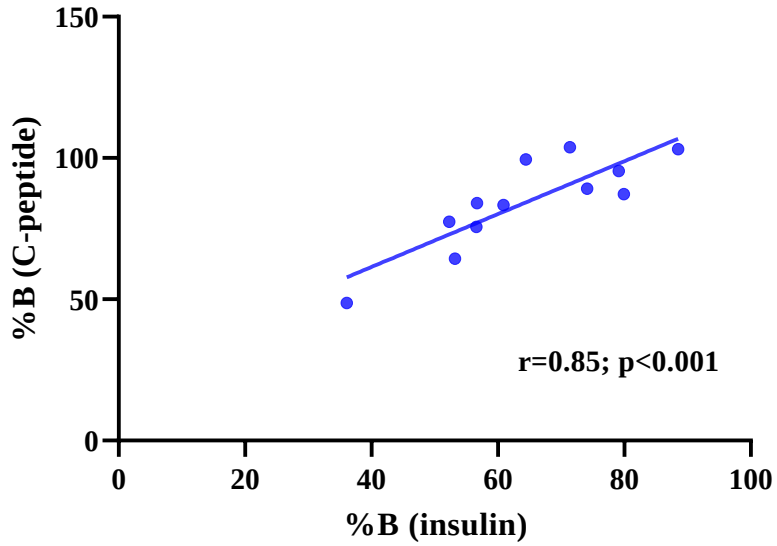


Figure 3.19: Scatterplot demonstrating the relationship with HOMAtx %B when calculated from either serum insulin or C-peptide.

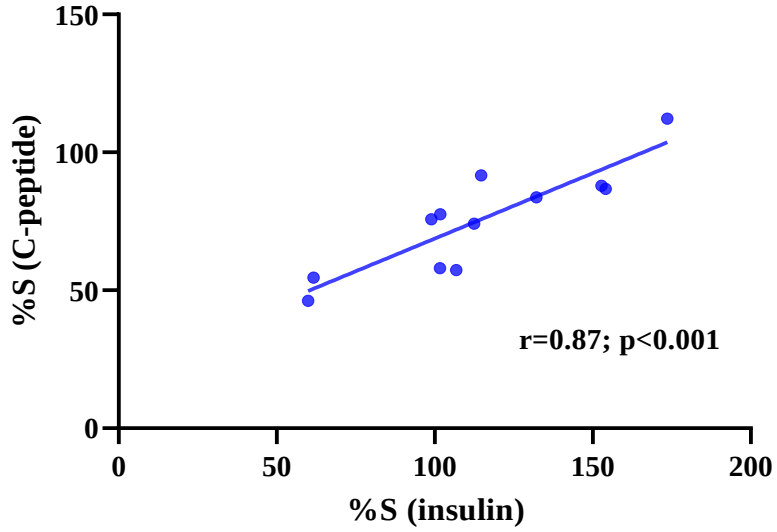


Figure 3.20: Scatterplot demonstrating the relationship with HOMAtx %S when calculated from either serum insulin or C-peptide.

Table 3.4: Characteristics of combined cohort of pancreas transplant recipients.

Participants	42
Age (years) †	47.5 (8.3)
Gender (Male)	31
BMI (kg/m^2) †	27.4 (5.15)
eGFR (ml/min/1.73m^2) †	67.2 (14.96)
CKD Stage (1/2/3A/3B)	4 / 25 /10 / 3
HbA$_{1c}$ (mmol/mol) †	34.6 (5.13)
HOMAtx %B †	96.1 (52.86)
HOMAtx %S †	112.4 (80.13)
Transplant (SPK/PTA/PAK)	37 / 3 / 2
fsOGTT	100
Normal OGTT	71
Impaired OGTT	21
Diabetic OGTT	8

† Values are mean (SD)

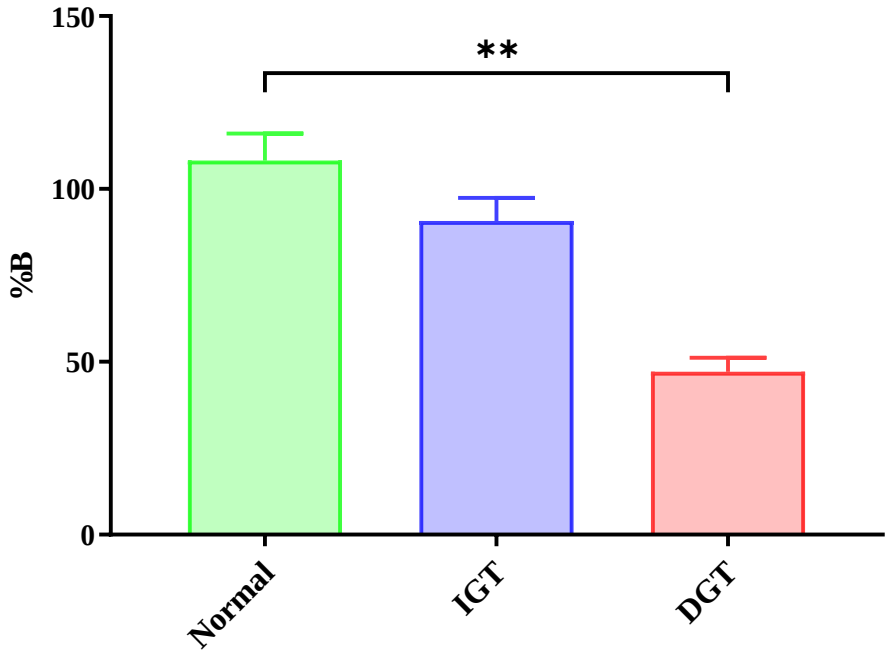


Figure 3.21: Bar chart showing mean HOMAtx %B (SEM) according to OGTT group (ANOVA: F = 5.03, p = 0.009; ** t test of normal vs DGT: t = 2.84, p = 0.006).

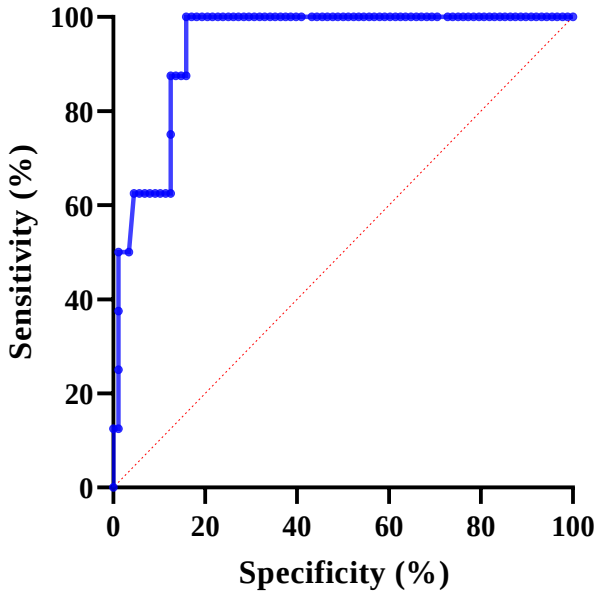


Figure 3.22: ROC curve analysis for HOMAtx %B and diabetic glucose tolerance during OGTT (AUC = 0.94, $p < 0.001$).

3.5 Discussion

The HOMA model has evolved over several iterations during the last 30 years to allow assessment of beta cell function and insulin sensitivity. It calculates these in the steady state of insulin and glucose concentrations i.e. either when fasting or during artificial glycaemic clamp techniques. The model uses physiological dose response curves to glucose uptake and insulin production and at the heart of this is hepatic glucose uptake and efflux based on portal venous endocrine drainage of the pancreas[273]. Despite this, several groups have reported the use of HOMA in the assessment of pancreas graft function and have even used HOMA to directly compare graft function between portal and systemically drained groups[275-277]. In their study, Aguilera et al.[277] concluded that 'the HOMA test should be routinely used in metabolic follow-up of patients with pancreas-kidney transplantation' based on correlation ($r = 0.64$) of HOMA %B and AUC insulin over a 5 point oral glucose tolerance test. They acknowledge some of the methodological weaknesses yet suggest that 'it is remarkable that mean HOMA-B levels were higher than 100%'; this implies a poor understanding of the HOMA model and the hyperinsulinaemia observed in systemically drained pancreas grafts.

This baseline hyperinsulinaemia (up to threefold higher than controls) has been described multiple times over the last few decades and was also observed in this study[278-280]. Three potential explanations for this have been proposed. Firstly, that the hyperinsulinaemia is caused by immunosuppressant medication such as steroids and calcineurin inhibitors. Whilst such medication is known to contribute to glucose intolerance, Diem et al. demonstrated that a comparable population of renal transplant recipients on similar medication do not have a significantly higher baseline level of serum insulin than normal non-diabetic controls[278]. Secondly, it has been suggested that the lack of high portal concentrations of insulin results in increased hepatic glucose output to which the pancreas

graft responds by increasing insulin production thereby resulting in baseline hyperglycaemia. However, Diem et al. countered this by analysing C-peptide production (minimal hepatic metabolism) between pancreas transplant recipients and control subjects – the result being that C-peptide levels were similar despite the difference in baseline and stimulated insulin levels. Thirdly and most likely, is that by positioning the pancreas graft in a heterotopic position with systemic venous endocrine drainage, there is a loss of first pass hepatic insulin metabolism which would usually result in removal of 40 - 80% of the insulin produced[281-283]. Despite hyperinsulinaemia, most pancreas transplant recipients will maintain normoglycaemia. The elevated insulin levels are thought to be offset by reduced peripheral insulin sensitivity. Support for this comes from a study by Elahi et al. who observed a 55% reduction in GLUT4 insulin transporter levels in the muscles of patients with a systemically drained pancreas graft[284]. It is important to note that the portal and peripheral drainage in the HOMA2 and HOMAtx models is sufficient to account for the hyperinsulinaemia seen in pancreas transplant recipients without any need to suspect any additional drive from impaired insulin sensitivity. For example, for %B and %S of 100%, the models would predict glucose and insulin concentrations for 4.8 mmol/l and 82 pmol/l in pancreas transplant patients and 4.9 mmol/l and 46.9 pmol/l in the normal patients.

Multivariate analyses in several older studies have suggested that hyperinsulinaemia may be an independent risk factor for poor metabolic and cardiovascular outcomes[285-289]. This has fuelled debate in the field of pancreas transplantation as to the optimal method of venous drainage and has driven some groups to perform portal venous drainage of the pancreas graft[107,290]. In addition to the proposed advantage of a more physiological glycaemic control, it has been suggested that there might be an immunological benefit of portal venous drainage with early hepatic alloantigen presentation and moderation of the immune response[108]. The most compelling evidence comes from the only RCT performed on the topic[291] and a recent

systematic review and meta-analysis from Oliver et al.[110] who analysed all papers since 1989 and found no difference in graft and patient survival or metabolic control (fasting glucose, HbA_{1c}, cholesterol, lipids) between portal and systemic drainage of the pancreas graft. Systemic venous drainage remains the preferred technique in the majority of institutions in Europe and the USA.

This is the first time that the HOMA model has been modified to allow for systemic venous drainage of the pancreas. The results show excellent correlation between HOMAtx derived beta cell function and hyperglycaemic clamps (Figure 3.8). This is an improvement over the original study by Matthews et al.[259] where r = 0.61 when using hyperglycaemic clamps. It is also comparable with several other hyperglycaemic clamp studies that included subjects with both normal and diabetic glucose tolerance where the r coefficients ranged from 0.62 – 0.90[292-294].

Correlation was better at the 8 mmol clamp stage in all of the clamp studies that we performed. This is most likely due to the increased intra-subject variation seen at the 12 mmol clamp in terms of actual serum glucose measured and corresponding glucose infusion rates (coefficient of variation for the transplant group was paradoxically better at the 12 mmol clamp than at 8 mmol).

HOMAtx derived insulin sensitivity did show some correlation with the hyperglycaemic clamp, however it was limited to the 8 mmol clamp stage and weaker than observed in other studies. Given that the HOMA2 model has previously been validated in multiple studies it is not clear whether this is a limitation of the HOMAtx model or the methodology we used. The main concern is that the hyperglycaemic clamp is not the optimal way of measuring insulin sensitivity – the gold standard is the hyperinsulinaemic euglycaemic clamp (a follow-up study using this methodology to assess HOMAtx %S is being planned). In

addition, the HOMAtx has not been altered to allow for peripheral hyperinsulinaemia in the range observed in pancreas transplant recipients. The model includes calculations for glucose uptake in peripheral fat and muscle which is insulin dependent, however, it is conceivable that the relationship (and therefore algorithms used in HOMAtx) are different in the context of persistent hyperinsulinaemia. There is evidence for reduced peripheral insulin sensitivity in pancreas graft recipients[284] and the insulin-glucose relationship has not been modelled in this context for inclusion in HOMAtx. However, theoretically this shouldn't matter because the correlation between peripheral and hepatic insulin sensitivity has not been disturbed.

Immunosuppressive drugs used in transplantation can also influence glucose homeostasis and this may have influenced the results in the transplant cohort of this study. All patients in this study were being treated with mycophenolate and tacrolimus maintenance therapy. Mycophenolate is not known to have any influence on glucose haemostasis[295]. There is however significant evidence that calcineurin inhibitors may contribute to impaired glucose tolerance and the development of diabetes after transplantation (tacrolimus more than cyclosporin[296]). The mechanism is unclear but numerous animal and human studies have suggested that tacrolimus predominantly influences insulin secretion although there is also some evidence to support a change in insulin sensitivity[297-301]. Any immunosuppressive effect would expect to be applied across the cohort and it is unlikely that the effects of variable tacrolimus levels alone can explain the poor correlation of HOMAtx-derived sensitivity in this study.

The results from HOMA2 in the control group are interesting. The correlation of %B with the hyperglycaemic clamp ($r = 0.60$, $p = 0.041$) is similar to the original study by Matthews et al.[259], although there have been several studies since with stronger correlation coefficients up to 0.9[292]. There are several potential factors that may have influenced this. Our control

cohort were matched with the transplant cohort thus creating selection bias with a less heterogeneous group. The control group had a mean %B of 71.5% and %S of 138.2% suggesting that they were different from the 'normal subjects' that give a mean %B and %S of 100%. The larger group of pancreas transplant recipients recruited for the UCPCR study described in Chapter 3 had a mean %B of 100.7% and %S of 112.1% which is clearly different to the cohort recruited for this study. The mean BMI of the control cohort was 26.5 kg/m^2 and this may explain the difference when compared to control subjects in the 1980s and 90s. Nevertheless, in contrast to HOMAtx %S in the transplant group, there was excellent correlation with HOMA2 %S in the control group. This suggests that the methodology is sound, and although not the gold standard for measuring insulin sensitivity, the hyperglycaemic clamps used have provided useful and meaningful data for this purpose. The poor correlation observed with HOMAtx %S cannot therefore be put down to methodology alone. Nevertheless, a follow-up study using hyperinsulinaemic euglycaemic clamps for assessing HOMAtx %S is being planned.

In addition to the study limitations already discussed, there are other important factors to consider when interpreting the results. The numbers in this study are small which makes analysis very sensitive to outliers. For example, there were 2 control males who had almost identical baseline parameters (age, weight, height, fasting glucose) yet dramatically different clamp results – one subject with %B = 39.3% and %S = 295.9%, and one with %B = 78.3% and %S = 115.8%. Whilst the first subject's results are clearly an 'outlier' in the context of this group, this individual had extremely tight glycaemic control during his oral glucose tolerance test. An increase in numbers should reduce the effect of such outliers and could improve the correlation observed. Numbers of participants in previous studies have varied from 7 to more than 100, yet larger cohorts do not seem to have always yielded an improved correlation[293,302]. Although not a direct limitation of this study, HOMA is only

applicable to the steady state i.e. during fasting or a glycaemic clamp. There were 3 pancreas transplant subjects that had either impaired or diabetic glucose tolerance during oral glucose tolerance testing. However, their HbA_{1c} values were all under 40 mmol/mol (normal) and their HOMAtx %B were 53 – 57% which is higher than several other subjects who had normal oral glucose tolerance tests.

Notwithstanding a lack of statistically significant correlation between HbA_{1c} and %B, there was an apparent trend evident between them. This may represent a lack of subject numbers or study timepoints though because it is discernible that as pancreas function declines, HbA_{1c} will start to rise at some point. It is important to note that HbA_{1c} is an assessment of overall glycaemic control which is a function of both %B and %S. Therefore, within the range of 'normal' glycaemic control we should not expect correlation of HbA_{1c} with either %B or %S alone. Nevertheless, as beta cell capacity reduces to a critical level, we would expect %B to reflect this. This is clearly shown in Figure 3.21 which demonstrates a significant difference in %B between those with normal and diabetic glucose tolerance during OGTT. ROC curve analysis has produced a highly sensitive and specific cut off for %B that predicts OGTT outcome from HOMAtx %B (Figure 3.22). This has significant implications for clinical practice, being potentially very useful for the early diagnosis of graft dysfunction at a time when other parameters (such as HbA_{1c}) may be normal. The numbers involved in this analysis are however small and we should therefore proceed with caution. These findings need to be confirmed in larger prospective studies that are specifically designed for this purpose.

3.6 Conclusion

In conclusion, this is the first study to develop and validate a model to assess pancreas allograft function. HOMAtx specifically addresses the need to allow for systemic venous drainage of the transplanted pancreas. The study shows that HOMAtx %B correlates well with graft function when compared with the gold standard of assessment (hyperglycaemic clamp). Therefore, a simple fasting blood test can give a useful interpretation of graft function which can be monitored over time as part of routine surveillance. Furthermore, there appears to be a clear level of %B that has a high sensitivity and specificity for predicting diabetic glucose tolerance at a time when other markers of graft function are normal. In the clinical setting, this may allow early identification of graft dysfunction with subsequent directed intervention aimed at preserving graft function. Future studies should aim to prospectively assess intra and inter subject variation in HOMAtx to evaluate whether it correlates with clinical deterioration in graft function in a timely manner.

Chapter 4

Urinary C-peptide in Pancreas Transplantation

4.1 Introduction

It is widely believed that the main reason for the high rate of graft loss after pancreas transplantation is the lack of clinically useful biomarkers. Current post-operative monitoring of graft function involves regular biochemical analysis of injury markers (serum amylase, lipase), and functional markers (C-peptide, insulin, glucose and glucose tolerance testing). Nevertheless, in clinical practice, monitoring of graft function is unsatisfactory because by the time abnormal endocrine function is detected, the damage is often irreversible. Early predictors of pancreas graft failure have been poorly defined such that a return to exogenous insulin injection is frequently the first indication of graft dysfunction[303].

As discussed in Chapter 3, the best way to directly assess beta cell function is with clamp studies[257,258]. However, these are not applicable to monitoring patients in a clinical environment. Attention therefore needs to turn towards other biomarkers or surrogates of graft function. C-peptide is one such biomarker that could fulfil this role and has been widely used as a method of assessing pancreatic beta cell function. Proinsulin is cleaved into insulin and C-peptide in the Golgi apparatus of pancreatic beta cells. C-peptide is produced in equimolar amounts to insulin, yet it doesn't suffer many of the disadvantages of insulin when used for assessing beta cell function. Insulin has a very short half-life (3 - 5 minutes), is metabolised variably in several different tissues, and is limited as a marker of endogenous beta cell function because of cross-reactivity with proinsulin and exogenous insulin. In contrast, C-peptide has a half-life of 20 - 30 minutes and is cleared from the peripheral circulation at a constant rate. This is predominantly via the kidneys through a combination

of metabolism (85%) and urinary clearance (5 - 14%)[304-306]. This can make C-peptide measurement in individuals with kidney disease inaccurate. In addition, C-peptide isn't thought to have any direct involvement in glucose metabolism although there is increasing evidence to suggest that it does have some physiological effects and may be a useful therapeutic adjunct in the treatment of diabetes[307].

Serum C-peptide is frequently tested in pancreas transplant recipients, yet isolated non-fasting values rarely provide significant insight or clinical benefit in monitoring graft function or providing early warning of impending graft failure. C-peptide is useful for the diagnosis of T1DM, in particular its differentiation from T2DM[63,308]. It may also correlate with diabetic complications as well as predict likely response to therapies and future use of insulin therapy[63,309-311].

In islet cell transplantation a clear correlation between beta cell function and serum C-peptide levels has been demonstrated[312-314]. A decline in serum C-peptide levels is associated with reduced islet mass and/or function, hyperglycaemia, and subsequent insulin dose. As such, measurement of C-peptide after carbohydrate challenge is a core test of islet cell transplant function[197,315,316]. This has recently been extended further in the BETA2 score that uses a fasting rather than stimulated C-peptide to reduce investigative burden[317].

Frequent fasting or stimulated serum C-peptide measurements could theoretically provide a more reliable method for detecting beta cell decline in solid organ pancreas transplantation. In clinical reality, however, this is intrusive and inconvenient for patients who often live significant distances from their transplant centre. Patients do not like fasting for samples nor prolonged visits for stimulated tests and therefore compliance with such techniques tends to be poor. Serum C-peptide samples need to be placed on ice and processed within hours of being taken (when taken in gel SST tubes) and this therefore prohibits local primary care

based sampling. Opportunities for samples are also sporadic with increasing intervals between patient visits after the first few months from transplantation. After one year many pancreas transplant patients with stable graft function may only be seen quarterly and this therefore presents a window where graft injury and failure could occur between visits.

Such problems with serum C-peptide sampling have prompted Hattersley et al. to investigate the use of urinary C-peptide as a surrogate for serum C-peptide and therefore beta cell function[318]. Whilst 24-hour urine collection might be optimal, this is time-consuming, requires good patient compliance and is still affected by renal function. The Exeter group have therefore used urinary C-peptide creatinine ratio (UCPCR) to allow for quick and easy spot sampling of urine specimens that are stable for up to 72 hours in boric acid[305]. They have validated this technique in a variety of cohorts including patients both with and without diabetes and also in patients with islet cell transplants[318-321]. C-peptide is eliminated by the kidneys and therefore one limiting factor is the lack of correlation between urinary and serum C-peptide in patients with severe renal impairment (correlation was preserved in those with mild to moderate impairment). This is pertinent in the context of pancreas transplantation where a significant proportion of patients have impaired renal function, even after concurrent kidney transplantation. There is also significant variation in renal function between and within individuals after transplantation due to the effects of immunosuppression, rejection, infection, and chronic nephropathy.

The use of a urine sample for assessment of pancreas endocrine function has several significant advantages. It is quick, simple, cheap and can be done remotely and frequently. Furthermore, the timing of the sample can be tailored relative to meals to give a practical home-based stimulated sample if required. Oram et al. investigated this in islet transplant recipients and demonstrated that UCPCR in urine samples taken 2 hours after breakfast

correlated well with serum C-peptide during mixed meal tolerance tests as well as other clinical means of assessing islet graft function[322].

Pancreas transplantation is offered to similar groups of patients as islet cell transplantation; specifically, they typically have type 1 diabetes with either hypoglycaemic unawareness (the commoner patient for islet transplantation) or end stage renal disease (the commoner patient for solid organ transplantation). Similar immunosuppressive regimes are used for both solid organ and islet cell transplantation. It can therefore be hypothesised that a similar correlation should exist between C-peptide production and beta cell function in solid organ transplantation.

UCPCR potentially offers a simple non-invasive test, requiring a urine sample that can be sent from a patient's home. If successful, it will improve longitudinal assessment of pancreas graft function whilst potentially allowing earlier identification of dysfunction and thereby permitting earlier assessment and intervention.

4.2 Aims and Objectives of Chapter 4

The aim of this chapter was to assess the use of UCPCR in pancreas transplant recipients and is divided into two components. Firstly, we aimed to establish whether UCPCR can be used as a surrogate marker for serum C-peptide in patients who have undergone pancreas transplantation. Secondly, we investigated the use of home urine samples analysed for UCPCR. The objectives of this chapter were:

Primary:

- To assess if UCPCR is a valid reflection of serum C-peptide during an oral glucose tolerance test in pancreas transplant recipients.

Secondary:

- To assess if a post-meal UCPCR taken at home can be used to remotely assess pancreas graft function.

4.3 Methods

4.3.1 Study design

30 pancreas transplant recipients were invited to participate in the study.

Participants attended three research visits at 0, 3 and 6 months. At each visit, participants underwent an oral glucose tolerance test with 75g glucose solution.

In addition to the 3 described research visits, participants were asked to provide a urine sample on 3 consecutive days a month for 6 months. These urine samples were taken 2 hours after breakfast to recreate a 'real-life' mixed meal tolerance test.

Research ethics and Trust approvals were gained for this study prior to recruitment (Appendix F).

4.3.2 Outcome measures

Primary:

- Correlation of 120-minute UCPCR with serum C-peptide during oral glucose tolerance testing at 0, 3, and 6 months.

Secondary:

- Correlation of mean post breakfast UCPCR with serum C-peptide from a recent oral glucose tolerance test.

4.3.3 Study participants

All patients who had received a pancreas transplant that was enterically drained were eligible for this study. Subjects were identified by the clinical team from a database of pancreas transplants performed at the Oxford Transplant Centre.

4.3.3.1 Inclusion criteria

- Aged 18 years or above
- Recipient of a pancreas transplant (SPK, PAK, PTA)

4.3.3.2 Exclusion criteria

- Any patient with a pancreas transplant that is drained via the bladder
- Poor renal function (eGFR < 30)
- Patient is taking oral hypoglycaemic agents
- Patient is taking insulin
- Inability to give informed consent
- Likely failure to cannulate
- Patient has had any investigational medical drug within 30 days of recruitment
- Pregnancy

4.3.4 Study procedures

Clinical data:

For each participant, clinical data was collected against a unique study identifier. Demographic and observational data was recorded for date of birth, gender, BMI, blood pressure, heart rate, comorbidities, details of transplant and medications.

Frequently sampled oral glucose tolerance test (fsOGTT):

Participants were asked to starve from midnight the night before the research visit and to avoid strenuous exercise or alcohol the preceding evening and morning of the research visit. At the research visit, a peripheral intravenous cannula was sited through which blood samples were taken according to the sampling protocol (Figure 4.1).

75g of anhydrous glucose was dissolved in 250 ml of water and this was drunk by the research participant over 4-5 minutes. A timer was started when drinking commenced, and participants were asked to remain resting in bed whilst blood samples were taken over 180 minutes after the glucose drink was consumed. In addition, participants gave a urine sample at 0 and 120 minutes for UCPCR and glucose testing. The oral glucose tolerance test was complete after 180 minutes and participants were provided with something to eat and drink and were observed for another 30 minutes until they achieved a stable glucose level without exogenous support. The peripheral cannula was removed, and participants were free to leave.

Home urine samples:

Participants were asked to provide a home urine sample on 3 consecutive days a month for 6 months. A pre-breakfast sample and a 2 hours post-breakfast sample were required. Samples were stored in the participants' freezer until all three days' worth of samples were complete. They were then posted directly to the laboratory for analysis of urine C-peptide, creatinine and glucose. The home urine samples started in the week after Visit 1 and then continued monthly until the 7^{th} set of urine samples were completed 6 months later (Figure 4.2).

Sampling protocol:

Blood samples were taken at each time-point as shown in Figure 4.1. 1 ml of blood was discarded at the beginning of each sample and the cannula was flushed with 1 ml saline at the end of each sample. At all timepoints 8 ml of blood was taken into a heparinised plasma tube, for testing of glucose, C-peptide and insulin. All samples were placed on ice and centrifuged at 4 °C and 3000 rpm for 10 minutes. Plasma was then separated into aliquots which were stored at -80 °C, pending assaying in batches in order to minimise batch and kit

variability. Samples were analysed with commercial chemiluminescent (glucose) or immunoassay (specific insulin and C-peptide) kits (Architect/Abbott Laboratories, Illinois, USA). Samples were processed by me at the Oxford University Hospitals NHS Foundation Trust biochemistry laboratories.

Urine samples (approximately 10 ml) were collected and stored in urine containers with boric acid preservative. They were sent directly to the Royal Devon and Exeter NHS Foundation Trust clinical and research laboratory in padded envelopes. Urine samples were pre-diluted 1:10 with equine serum albumin (diluent multianalyte, Roche diagnostics, Manheim, Germany) and C-peptide analysis was performed by electrochemiluminescence immunoassay (Roche Diagnostics E170 C-peptide assay, Manheim, Germany). Creatinine was analysed on the Roche P800 modular platform using creatinine Jaffé reagent (standardised against isotope dilution mass spectrometry)[305].

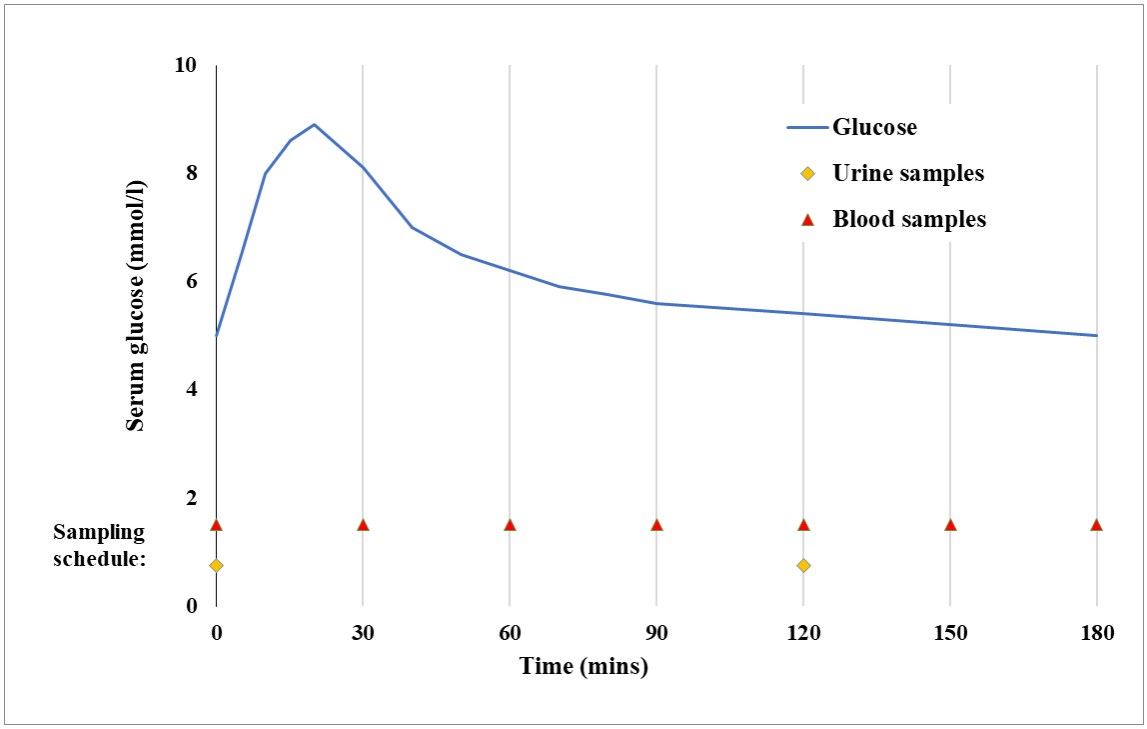


Figure 4.1: Schematic of frequently sampled oral glucose tolerance tests with sampling timepoints.

	Visits									
	1	Home	Home	Home	2	Home	Home	Home	3	Home
Week	0	1	5	9	12	13	17	21	24	25
Baseline bloods	X				X				X	
fsOGTT	X				X				X	
Home urine samples over 3 consecutive days		X	X	X		X	X	X		X

Figure 4.2: Schedule of study procedures for participants.

4.3.5 Statistical analyses

Data was tested for normality and then analysed using Pearson's or Spearman's rank correlation co-efficient between individual values. Specifically, the following data was compared:

- Fasted 0-minute UCPCR and fasted serum C-peptide/insulin
- 120-minute UCPCR and serum c-peptide/insulin at different OGTT time points
- 120-minute UCPCR and area under the curve serum C-peptide/insulin
- 120-minute UCPCR and HbA_{1c} (including grouped analysis e.g. tertiles)
- 120-minute UCPCR and OGTT result
- Mean monthly home post breakfast UCPCR and serum C-peptide at different OGTT time points (including grouped analysis e.g. tertiles)

Further analysis for renal function and glucose tolerance subgroups were performed where data permitted.

Statistical analyses were performed using SPSS version 25 for Windows (IBM SPSS Statistics, Armonk, New York, USA) and Graphpad Prism version 8 for Windows (GraphPad Software, La Jolla, California, USA). A p value of <0.05 was considered to be statistically significant.

4.4 Results

4.4.1 Demographics and baseline graft function

30 participants with pancreas transplants were recruited to the study (Table 4.1). One participant dropped out of the study after the first visit, but data acquired to this point was included for analysis. The group consisted of 21 men and 9 women. The mean age was 46.7 years (SD = 8.12) with a BMI of 27.4 kg/m^2 (SD = 5.74). The group consisted of 25 SPK, 3PTA, and 2 PAK transplants. The median time after transplantation that participants were recruited was 22 months (range 2 - 128).

Baseline pancreas graft function was good - all participants had a normal HbA_{1c} at the time of recruitment with a mean of 34.3 mmol/mol (SD = 5.52). Mean fasting glucose was 5.2 mmol (SD = 0.77). HOMAtx derived measures of graft function revealed a mean %B of 100.7% (SD = 54.86) and mean %S of 112.1% (SD = 84.90).

According to KDIGO guidelines[323], only one participant had normal renal function; 16 had mild CKD whereas 13 had moderate CKD. Mean glomerular filtration rate, calculated using the MDRD equation (eGFR), was 64.3 $ml/min/1.73m^2$ (SD = 15.29).

Table 4.1: Demographic and baseline data for study participants.

Participants	30
Age (years) †	46.7 (8.12)
Gender (Male)	21
BMI (kg/m^2) †	27.4 (5.74)
eGFR (ml/min/1.73m^2) †	64.3 (15.29)
CKD Stage (1/2/3A/3B)	1 / 16 /10 / 3
HbA_{1c} (mmol/mol) †	34.3 (5.52)
Fasting Glucose (mmol/l) †	5.2 (0.77)
Fasting Insulin (pmol/l) †	104.6 (74.44)
Fasting C-peptide (pmol/l) †	671.6 (302.88)
HOMAtx %B †	100.7 (54.86)
HOMAtX %S †	112.1 (84.90)
Transplant (SPK/PTA/PAK)	25 / 3 / 2

†Values are mean (SD)

4.4.2 UCPCR in pancreas transplantation

88 frequently sampled oral glucose tolerance tests were successfully performed in 30 participants between November 2015 and June 2017 (Figure 4.3 and Table 4.2). Two urine samples from these visits were not processed due to transportation problems (i.e. leaked or lost in transit). According to WHO criteria[324], 62 of the OGTTs performed were normal whereas 26 were either impaired or diabetic. Only 3 of these had a corresponding HbA_{1c} of $\geq$ 48 mmol/mol.

UCPCR and serum C-peptide and insulin results were analysed for a range of timepoints (Table 4.3). The strongest correlation was observed between the 120-minute UCPCR and the 120 minute serum C-peptide (Figure 4.4). Pearson correlation analysis confirmed an r coefficient of 0.62 ($p < 0.001$).

Table 4.2: fsOGTT and UCPCR results.

fsOGTT (n)	88
UCPCR results	85
UCPCR lost/leaked/lab error	3
UCPCR at 120 mins (nmol/mmol) †	1.8 (1.26)
Normal OGTT	62
Impaired OGTT	19
Diabetic OGTT	7

†Values are mean (SD)

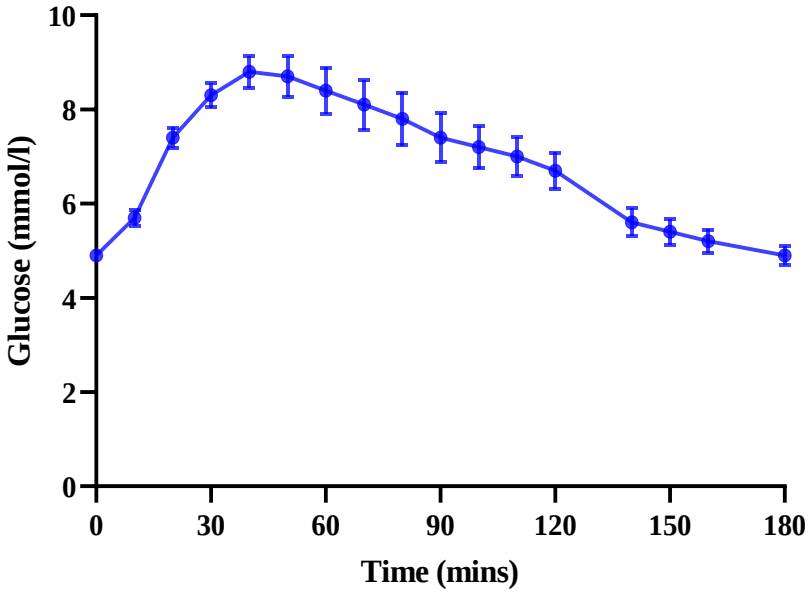


Figure 4.3: Oral glucose tolerance tests showing mean glucose for all participants. Bars represent SEM.

Table 4.3: Correlation between UCPCR and serum C-peptide or insulin during fsOGTT.

UCPCR timepoint (mins)	Serum C-peptide timepoint (mins)	R value	P value
0	**0**	0.54	< 0.001
0	**120**	0.45	< 0.001
120	**60**	0.15	0.170
120	**90**	0.33	0.002
120	**120**	0.62	< 0.001
120	**150**	0.33	0.002
120	**AUC 0-120**	0.36	< 0.001

UCPCR timepoint (mins)	Serum insulin timepoint (mins)	R value	P value
120	**90**	0.48	< 0.001
120	**120**	0.57	< 0.001

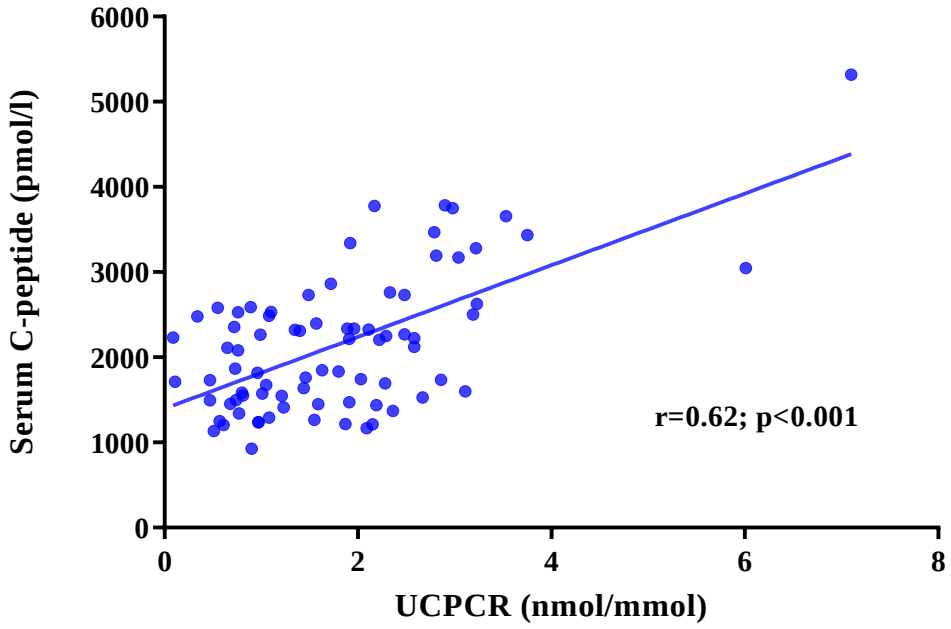


Figure 4.4: Scatterplot demonstrating relationship between 120-minute serum C-peptide and 120-minute UCPCR during OGTT (r= 0.62, p < 0.001).

4.4.3 UCPCR in pancreas transplant recipients with CKD

40 of the 88 OGTTs were performed in patients with moderate or severe CKD (Stage 3A = 28, Stage 3B = 11, Stage 4 = 1). These are highlighted in the context of all patients in Figure 4.5. If these participants are removed from the correlation analysis of serum C-peptide and UCPCR at 120 minutes, then correlation weakens slightly with $r = 0.59$ ($p < 0.001$). Linear regression analysis confirmed that the association between UCPCR and 120-minute serum C-peptide was not different between those with and without renal impairment ($p = 0.43$)

4.4.4 UCPCR and glycaemic control

The data was analysed for associations between UCPCR and glycaemic control (HbA_{1c} and OGTT result). There was a significant correlation observed when UCPCR was analysed against participants that only had an HbA_{1c} of more than 40 mmol/mol ($r = 0.77$, $p = 0.004$; Figure 4.6). There was no significant difference between HbA_{1c} and UCPCR tertiles ($p = 0.38$; Figure 4.7A), nor UCPCR and HbA_{1c} tertiles ($p = 0.78$; Figure 4.7B).

There was, however, a significant difference in UCPCR according to OGTT result group ($p = 0.006$; Figure 4.8). Receiver operator characteristic curve (ROC) analysis demonstrated an area under the curve of 0.86 ($p = 0.002$; Figure 4.9). A UCPCR value of 1.165 nmol/mmol had a 100% sensitivity and 64.1% specificity for having diabetic glucose tolerance during OGTT.

4.4.5 UCPCR and HOMAtx

The HOMAtx model was used to calculate %B and %S for all participants and each visit (Table 4.1). Weak correlation between UCPCR and %B was confirmed ($r = 0.31$, $p = 0.008$; Figure 4.10).

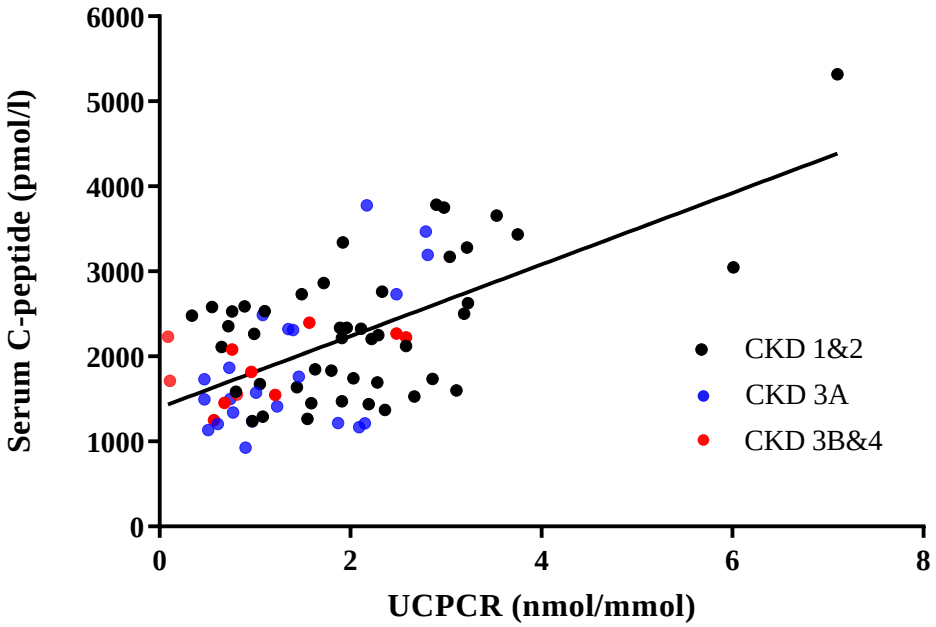


Figure 4.5: Scatterplot demonstrating relationship between 120-minute serum C-peptide and 120-minute UCPCR during OGTT with CKD participants highlighted.

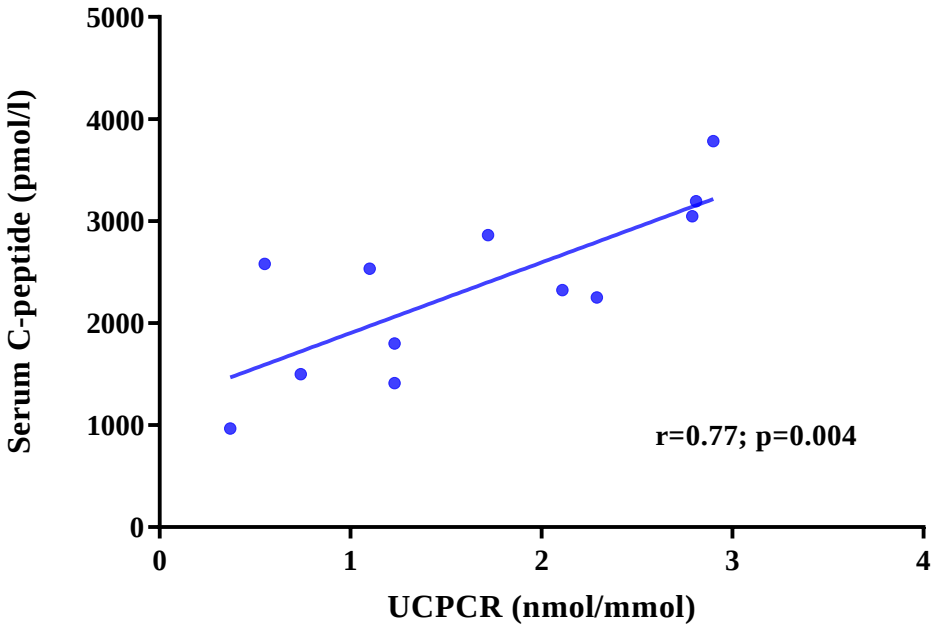


Figure 4.6: Scatterplot demonstrating relationship between 120-minute serum C-peptide and 120-minute UCPCR during OGTT for participants only with HbA_{1c} > 40 mmol/mol (r = 0.77, p = 0.004).

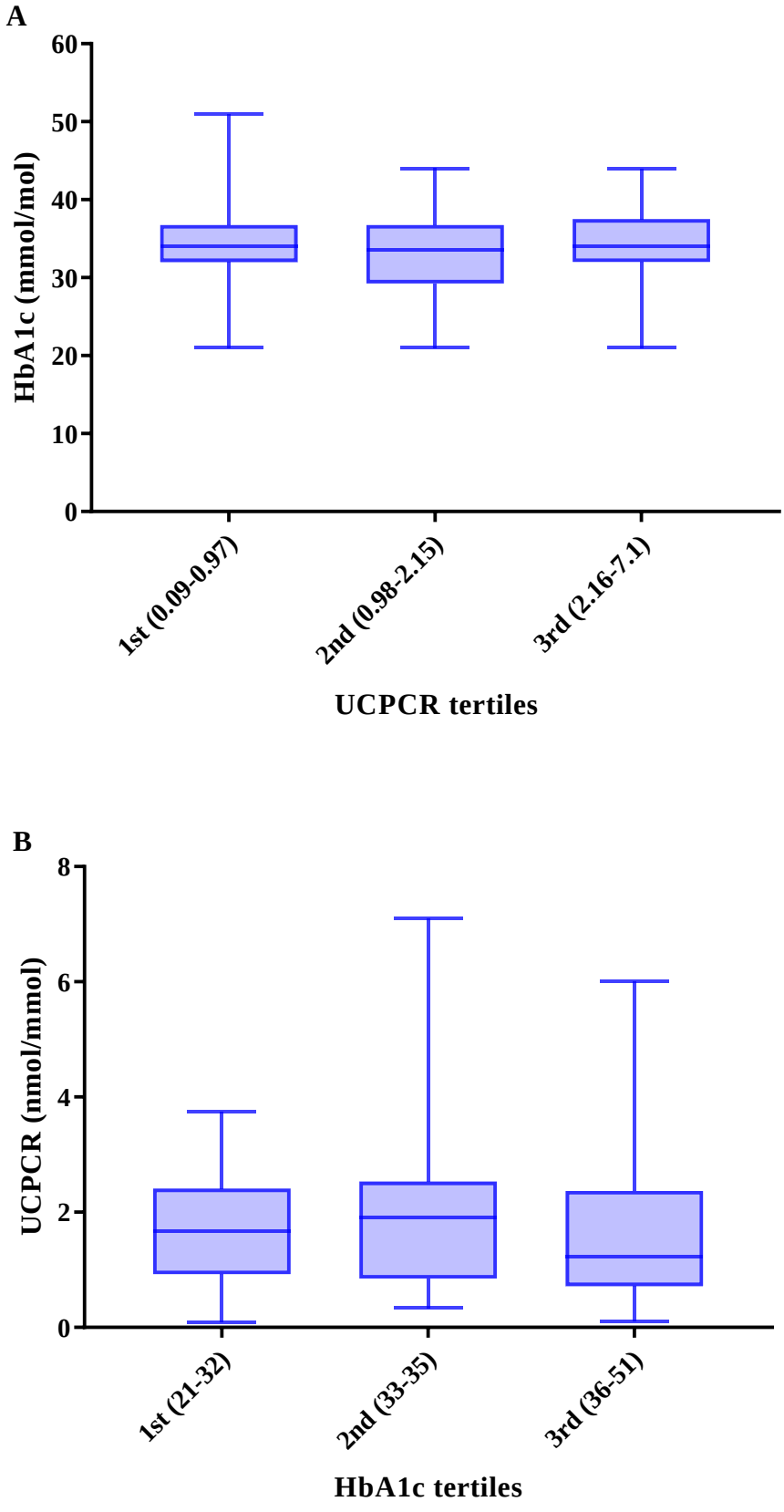


Figure 4.7: Box and whisker plots. (A) HbA_{1c} according to UCPCR tertiles (p = 0.38) (B) UCPCR according to HbA_{1c} tertiles (p = 0.78).

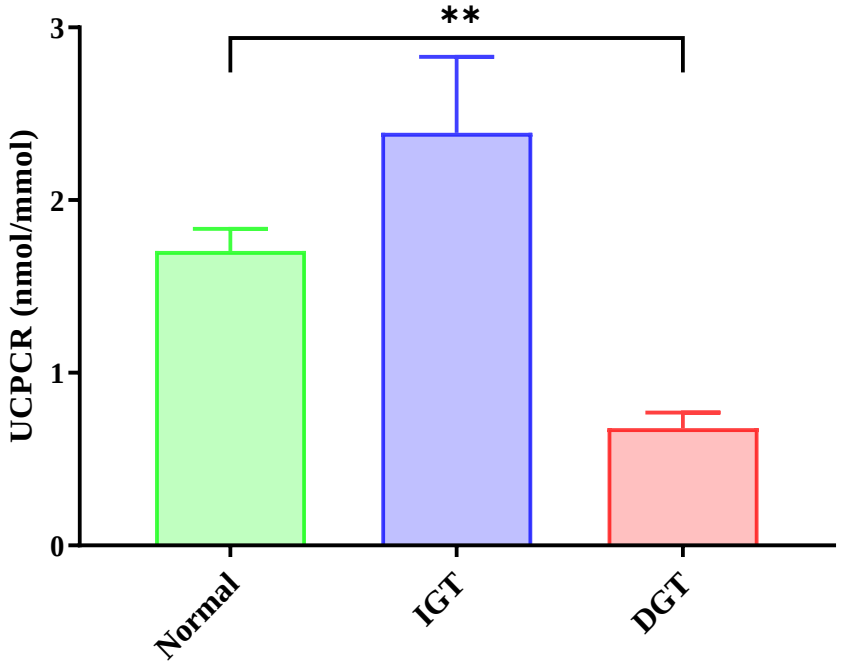


Figure 4.8: Bar chart showing mean UCPCR (SEM) according to OGTT group (ANOVA: F = 5.40, p = 0.006; ** t test of normal vs DGT: t = 2.69, p = 0.009).

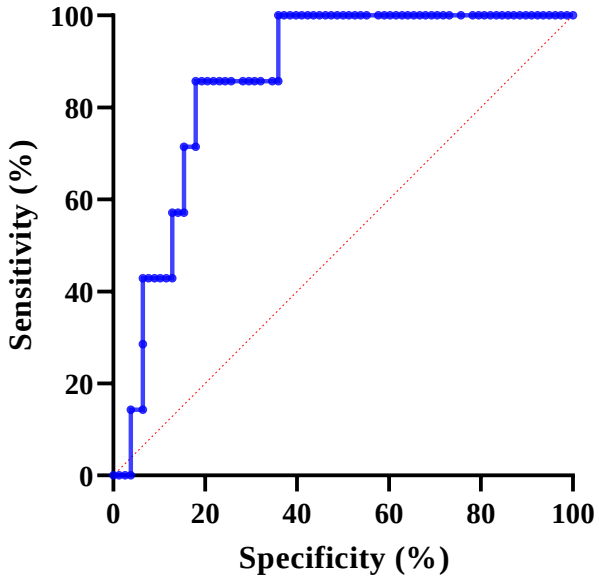


Figure 4.9: ROC curve analysis for UCPCR result and diabetic glucose tolerance during OGTT (AUC = 0.86, p = 0.002).

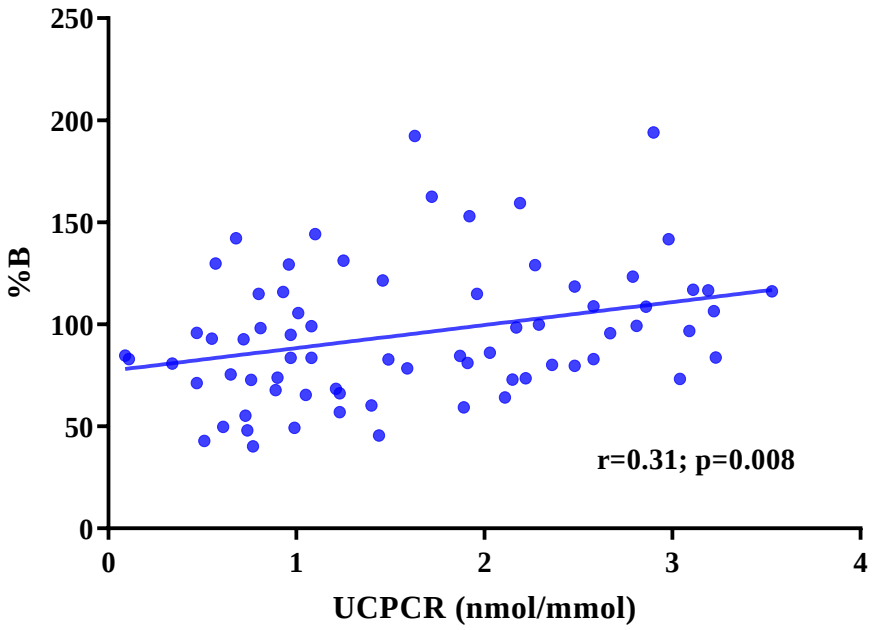


Figure 4.10: Scatterplot demonstrating relationship between HOMAtx %B and 120-minute UCPCR during OGTT (r = 0.31, p = 0.008).

4.4.6 Home UCPCR

The 30 participants sent a total of 579 home urine samples for UCPCR (2 hours post breakfast). 25 of these were not processed due to transportation or sample problems, leaving results available for 554 samples (Table 4.4).

The mean post-breakfast home UCPCR was significantly less than the mean 120-minute OGTT UCPCR (1.3 vs 1.8 nmol/mmol; $t = 5.22$, $p < 0.001$). Home UCPCR results were analysed against serum C-peptide results from different timepoints during the OGTTs. There was a significant correlation between home UCPCR and the 120 minute serum C-peptide ($r = 0.31$, $p < 0.001$), however, in contrast to the OGTT UCPCR samples, the strongest home UCPCR correlation was observed with the 90 minute serum C-peptide ($r = 0.32$, $p < 0.001$; Figure 4.11).

Home UCPCR results from the 1st, 4th, and 7th months were analysed against the C-peptide results from OGTTs that had taken place in the preceding week (i.e. these specific home urine samples were always sent approximately 1 week after an OGTT). The strongest correlation was observed with the 120-minute serum C-peptide ($r = 0.37$, $p = 0.001$; Figure 4.12).

Receiver operator characteristic curve (ROC) analysis of mean home UCPCR and OGTT result group demonstrated an area under the curve of 0.80 ($p = 0.002$; Figure 4.13). A UCPCR value of 1.140 nmol/mmol had a 100% sensitivity and 58.9% specificity for having diabetic glucose tolerance during OGTT.

Table 4.4: Home UCPCR results.

Home UCPCR (total possible)	609
Home UCPCR results received	554
UCPCR leaked/lost/mislabelled	25
UCPCR missed	30
UCPCR at 120 mins (nmol/mmol) †	1.3 (0.71)

†Values are mean (SD)

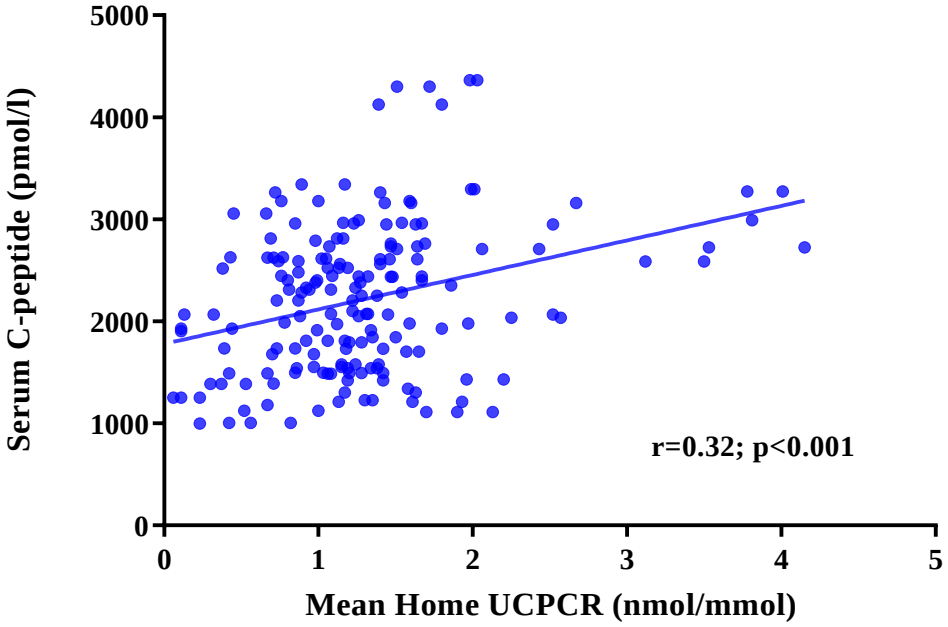


Figure 4.11: Scatterplot demonstrating relationship between 90-minute serum C-peptide and mean home post-meal UCPCR (r = 0.32, p < 0.001).

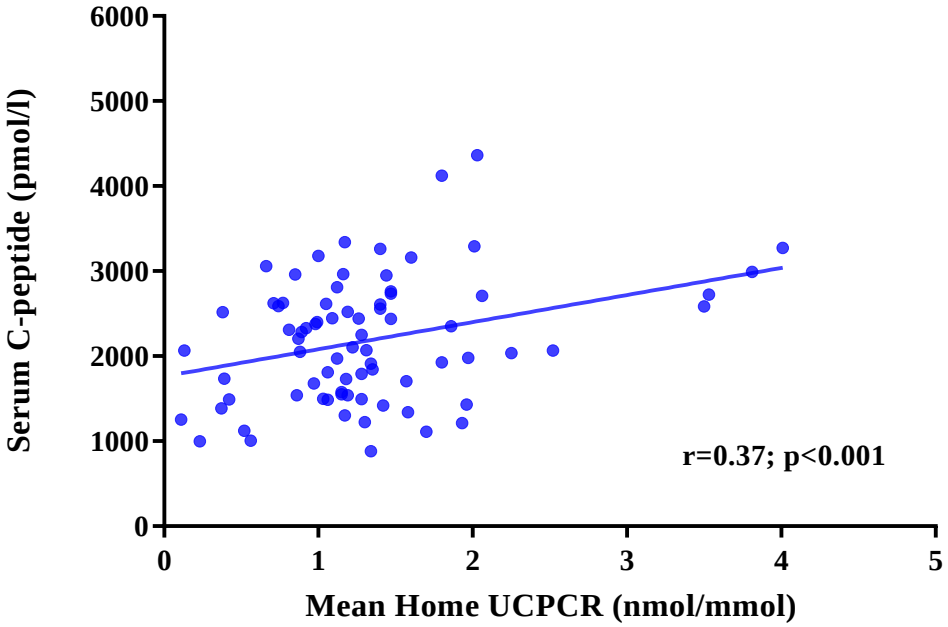


Figure 4.12: Scatterplot demonstrating relationship between 120-minute serum C-peptide and mean home post-meal UCPCR samples sent in the week immediately after an OGTT (months 1, 4 and 7; r = 0.37, p < 0.001).

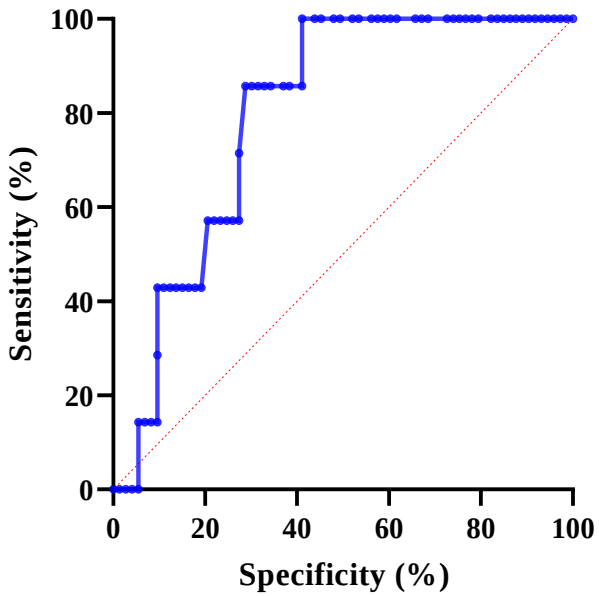


Figure 4.13: ROC curve analysis for mean home UCPCR result (H1, H4, H7) and diabetic glucose tolerance during OGTT (AUC = 0.80, p = 0.009).

4.5 Discussion

The use of urinary C-peptide quantification for assessing beta cell function is not novel, having first been described over 40 years ago[325,326]. Nevertheless, clinical interest was revitalised when McDonald et al. demonstrated that a single urine sample preserved in boric acid could be stable and reproducible for up to 72 hours when tested for C-peptide creatinine ratio[305]. This spot sample correlated well with 24 hour urine C-peptide which itself had previously been shown to be a good measure of beta cell function[326,327]. There has now been nearly a decade of research into the use of UCPCR for assessing beta cell function and it has been validated in various patient cohorts including adults with type 1 & 2 diabetes mellitus[304,319,321], paediatric diabetes mellitus[318], maturity onset diabetes of the young (MODY)[328,329], pregnancy[330], and islet cell transplantation[322]. It is now available for routine clinical practice in several NHS Trusts in the United Kingdom.

The 30 participants in this study form a heterogeneous group that is a realistic representation of our pancreas transplant recipient cohort in terms of type of pancreas transplant, BMI, variation in CKD, and time after transplantation. There is clearly variation in graft function and glucose homeostasis with HbA_{1c} values ranging from 21 to 51 mmol/mol and there are also significant differences in both fasting glucose and HOMAtx %B compared with the cohort of participants in the HOMA study described in Chapter 3 ($p = 0.010$ and $p = 0.030$ respectively). It is interesting to see the disparity that can exist between OGTT result and HbA_{1c}. There are several participants in this cohort that had an HbA_{1c} of between 32 and 37 mmol/mol yet had frankly diabetic glucose tolerance tests with 120-minute glucose values in excess of 11.1 mmol/l. The use of HbA_{1c} for diagnosing diabetes has been mired in controversy for decades yet it has now been widely adopted for use in the general population due to its ease of use and overall reproducibility[331,332]. None of the patients with either impaired or diabetic OGTT results and normal HbA_{1c} subsequently developed significant

deterioration of their HbA_{1c} over the 6 months of the study and therefore the relevance of this test in the diagnosis of acute pancreas graft dysfunction must be questioned. Several of these patients did however have objective evidence of progressive graft dysfunction by other measures such as OGTT and HOMAtx. This further highlights the need for validated biomarkers that can be reliably used for pancreas allograft surveillance.

This study demonstrates a good correlation between 120-minute UCPCR and 120 minute serum C-peptide during OGTT (r = 0.62). Bowman et al. [321] reported a similar correlation in 77 adults with type 2 diabetes (r = 0.64) whereas Besser et al. [318] reported excellent correlation in 72 children and adults with type 1 diabetes (r = 0.91). Oram et al. [322] also reported good correlation in a cohort of 17 islet transplant recipients (r = 0.73). Interestingly, the two cohorts with the strongest correlation between UCPCR and serum C-peptide are also those with the lowest amount of beta cell capacity (i.e. type 1 diabetes and islet transplant recipients). One explanation for this is that serum and urinary C-peptide are less variable when absolute levels are lowest. The median UCPCR in T1DM and islet recipients was 0.34 nmol/mmol[318] and 0.74 nmol/mmol[322] respectively whereas in this study it was 1.58 nmol/mmol. There is clearly a substantial beta cell reserve in most pancreas allografts that produces significant variation in response to stimulation whereas most islet transplant recipients and patients with type 1 diabetes are already operating at the limit of their beta cell secretion capacity. This is also reflected by the fact that all pancreas transplant recipients in this study were insulin independent.

There was a significant correlation between fasting UCPCR and 120-minute serum C-peptide in this study (r = 0.45; Table 4.3). This was also reported by Besser et al.[318] in their study of patients with type 1 diabetes (r = 0.94). This has significant implications for the clinical use of UCPCR where patients are generally averse to stimulated tests and prolonged waiting in secondary care environments. Therefore, if a single fasting sample gives a

reasonable representation of the stimulated sample, there is arguably little additional benefit of undergoing the full stimulated test. This should also be treated with caution though because it may not accurately represent those patients with graft dysfunction where a fasting sample does not correctly reflect the limited secretory capacity that would be highlighted by a stimulated test.

Correlation between UCPCR and serum C-peptide in this study was preserved in patients with renal dysfunction, notably even in those with a GFR between 30 and 44 ml/min/1.73m^2 (CKD stage 3B). It is clear from Figure 4.5 that these patients are not significant outliers and regression analysis confirmed no significant difference in the association between UCPCR and serum C-peptide at 120 minutes. This study contains the largest number of participants with renal impairment and our findings confirm those of Bowman et al.[321] and Oram et al.[322] who also observed no difference in UCPCR correlation in those with moderate CKD. It would be expected that severe renal impairment would have a significant effect on UCPCR; specifically, a higher serum C-peptide with lower UCPCR would be anticipated and this should be assessed in future work.

Oram et al. demonstrated a lower threshold of UCPCR that had clear implications for islet graft function, clinical scores of beta cell function, and ultimately insulin requirements[322]. Similar to that seen in islet transplantation, there was a sensitive lower value of UCPCR in this study that could predict diabetic glucose tolerance during OGTT (UCPCR <1.165 nmol/mmol had a sensitivity of 100% and specificity of 64.1%; Figure 4.9). In clinical practice this would mean that UCPCR values > 1.165 nmol/mmol would be very likely to reflect good graft function whereas lower UCPCR values might alert us to potential graft dysfunction requiring further investigation.

There was no clear relationship between UCPCR and HbA_{1c} in this study. It is likely that this reflects the diversity of pancreas transplant recipients and the cause of their poor glycaemic control. Two participants with either impaired or diabetic glucose tolerance and an HbA_{1c} of 32 mmol/mol highlight this. One participant had a BMI of 19, a HOMAtx %B and %S of 40.2% and 299% respectively and low UCPCRs of approximately 0.6 nmol/mmol. The second participant had a BMI of 33, a HOMAtx %B and %S of 123.3% and 55.5% respectively, and high UCPCRs of more than 2.5 nmol/mol. The first participant is behaving like someone with beta cell insufficiency whereas the second is more akin to insulin resistance. In this context UCPCR could have a role in guiding management of these patients, particularly when taken in combination with other measures such as HOMAtx and HbA_{1c}. A patient with high UCPCRs is likely to have significant beta cell capacity and may benefit from treatment similar to that offered to those with T2DM. Indeed, there have been 2 studies suggesting that UCPCR can help predict which patients with type 2 diabetes will respond to GLP-1 receptor agonists[333,334]. Furthermore, UCPCR has also been used to monitor patients with type 2 diabetes for progression to absolute insulin deficiency[335]. It is interesting to note that the correlation between UCPCR and serum C-peptide was preserved and indeed was strongest when looking specifically at participants with an HbA_{1c} of more than 37 mmol/mol ($r = 0.69$). These patients are likely to already have some degree of graft dysfunction and is the cohort most at risk of graft failure within 12 months[202].

Home UCPCR did not perform as well as seen in other studies. Correlation was observed with serum C-peptide from the OGTTs but this was not particularly strong ($r = 0.32$; Figure 4.11), although highly statistically significant ($p < 0.001$) and this was improved by using just the UCPCR from samples taken the week after an OGTT ($r = 0.37$; Figure 4.12). Oram et al.[322] described a stronger correlation between their home post-breakfast UCPCR samples and the 90 minute serum C-peptide ($r = 0.73$) in their cohort of islet transplant patients whilst

Besser et al.[318] observed a very strong association between UCPCR after a standardised breakfast with 90 minute MMTT C-peptide (r = 0.94). There are several explanations for this. Patients were requested to consume their 'normal breakfast' with no other stipulations. It is likely that this led to significant variability whereas Besser et al. used a standardised breakfast meal that contained a specified amount of carbohydrate. Compliance may have also contributed to home sample variability. Several participants informed the research team that they had taken the urine sample at the wrong time or not at all and therefore these results were discarded. It is therefore possible that this happened to others without the research team being informed. We attempted to allow for this variability by taking the mean of samples taken over 3 consecutive days. This part of the study was intentionally designed to assess applicability of the technique in 'real world' conditions and it is interesting to note that only 5% of samples were missed by patients. However, when combined with other causes of missing results such as mislabelling, postal losses, spillage and laboratory errors, a total of 9% of home UCPCR results were not available.

The mean UCPCR values from the home urine samples were significantly lower than those from the OGTT urine samples (1.3 nmol/mol vs 1.8 nmol/mol; $p < 0.001$). This difference may be due to the difference in carbohydrate load in the lab-based OGTT and home based autonomous MMTT (breakfast). Pancreas transplant patients as a group are often particularly wary of consuming carbohydrates due to many years of strict carbohydrate awareness pre-transplantation and their experience of diabetic complications. This is likely to influence the carbohydrate content of a typical breakfast in this cohort and would subsequently translate into the lower UCPCR values that were observed. A standardised breakfast may have alleviated some of this difference. Despite the potential weaknesses in performing home based MMTTs and urine sampling, ROC curve analysis demonstrated a clear lower threshold for predicting diabetic OGTT result (Figure 4.13). A value of < 1.140

nmol/mmol was 100% sensitive and 58.9% specific for detecting diabetic glucose tolerance during OGTT in the previous week. This reinforces our findings from the OGTT results and lends support for this technique to be investigated further for home based pancreas allograft surveillance.

The effect of renal transplantation and immunosuppression on UCPCR is unknown. Transplantation and subsequent ischaemia-reperfusion injury causes acute tubular necrosis of the kidney that may clinically manifest as delayed graft function. This typically takes several weeks to months to fully recover and may affect long-term graft function and graft survival[336]. There were 6 participants in this study who were recruited within 6 months of SPK transplantation. They all had excellent pancreas and renal graft function with a range of stimulated UCPCRs of 0.97 to 3.75 nmol/mmol. Calcineurin inhibitors are known to cause nephrotoxicity as well as impair beta cell insulin secretion and increase insulin resistance[337,338]. Yet, how these interactions may affect overall C-peptide production and subsequent renal clearance remains unclear. This study, alongside that of Oram et al., suggests that any direct effect of immunosuppression or transplantation on UCPCR is modest and that it can be used as a relatively simple surrogate of C-peptide production.

4.6 Conclusion

In conclusion, this is the first study to assess the use of urinary C-peptide creatinine ratio in pancreas transplant recipients. It confirms that UCPCR correlates well with serum C-peptide during fasting and stimulated testing such as an OGTT. It can therefore be used as a simple, cheap, and non-invasive surrogate for pancreas graft function. A lower threshold for stimulated samples has been identified that can accurately identify graft dysfunction in the presence of more traditional markers such as HbA_{1c} that may be normal. Home based urine samples demonstrate the same high sensitivity for predicting graft dysfunction and could provide an invaluable tool for remote graft surveillance. Future prospective studies are required to assess whether UCPCR can identify graft dysfunction as part of a surveillance package that incorporates both remote and secondary care based assessment.

Chapter 5

Sentinel Skin Flaps in Pancreas Transplantation

5.1 Introduction

The overall rate of pancreas graft loss remains high when compared with other solid organ transplants (such as liver and kidney). More than 48% of isolated pancreas recipients and 22% of simultaneous pancreas kidney recipients will have returned to insulin therapy within 5 years of transplantation[101]. This high rate of graft loss reduces the benefit of pancreas transplantation and restricts its application to patients with the most severe symptoms. Failure of the pancreas graft results in significant morbidity and an increased risk of loss with any further transplant due to allosensitisation.

There are many potential causes of pancreas graft loss that have already been described in Chapter 1.5. Although very early rejection has been reported[175], most graft loss in the immediate post-operative period is not due to rejection. However, beyond the immediate post-operative period it is highly likely that the predominant cause of graft loss is immunologically mediated. Any sustained immunological assault on the pancreas allograft will eventually produce graft dysfunction and then failure; therefore, early diagnosis and treatment of rejection is critical to preventing and reversing transplant damage caused by the immune response. We have no validated biomarkers to identify or predict early rejection or graft failure in pancreas transplantation, such that by the time abnormal endocrine or exocrine function is detected, the damage is often irreversible with patients returning to insulin therapy[200,339]. Chapters 3 and 4 have focused on the use of novel techniques to monitor endocrine function more sensitively and thereby identify pancreas graft dysfunction earlier than is currently possible in clinical practice. Whilst these techniques show

significant potential for use in graft surveillance, the primary cause for dysfunction still needs to be investigated and managed.

Immunological causes of graft dysfunction are difficult to identify definitively without pancreas allograft biopsies. As discussed in Chapter 1.6, direct pancreas biopsies are a topic of controversy with many units arguing that the potential complications and morbidity outweigh the benefit of histopathological assessment. Conversely, it is difficult to justify the potential morbidity of escalating immunosuppression without histological confirmation of rejection; thereby a dilemma arises that ultimately may contribute to conservatism, management variability, and poorer outcomes. Some units have used donor duodenal biopsies in PTA transplants and kidney biopsies in SPK transplants as surrogates of the pancreas allograft. However, these techniques also have associated morbidity and discordant rejection has been reported with rates of up to 60%, meaning that isolated rejection of the pancreas may be missed[170,172,216,340].

The development of accurate techniques that predict rejection before the pancreas graft sustains any injury whilst also recognising the absence of rejection is therefore crucial. Such techniques would allow the use of targeted immunosuppression according to the needs of each transplant recipient. Those at risk of rejection would receive increased immunosuppression before the onset of any damage to the allograft, whilst those with stable graft function would benefit from a reduction of the empirical immunosuppression, thus minimising the damaging side effects of this medication.

Vascularised composite allografts (VCAs) are transplants that contain multiple tissue components including skin, fat, fascia, vessels, muscle, nerve, tendon and bone. There have been approximately 200 limb, face, and abdominal wall VCA transplants performed worldwide since the first successful hand transplant in 1998[341,342]. The tissue components

of these VCAs are subject to a destructive alloimmune response in the absence of immunosuppression. Skin is regarded as the most susceptible of the VCA tissue components to rejection and it therefore allows prediction of rejection in the remaining components[343-346]. Rejection of the skin is typically easily visualised as a skin rash and can be easily biopsied. The skin biopsy is then assessed using the Banff VCA working classification system for rejection and patients can be treated depending on the severity of rejection[344].

Several groups have co-transplanted donor skin to remote recipient locations in addition to the primary VCA to facilitate remote monitoring and to provide access for biopsies away from the primary VCA[347-349]. Initially this was with non-vascularised skin grafts and then subsequently with vascularised skin flaps[347,350,351]. Experience from the VCA programmes suggests that rejection episodes are usually concordant between the primary VCA and vascularised skin flap[348].

Abdominal wall transplants are a type of VCA that were first performed with the sole intention of achieving primary abdominal closure after intestinal transplantation[352]. The Miami group that first described this type of transplant even utilised abdominal walls from different donors to the intestinal grafts in their quest to avoid an open abdomen and the inevitable comorbidities. Abdominal wall transplantation was subsequently adopted by the Oxford intestinal transplantation team[353], which performed direct microvascular anastomoses to the epigastric vessels[354], and also introduced remote revascularization of the abdominal wall transplant to upper limb vessels[355]. Based on their experience with simultaneous, single donor, intestinal and abdominal wall transplantation, it became increasingly apparent that the abdominal wall transplant might also function as a sentinel marker for rejection of the visceral allograft[356,357]. Historically, intestinal transplant patients presenting with bowel dysfunction can be very difficult to manage with immunological and infective aetiologies being most common, yet difficult to differentiate and at alternate ends

of the spectrum in terms of treatment strategies. In this context, the presence of a VCA seems to have significantly aided the management of these patients. The presence of skin rejection with bowel dysfunction is usually treated as acute rejection (albeit usually with confirmatory biopsies), whereas the absence of skin rejection combined with equivocal intestinal biopsies leads to investigation for other causes. The Oxford group has not reported any cases of discordant intestinal rejection in the absence of skin rejection[358]. In contrast, Selvaggi et al. did report discordant rejection occurring in both the intestine and abdominal wall transplants[359]. Crucially, however, 3 out of 4 of these patients had abdominal wall grafts from a separate donor to the intestinal graft.

Gerlach et al. reported a case controlled series of 15 patients at Oxford receiving donor-derived abdominal wall and highlighted that there was a significant reduction in bowel rejection episodes (7% vs 27%), yet the total number of rejection episodes were similar when including skin rejection[356]. This was presumed to be due to the treatment of skin rejection that pre-empted the development of intestinal rejection. Indeed, anecdotal evidence suggests that rejection of the VCA skin may precede rejection of the transplanted intestine by up to 10 days. As the experience with abdominal wall transplantation develops, clinicians are increasingly relying on the 'sentinel' aspect of the abdominal wall to aid in the clinical management of patients with suspected rejection or infection[356]. However, many patients undergo intestinal transplantation without concurrent abdominal wall transplantation. This may be because it is not required to obtain adequate primary closure, donor consent is not available, or because there are significant size or aesthetic differences. The solution in these cases may lie in the use of a remote section of vascularised skin as a sentinel skin flap from the same donor as the intestinal graft. Several such cases of remote sentinel skin flaps (SSF) have been performed by the Oxford team in intestinal transplant patients (unpublished).

Using the skin as a sentinel marker of rejection has the potential to benefit other types of visceral transplantation where there is immunological complexity or difficulty in monitoring the primary allograft. Pancreas transplantation is one such example where there is significant difficulty in monitoring the pancreas allograft and where a robust method for detecting early rejection could significantly reduce rates of graft loss secondary to immunological events.

5.2 Aims and Objectives of Chapter 5

The aim of this chapter was to assess the use of vascularised donor-derived sentinel skin flaps for diagnosing and monitoring rejection in pancreas transplantation. The study investigated the use of sentinel skin flaps in clinical practice and evaluated whether rejection occurs concordantly or discordantly between the sentinel skin flap and the transplanted abdominal organs. A series of more detailed immunological investigations were also performed during this study and these will be described separately in Chapter 6.

The objectives of this chapter were:

Primary:

- To establish if a sentinel skin flap acts as a marker of rejection of the transplanted pancreas/kidney.

Secondary:

- To establish if discordant rejection occurs between the sentinel skin flap and the pancreas/kidney.
- To establish if treatment of skin rejection prevents rejection of the pancreas/kidney.
- To establish if the use of a sentinel skin flap is associated with improved pancreas graft survival at 12 months.

5.3 Methods

5.3.1 Study design

This study has two components. The first is a clinical pilot study that investigated whether sentinel skin flaps could be used to detect rejection in pancreas transplantation: this will be the focus of this Chapter. The second component is a series of immunological studies to investigate mechanisms that take place during visceral and skin rejection. These will be the focus of Chapter 6.

Twenty patients were recruited to this study and had a sentinel skin flap implanted at the same time as pancreas transplantation. The study lasted for 12 months from the day of transplantation for each patient. Research ethics, NHSBT, and Trust approvals were gained for this study prior to recruitment (Appendix F)

The patients underwent routine clinical follow-up after transplantation. The Oxford Transplant Centre has standard protocols for the management of patients after pancreas transplantation and for patients with a VCA. Typically, such patients are reviewed in an outpatient clinic at least 20 times in the first 6 months and then 6 times in the second 6 months (more frequently if problems arise). In addition to routine clinical visits, patients attended for 5 specific research visits as well as any additional ‘event triggered visits’ (see Section 5.3.4).

5.3.2 Outcome measures

Primary:

- Correlation of histological rejection in the skin flap and the pancreas/kidney over 12 months.

Secondary:

- Discordant rejection in the skin flap and the pancreas/kidney over 12 months.
- Episodes of biopsy proven acute rejection of the pancreas/kidney and sentinel skin flap over 12 months
- Total dose immunosuppression over 12 months
- Insulin independence over 12 months
- Graft survival at 12 months

5.3.3 Study participants

All patients who were on the waiting list to receive a pancreas transplant were assessed for eligibility by a member of the healthcare team (via clinical information held electronically for patients awaiting transplantation). All patients who were thought to fulfil the inclusion and exclusion criteria were sent a letter of invitation and a patient information sheet.

5.3.3.1 Inclusion criteria

- Recipient of a pancreas transplant (SPK or PTA)
- Participant is willing and able to give informed consent for participation in the study
- Male or Female, aged 18 years or above

5.3.3.2 Exclusion criteria

- Patient does not have a patent ulna artery in either forearm
- Recipient of an enterically drained PTA

- Patient has received any investigational medical drug within 30 days of transplantation
- Patient is not willing to return to Oxford for follow-up for 12 months
- Any other significant disease or disorder which, in the opinion of the Investigator, may either put the participants at risk because of participation in the study, or may influence the result of the trial, or the participant's ability to participate in the study.

5.3.4 Study procedures

Pancreas transplantation is most commonly performed simultaneously with a kidney transplant, however less frequently the pancreas is transplanted alone. The exocrine drainage of the pancreas is different between the techniques (enteric drainage for SPK, bladder drainage for PTA and PAK), and therefore post-operative monitoring also differs (as previously described in Section 1.6). Therefore, clinical triggers for the study were dependent on the type of pancreas transplant that the recipients received.

A specific sequence of clinical investigations was performed in participants of the study according to triggering events that may signify graft rejection (Figure 5.1).

In simultaneous pancreas-kidney transplant patients, a skin biopsy **and** kidney biopsy were performed if any of the following events occur:

- Skin rash on sentinel skin flap confirmed as rejection on biopsy
- Significant rise in serum creatinine (> 20% from baseline with no other cause)
- Significant deterioration in oral glucose tolerance (new impaired glucose tolerance with no other cause)
- High amylase/lipase with radiological features of graft pancreatitis

In pancreas alone transplant patients, a skin biopsy **and** donor duodenum biopsy were performed if any of the following events occur:

- Skin rash on sentinel skin flap confirmed as rejection on biopsy
- Significant drop in urinary amylase (> 50% below baseline)
- Significant deterioration in oral glucose tolerance (new impaired glucose tolerance with no other cause)
- High amylase/lipase with radiological features of graft pancreatitis

In addition to biopsies required by clinical triggers, surveillance biopsies of the sentinel skin flap were performed during the 12-month period after transplantation. These were performed at: pre-reperfusion, 6 weeks, 3, 6, and 12 months.

Patients found to have rejection on any biopsies were treated as detailed in Section 5.3.5.

Donor specific antibody tests (DSA) were performed every 3 months and at any time that there was a clinical trigger as described above.

A 50ml blood sample for research studies was also taken each time a skin biopsy was performed as described in Section 5.3.4.

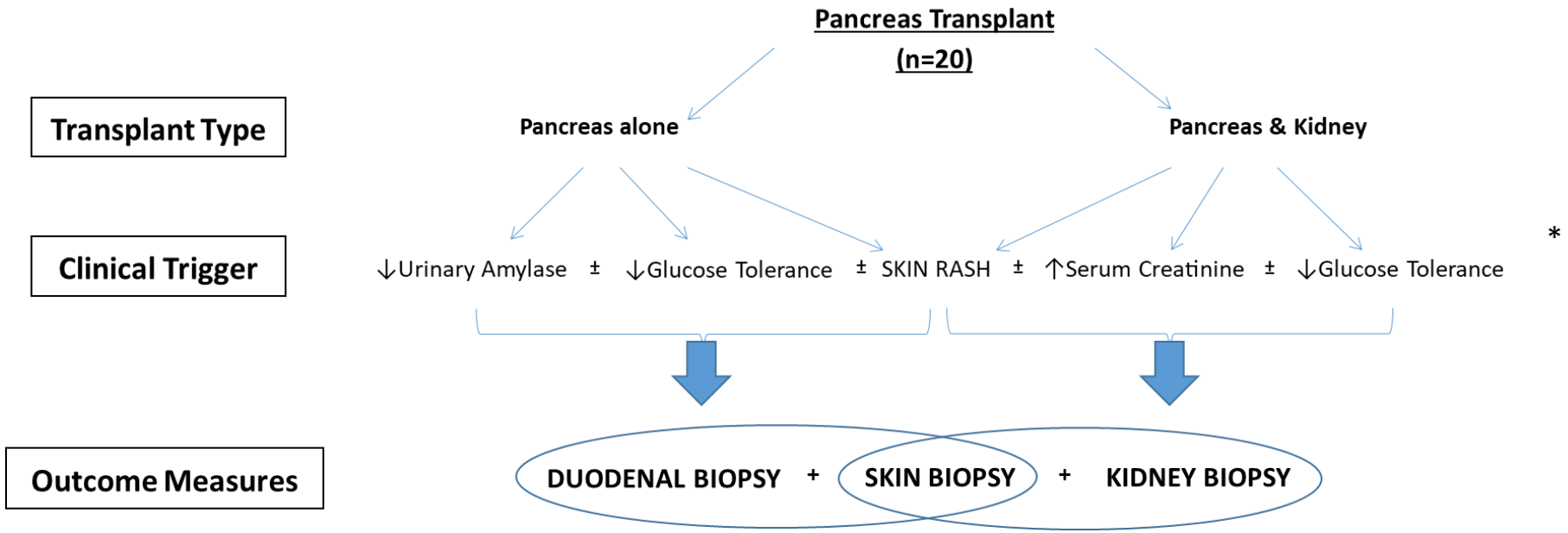


Figure 5.1: Clinical procedures according to pancreas transplant type (* in addition high amylase/lipase with radiological features of graft pancreatitis).

5.3.4.1 SSF transplantation

A donor-derived vascularised skin flap was taken from the donor at the same time as pancreas retrieval (Appendix B describes retrieval procedures). The SSF is based on the donor's radial artery with venous drainage via the venae comitantes and cephalic or superficial veins if present (Figure 5.2 A & B). The SSF was implanted by a microvascular surgeon onto the recipient's non-dominant forearm (where possible) at the same time as pancreas transplantation (Figure 5.2 C). The donor radial artery was anastomosed onto the ulnar artery as an interposition run-through graft using interrupted 10/0 ethilon sutures. The proximal venae comitantes of the donor radial flap were anastomosed to the recipient's venae comitantes using interrupted 10/0 ethilon sutures. The donor cephalic vein of the radial flap was anastomosed onto any nearby recipient superficial vein using interrupted 10/0 ethilon. Pancreas and SSF transplantation were performed simultaneously to ensure that total operative and anaesthetic time was not prolonged.

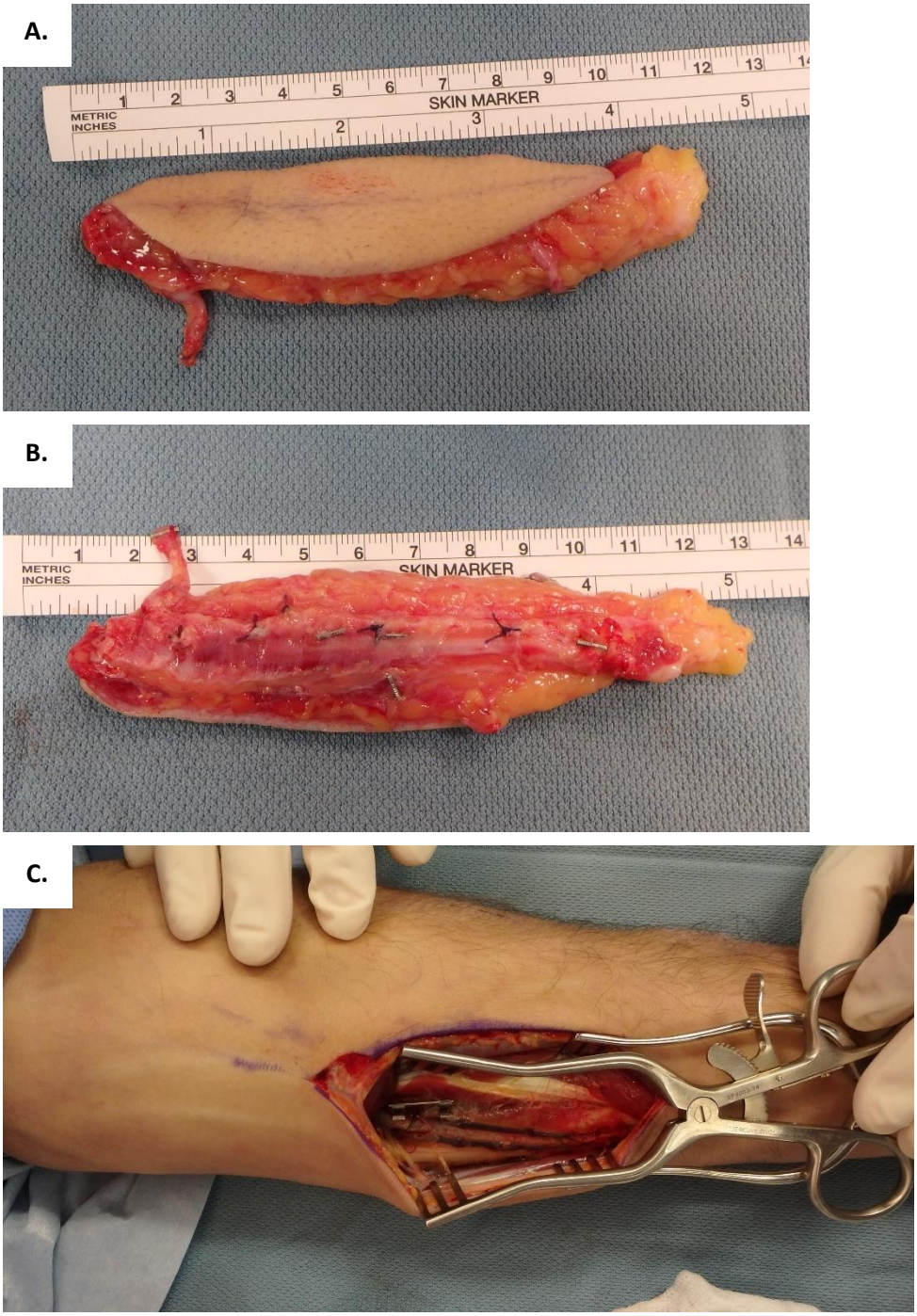


Figure 5.2: A retrieved sentinel skin flap prior to transplantation (A & B). The skin is marked at 12 x 4 cm on the donor but shrinks without tension until transplantation. The recipient's ulna artery and vena comitantes are used for microvascular anastomoses to the sentinel skin flap (C). See Figure 5.3 for post-transplantation pictures.

5.3.4.2 Visit 1

This was performed at the time of transplantation.

For each participant, clinical data was collected against a unique study identifier. Demographic and observational data was recorded for date of birth, gender, BMI, blood pressure, heart rate, comorbidities, details of transplant and medications.

The following routine clinical baseline information was collected: full blood count, renal function, liver function tests, C-peptide, glucose, HLA crossmatch, and DSA status.

The patient undergoes pancreas and sentinel skin flap transplantation. A small part of the excess skin tissue from the SSF was taken as a biopsy prior to revascularisation of the SSF and sent for clinical histopathology and research study investigations.

50mls of blood was taken from the patient's central line whilst under general anaesthetic but before any immunosuppression was given or any tissue/organs have been implanted. This blood sample was for research investigations as per Section 5.3.4.5.

Following transplantation, the following information was obtained from the healthcare team:

donor type, ischaemic times of the transplanted organs and post-operative complications.

5.3.4.3 Visits 2, 3 ,4, and 5

These visits took place at 6 weeks, 3 month, 6 months and 12 months.

Demographics, medical history, concomitant medication, vital signs, eligibility and adverse events were recorded.

The following routine clinical information was collected: full blood count, renal function, liver function tests, C-peptide, insulin, OGTT glucose, HbA_{1c}, amylase, lipase, C-reactive protein, tacrolimus levels and DSA status.

At the same time that blood tests were performed by the clinical team, 50mls of blood was taken for research investigations (Section 5.3.4.5).

A 4mm punch biopsy was taken from the sentinel skin flap (Section 5.3.4.5).

5.3.4.4 Event triggered visits

Certain events that indicate possible rejection triggered an additional 'event triggered visit' and associated procedures described below (see Section 5.3.4 for triggers, Figure 5.1).

Demographics, medical history, concomitant medication, vital signs, eligibility and adverse events were recorded.

The following routine clinical information was collected: full blood count, renal function, liver function tests, C-peptide, insulin, OGTT glucose, HbA_{1c}, amylase, lipase, C-reactive protein, tacrolimus levels and DSA status.

At the same time that blood tests were performed by the clinical team, 50mls of blood was taken for research investigations (Section 5.3.4.5).

A 4mm punch biopsy was taken from the sentinel skin flap (Section 5.3.4.5).

5.3.4.5 Sample handling

NHS Blood tests:

The following blood tests were performed as part of routine clinical care and were processed by haematology, biochemistry, and immunology laboratories at OUH NHS Trust:

Full blood count, renal function tests, liver function tests, amylase, lipase, glucose, HbA_{1c}, insulin, C-peptide, CRP, tacrolimus levels, HLA cross-matching, and DSA status.

The results of these tests were provided by the healthcare team where indicated by the study protocol. All results were stored by the research team in an anonymised form against a unique study ID.

Research blood tests:

A research blood sample of 50mls was taken at 5 time points during the study as well as at each event triggered visit. This is in addition to approximately 20mls of blood that was being taken for routine clinical tests (i.e. 70mls in total at each visit). Blood was stored in EDTA and SST tubes on ice and then taken to the Transplant Research Immunology Group (TRIG) research laboratories at the University of Oxford. Further information on the methods and results of these blood samples is described in Chapter 6.

Tissue biopsies:

Biopsies of the sentinel skin flap and transplanted duodenum/kidney were processed by the histopathology department at OUH NHS Trust.

The skin biopsies were taken using a 4mm punch biopsy tool that is used in routine clinical practice. The punch biopsy was divided into 2 pieces. One half was placed in formalin and sent to OUH histopathology for analysis. The other half was further divided and snap frozen in 2 Eppendorf tubes (one dry and one with OCT) in liquid nitrogen and sent to the TRIG research laboratories at the University of Oxford for storage at -80 °C.

Percutaneous kidney biopsies were taken as per standard clinical practice at OUH and sent to histopathology in the usual manner by the healthcare team.

The healthcare team provided biopsy results to the research team where indicated in the study protocol. All results were stored by the research team in an anonymised form against a unique study ID.

5.3.5 Acute rejection

5.3.5.1 Pancreas and kidney rejection

Acute rejection of the pancreas and/or kidney was treated according to established practice at the Oxford Transplant Centre. First line therapy is 3x doses of intravenous methylprednisolone with escalation of daily immunosuppression if required. Any cases of resistant rejection would be treated on a case by case basis after discussion with the transplant multidisciplinary team.

5.3.5.2 SSF rejection

Biopsy proven acute rejection of the sentinel skin flap was treated according to established practice at the Oxford Transplant Centre. This is usually with 3x doses of intravenous methylprednisolone with escalation of daily immunosuppression if required. Occasionally, in addition, topical steroids are useful to expedite resolution of skin flap changes associated with rejection. Any cases of resistant rejection would be treated on a case by case basis after discussion with the transplant multidisciplinary team.

5.3.6 Immunosuppression

Immunosuppression was managed by the healthcare team according to standard practice. This involved induction with alemtuzumab whilst maintenance was with tacrolimus (target trough level 8 - 12 for the first 6 months, then 6 - 10 thereafter) and mycophenolate mofetil

750 mg twice daily. Maintenance immunosuppression was changed at the discretion of the clinical team according to factors such as rejection episodes, renal function, neutrophil count, viral infections, and side-effects.

5.3.7 Statistical analyses

Data was analysed after 5 and 10 patients to ensure patient safety. Data was then analysed at the end of the study. Graft and patient survival at 12 months along with different tissue/organ rejection episodes was determined (accounting for total dose immunosuppression where possible). Trough tacrolimus levels were analysed using 3 methods: coefficient of variation, tests in range, and time in range using the Rosendaal method[360]. Exploratory data analysis was performed by calculating summary statistics for various markers and changes thereof over time, stratified by rejection or non-rejection.

As time to rejection is also important, survival analysis models were used (such as Kaplan-Meier and Cox regression). For Kaplan Meier survival curves, logrank significance tests were employed. For simple grouped data, student t-test or Mann-Whitney U tests were employed.

5.4 Results

5.4.1 Demographics

20 participants were recruited to the study and received a sentinel skin flap VCA at the same time as pancreas transplantation (Table 5.1). One participant had sentinel skin flap necrosis requiring removal but remained in the clinical study. One participant who underwent SPK transplantation required early pancreatectomy but decided to keep his sentinel skin flap and remain in the study. The group consisted of 13 men and 7 women. The mean age was 46.6 years (SD = 7.58) with a BMI of 26.0 kg/m^2 (SD = 3.83). There were 17 SPK transplants, 1 PTA, 1 PAK, and 1 pancreas after SPK.

5.4.2 Pancreas and SSF offers

Given the complex logistics involved with this study, it was important to report the recruitment rates for guiding future studies and clinical practice. The 20 participants were transplanted over 20 months between May 2016 and January 2018. During this period 112 pancreas transplants were performed in total at the Oxford Transplant Centre. All patients were successfully recruited to the study where both donor and recipient consent existed. The most common reason for failure to recruit was lack of donor SSF consent which itself was multifactorial (e.g. family aesthetic concerns, language barriers, SNOD training). The second most common reason was that the location of the donor was out of region (i.e. more than 3 hours from Oxford, and therefore excluded due to retrieval logistics). Less than 10% of patients on the waiting list declined to take part in the study in advance. One of these patients subsequently received a pancreas transplant when donor consent was in place for SSF.

Table 5.1: Demographic and baseline data for study participants.

Participants	20
Age at transplantation (years) †	46.6 (7.58)
Gender (male / female)	13 / 7
Ethnicity (White / Asian)	18 / 2
BMI (kg/m^2) †	26.0 (3.83)
eGFR ($ml/min/1.73m^2$) †	9.8 (3.18)
DM type 1/2	19 / 1
Dialysis (pre / PD / HD)	6 / 4 / 10
Days from listing to transplant ‡	462 (1 - 680)
Transplant (SPK/PTA/PAK/PASPK)	17 / 1 / 1 / 1

† Values are mean (SD); ‡ Values are median (range)

5.4.3 Transplant factors

Donor demographic and transplant factors are summarised in Table 5.2. The mean age of the donors was 37.3 years which was significantly younger than the recipients ($p = 0.018$). There was no significant difference in the BMI of donors and recipients ($p = 0.078$). 75% of transplants were from DBD donors. 80% of donors were CMV negative compared with 65% of recipients. 2 recipients who were CMV negative received organs from CMV positive donors and required additional antiviral prophylaxis. 7 recipients had a calculated reaction frequency (cRF) of > 5% at the time of transplantation although none were highly sensitised (i.e. cRF ≥ 85%).

Table 5.2: Donor demographic and transplant data.

Participants			**20**
Donor age (years) †			37.3 (15.0)
Donor gender (male / female)			15 / 5
Donor ethnicity (White / Black)			19 / 1
Donor BMI (kg/m^2) †			24.0 (3.06)
Donor girth (cm) †			86.7 (8.75)
Donor eGFR (ml/min/1.73m^2) †			82.3 (14.6)
DBD / DCD			15 / 5
CMV status:	**D + / R +**		2
	D + / R -		2
	D - / R -		11
	D - / R +		5
Donor blood group:	**A**		8
	B		2
	AB		1
	O		9
Recipient blood group:	**A**		7
	B		3
	AB		2
	O		8
Kidney CIT (mins) †			691.5 (68.87)
Pancreas CIT (mins) †			596.7 (74.62)
SSF CIT (mins) †			577.5 (69.71)
cRF > 5%			7
Total HLA mismatch ‡			4 (3 – 5)
HLA mismatch	**Class I (A & B)**	**0/1**	0
		2	10
		3	10
		4	0
	Class II (DR)	**1**	16
		2	4
Length of stay (days) ‡			12.5 (6 – 57)
Blood units intraoperatively ‡			1 (0 – 3)
Total blood units ‡			2 (0 – 25)

† Values are mean (SD); ‡ Values are median (range)

5.4.4 Outcomes

Patient survival at 12 months was 100%. Pancreas and kidney allograft survival at 12 months was 95% and 100% respectively. Sentinel skin flap survival at 12 months was 95%. Figure 5.3 shows typical appearances of a normal SSF at different timepoints post-transplantation. Graft loss is further described in Section 5.4.7.

Estimated glomerular filtration rate (eGFR) derived using the MDRD equation was considered representative of kidney allograft function whilst HbA_{1c} and OGTT 120-minute glucose were used to represent pancreas allograft function (Figure 5.4 and Figure 5.5).

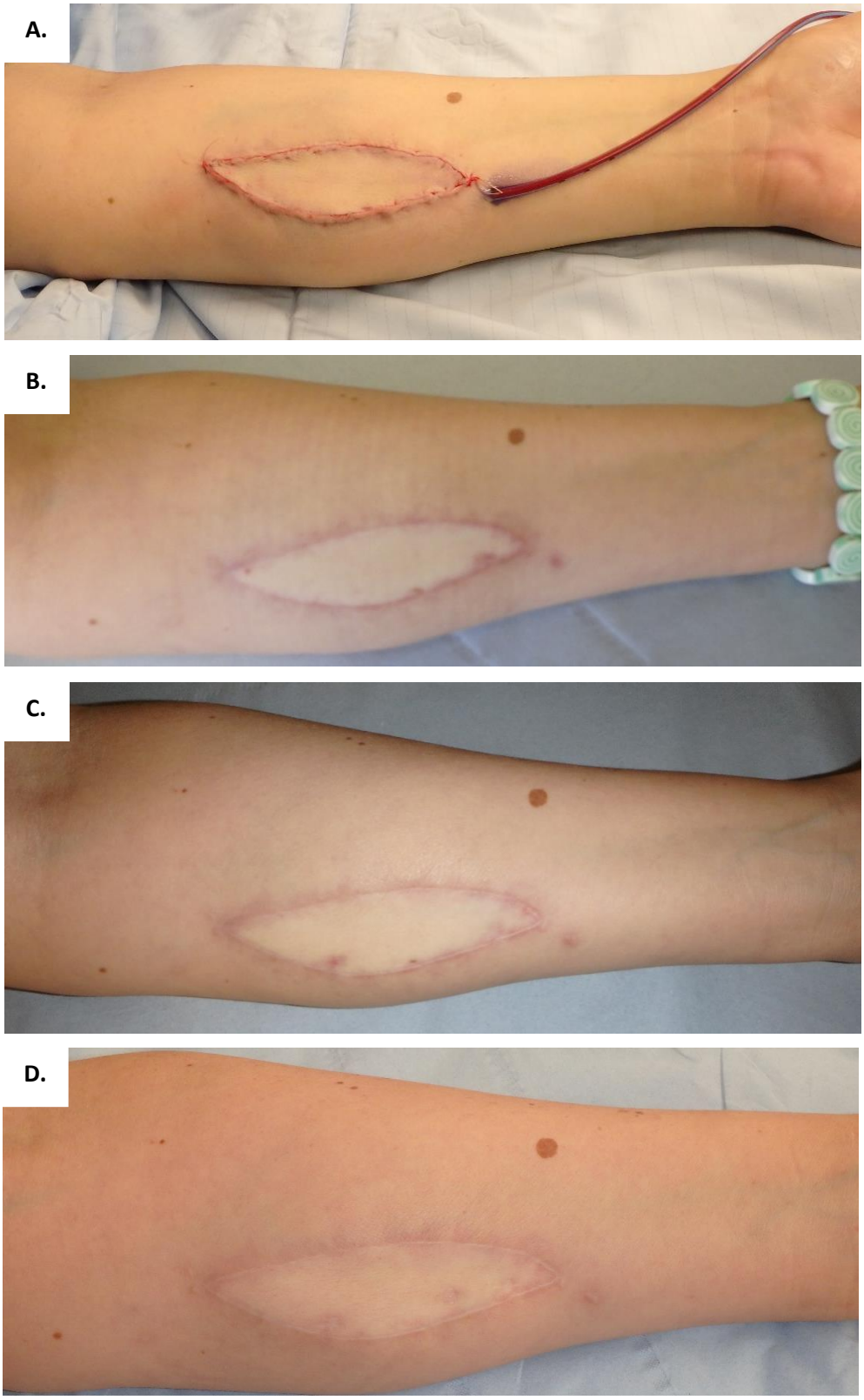


Figure 5.3: A healthy sentinel skin flap at different timepoints. A) transplantation B) 3 months C) 6 months D) 12 months.

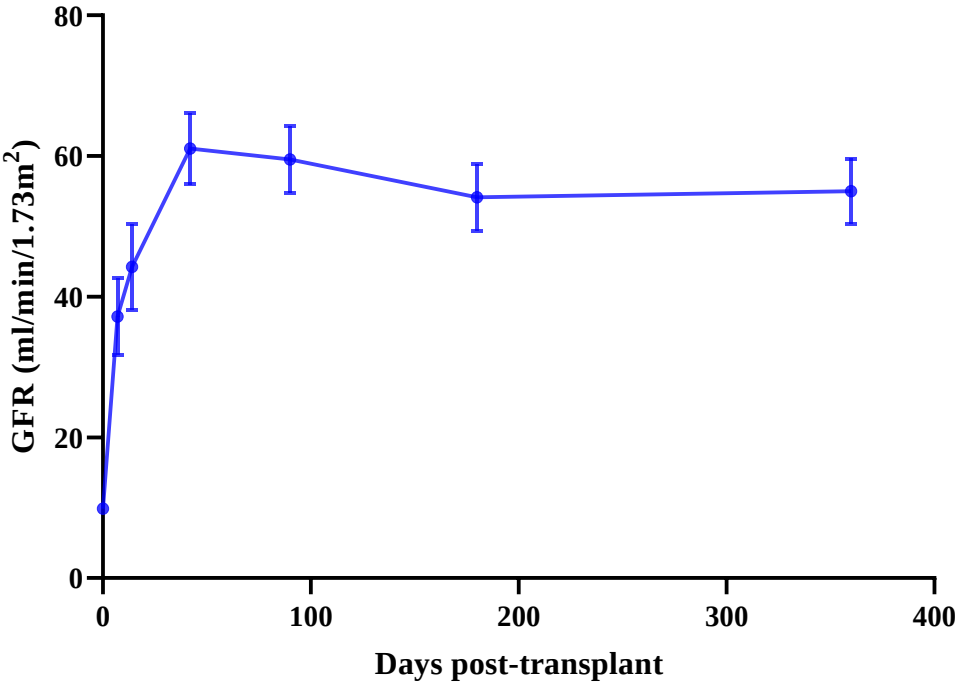


Figure 5.4: Mean eGFR of SPK patients during the first 12 months after transplantation (with SEM error bars).

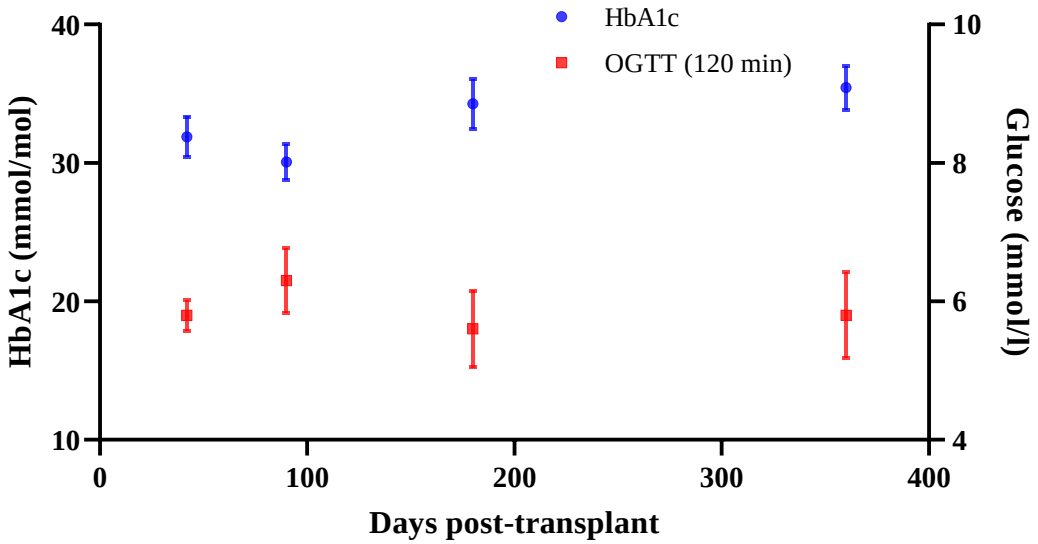


Figure 5.5: Mean HbA_{1c} and OGTT 120-minute glucose of all patients with a functioning pancreas allograft during the first 12 months after transplantation (with SEM error bars).

5.4.5 Immunosuppression

Alemtuzumab induction produces profound lymphocyte depletion which can take many months to recover. The mean lymphocyte number at 1-year post transplantation was 0.79 x 10^9 (SD = 0.484) and only 5 (25%) of the participants had returned to the normal range at this time (Figure 5.6).

Tacrolimus levels were closely monitored during the study period as part of routine clinical practice. 433 trough tacrolimus levels were available for analysis with a mean of 22 tests per patient (SD = 12.8) performed over the first 12 months after transplantation. Therapeutic efficacy was assessed using 3 techniques (Table 5.3): coefficient of variation, % of tests in range, % of time in range (Rosendaal method[360]).

Mycophenolate is routinely used for maintenance immunosuppression after pancreas transplantation, but serum levels are not usually monitored. The two most common complications from mycophenolate that are observed in clinical follow-up are gastrointestinal disturbance and myelosuppression. Myelosuppressive effects are generally monitored via the neutrophil count (Figure 5.7). Although mean neutrophil counts for the 20 participants were generally in the normal range, it is notable that from 3 - 12 months the mean count was approximately 3 x10^9/L with a SD of 1.5 – 1.7, suggesting that counts were generally towards the lower end of the normal spectrum. This is further highlighted by the fact that 65% of participants had a period of neutropaenia with an absolute neutrophil count of less than 2.0 x10^9/L that required a reduction of mycophenolate dose, whereas 25% of participants had a period of severe neutropaenia with an absolute neutrophil count of less than 1.0 x10^9/L that required cessation of mycophenolate.

Table 5.3: Trough tacrolimus level analysis.

Total tacrolimus results (n)	433
Tacrolimus results per patient †	22 (12.80)
Tacrolimus CV (%) †	30.0 (8.82)
Tests in range (%) ‡	75.5 (0 – 87.5)
Time in range (%) ‡	85.2 (0 – 97.5)

† Values are mean (SD); ‡ Values are median (range)

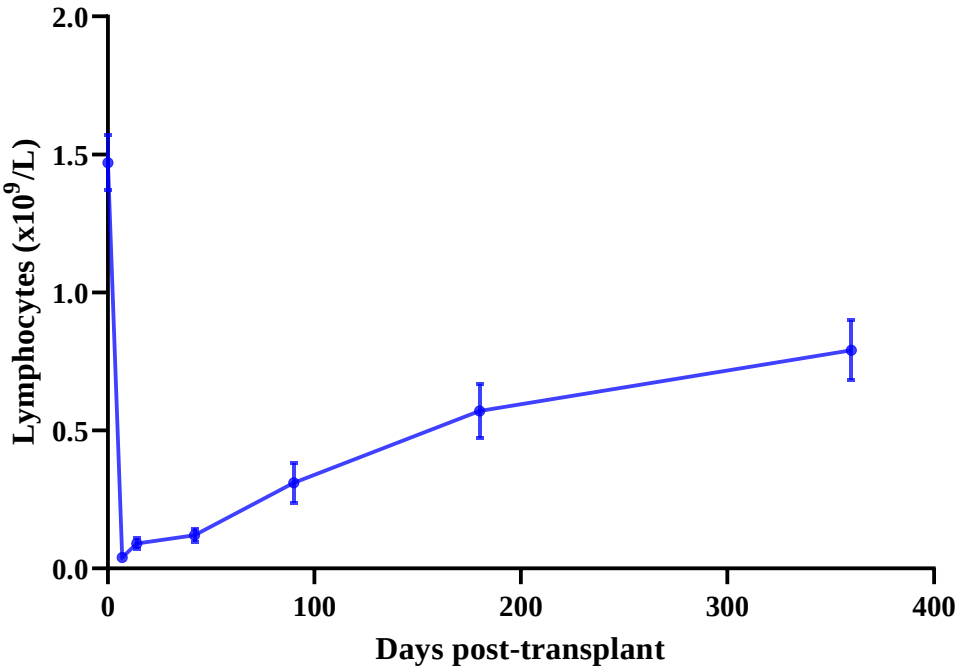


Figure 5.6: Mean lymphocyte count of all patients during the first 12 months after transplantation (with SEM error bars).

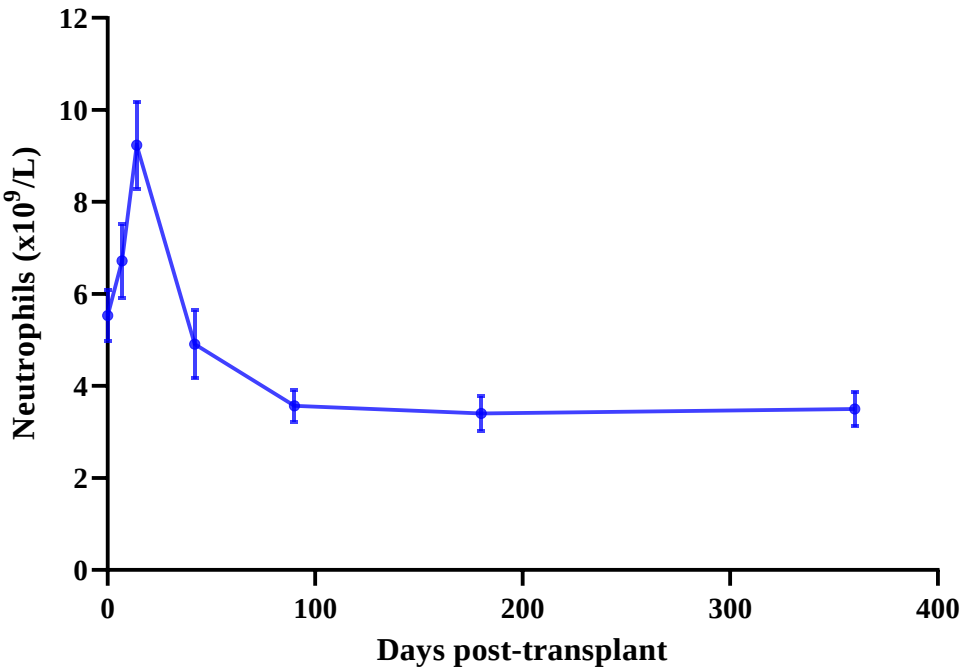


Figure 5.7: Mean neutrophil count of all patients during the first 12 months after transplantation (with SEM error bars).

5.4.6 Infections

Patients were screened for opportunistic infections according to clinical protocols. Four of the twenty patients (20%) developed CMV viraemia, although only 2 of these (10%) developed CMV symptoms or high titres that required antiviral treatment. The 2 who required antiviral treatment both had positive CMV serology prior to transplantation (D+/R+ and D-/R+). Four of the twenty patients (20%) developed BKV viraemia, 2 of which were associated with concordant CMV viraemia that required antiviral treatment. One patient developed primary EBV viraemia (D+/R-) that was associated with high viral titres ($3.07x10^5$) and a prolonged period of neutropenia.

Non-opportunistic infections were also observed in the participants. 5 participants (25%) developed a urinary tract infection during the first year after transplantation. There were no other non-opportunistic infections reported.

5.4.7 Complications

All participants were monitored for post-transplantation complications and these were managed according to clinical indication. Surgical complications are detailed in Table 5.4 – the last 4 rows pertain to complications that are purely due to transplantation of the sentinel skin flap. Figure 5.8 highlights some of these specific SSF complications and outcomes.

Table 5.4: Complications in the SSF recipients. Surgical complications are frequently classified according to the Clavien-Dindo system, as shown and further in Appendix G [361]**.**

Complication	No. of pts	Classification	Outcome
Pancreas allograft thrombosis	1	IVa	Pancreatectomy (Day 1)
Severe allograft pancreatitis	3	IVb	Returns to theatre
Recurrent admissions related to bladder drainage of PTA	1	IIIb	Enteric conversion (Week 8)
Post kidney biopsy bleeding	1	IIIb	Theatre for haemostasis
SSF failure & necrosis	1	IIIb	SSF excision (Day 11)
SSF basal cell carcinoma	1	IIIa	Excised (3 months)
Necrotic SSF wound	1	I	Healed after 6 weeks
Prolonged ulna nerve palsy	1	I	Resolved after 6 months

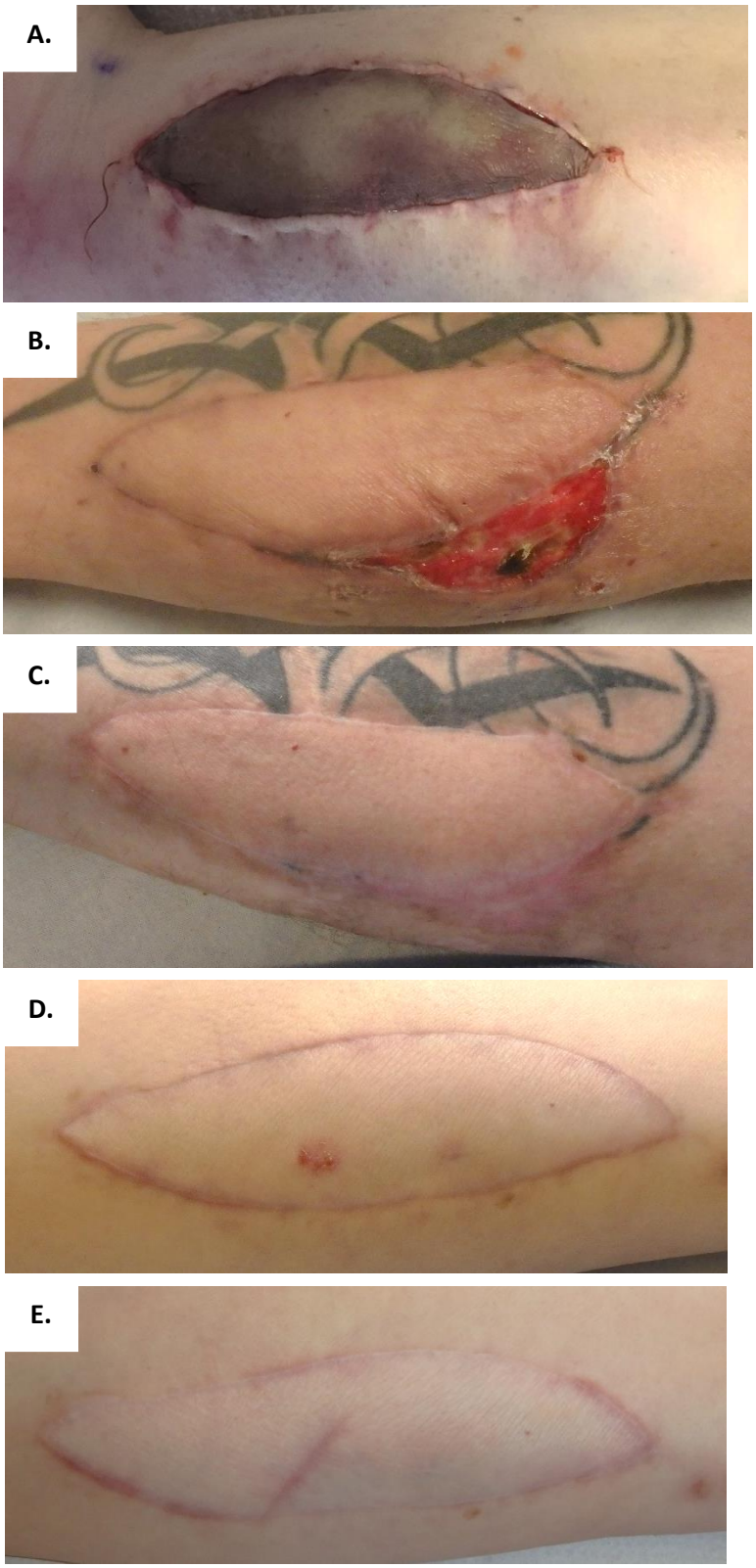


Figure 5.8: Complications after SSF transplantation. A) SSF failure & necrosis B) & C) recipient skin necrosis and then healed D) SSF basal cell carcinoma E) BCC excised.

5.4.8 Triggered events and rejection episodes

All 20 participants completed the study although only 19 had an intact sentinel skin flap at the end of the study. There were 83 sentinel skin flap protocol biopsies taken. None of these biopsies showed any evidence of subclinical rejection. There were 7 'event triggered' visits that lead to 7 additional sentinel skin flap biopsies and 6 kidney allograft biopsies in SPK patients. There were no events in the PTA/PAK/PASPK patients that would have required a trans-cystoscopic biopsy of the donor duodenum. These 7 triggered events are shown in Figure 5.9.

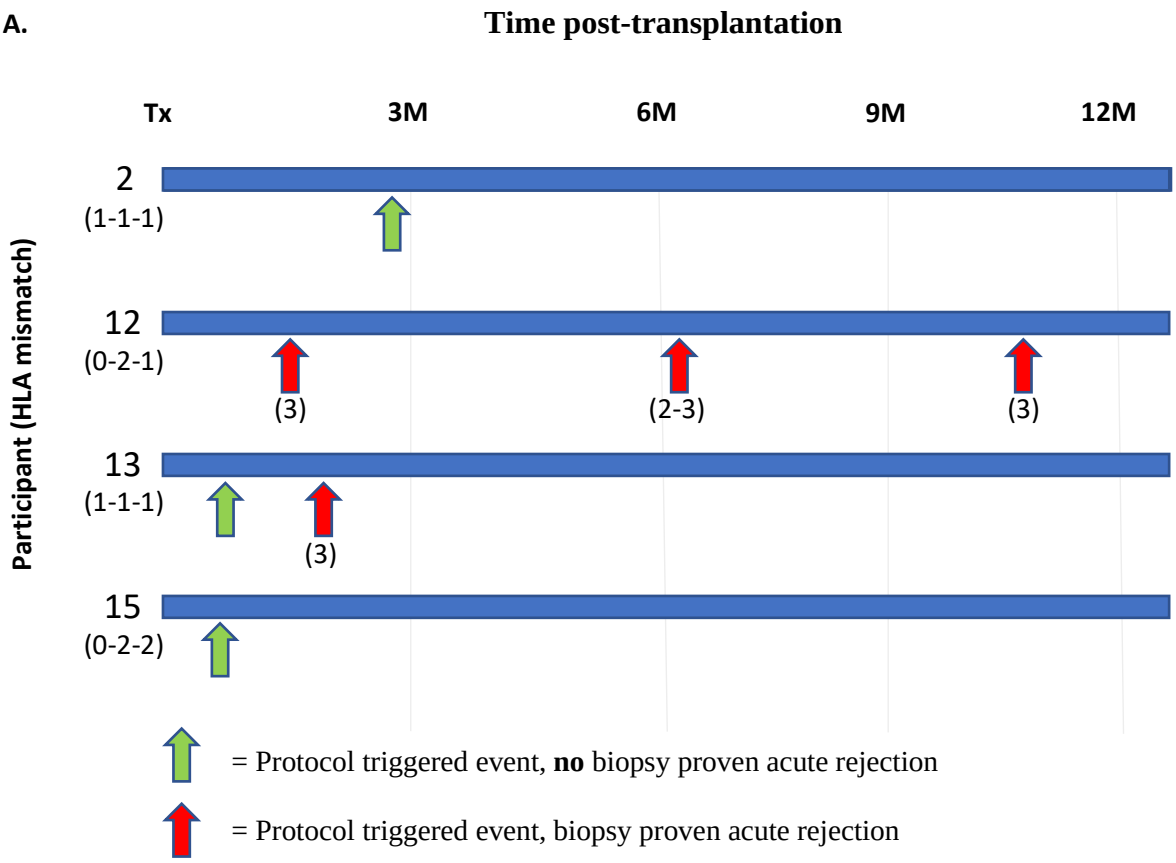
There were 4 episodes of acute rejection of the sentinel skin flap in two patients (1 patient experienced 3 episodes, the other a single episode). There was one episode of Grade II rejection and three episodes of Grade III rejection according to Banff criteria[344]. The macroscopic appearance of the sentinel skin flaps with acute rejection was variable; it ranged from a mild maculopapular rash to florid oedema, erythema and ulceration (Figure 5.10). Rejection free survival is shown in Figure 5.11. There were no episodes diagnosed of acute rejection of the pancreas or kidney allografts.

5.4.8.1 Participant 12

Participant '12' is a complex patient that needs further clarification – he experienced 3 episodes of SSF rejection as shown in Figure 5.9. Under-immunosuppression is likely to have contributed to these rejection episodes – each episode coincided with a tacrolimus level of less than 7 and his trough tacrolimus variability was the highest in the cohort (CV = 51%). His mycophenolate also had to be stopped due to an extended period of severe neutropenia. To complicate matters further he developed primary EBV with a prolonged period of high viral titres and lymphadenopathy that was difficult to distinguish from post-transplant lymphoproliferative disorder (PTLD). It is unclear whether this patient would have

developed rejection of his pancreas or kidney allografts if he had not been treated for SSF rejection. He did not develop any donor specific antibodies (DSA will be discussed further in Chapter 6).

A.



B.

Study ID	Day	Trigger	SSF Bx	Kidney Bx	Outcome
2	81	Abnormal OGTT	Normal	Normal	Y graft angioplasty
12	44	SSF rash	Grade 3 BPAR	Normal	Steroids
12	185	SSF rash, AKI	Grade 2 - 3 BPAR	CNI toxicity	Steroids
12	290	SSF rash	Grade 3 BPAR	Normal	Steroids
13	18	AKI	Normal	CNI toxicity	CNI reduction
13	55	SSF rash	Grade 3 BPAR	CNI toxicity	Steroids
15	15	SSF rash	Post-op changes	N/A	Resolved

Figure 5.9: Triggered events during the study. A) 4 patients experienced trigger events of which 2 had confirmed biopsy proven acute rejection (BPAR). Maximum grade of rejection according to Banff 2007 is in brackets. B) Causes of the trigger events with outcomes.

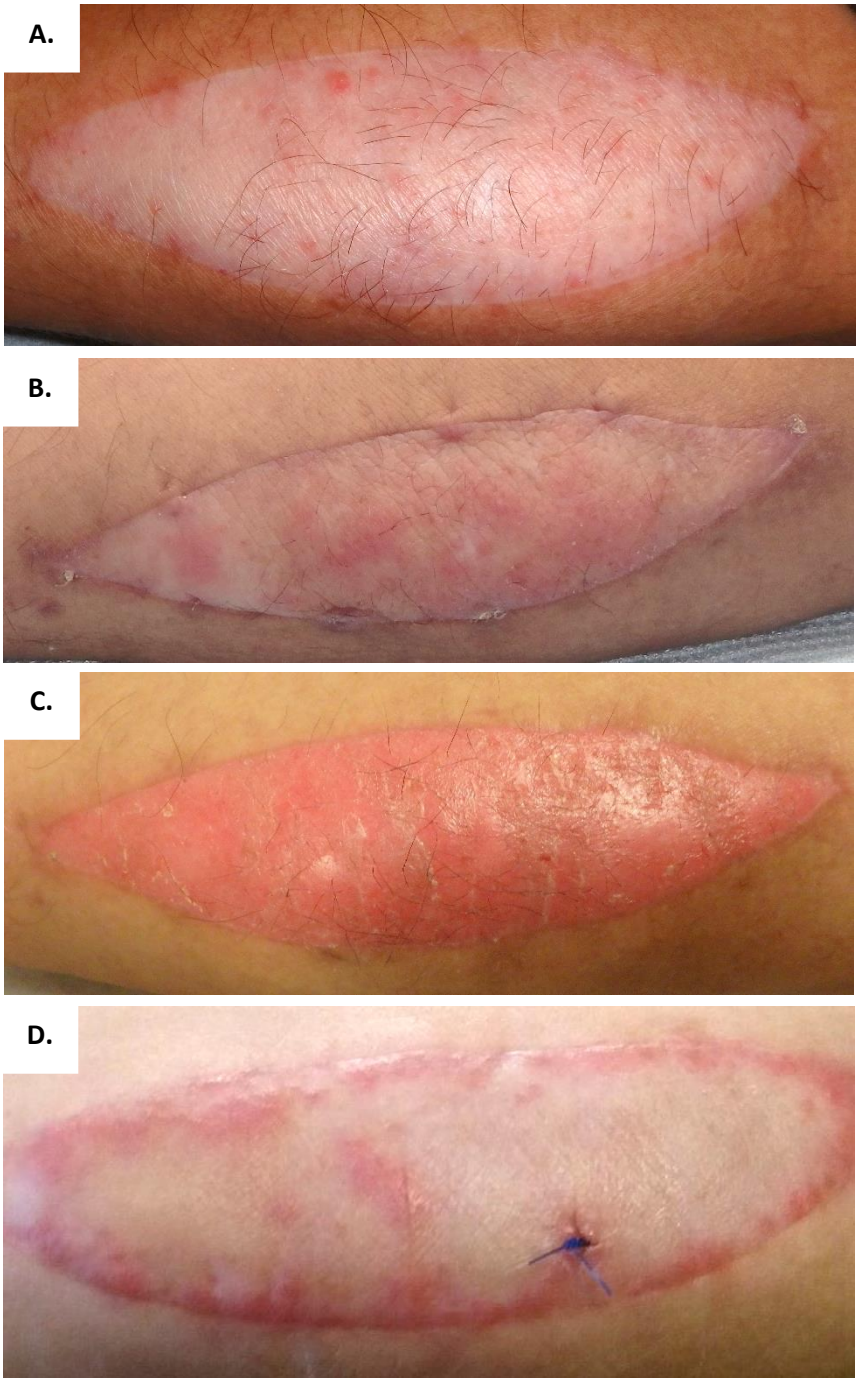


Figure 5.10: Acute rejection of the sentinel skin flaps demonstrating the variability in macroscopic appearances. Note the rash is always confined to the donor skin. Image A has Grade 2 rejection whereas images B - D have Grade 3 rejection according to Banff criteria for VCA rejection[344].

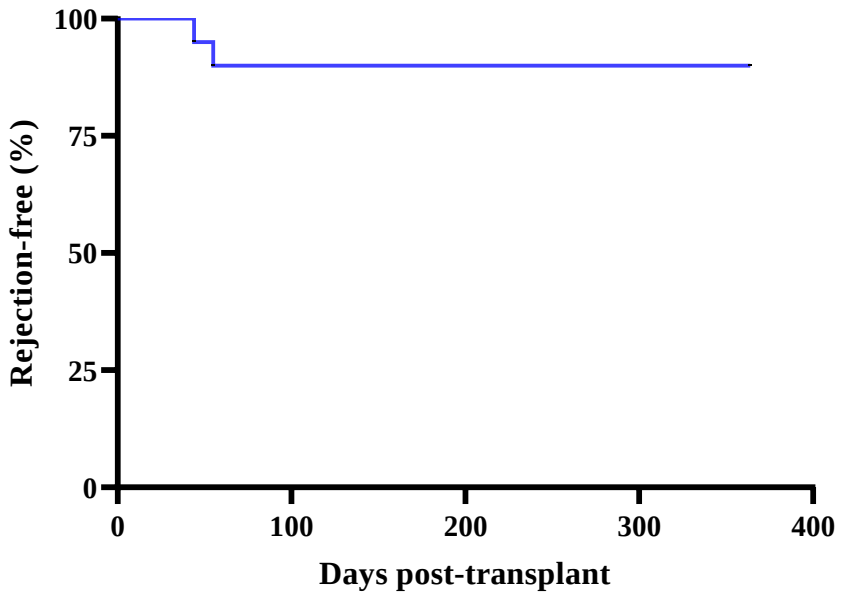


Figure 5.11: Kaplan-Meier graph showing rejection-free survival for all patients.

5.5 Discussion

The use of remote skin allografts as a marker of rejection in visceral transplantation is not entirely new. Haberal et al. reported the use of 9 skin allografts from living donors in ABO incompatible kidney transplants in 1989[362,363]. Although only used as a crude marker, they noted a parallel outcome for the skin and kidney allografts. The potential use of skin allografts in reconstructive plastic surgery stimulated further interest and Wendt et al. reported 6 cases of skin allografts transplanted at the same time as kidneys from 4 living and 2 cadaveric donors[364]. After 5 years they reported that skin allografts could survive indefinitely on patients taking immunosuppression regimes used in renal transplantation[365]. It is unclear why these early reports did not provoke more interest from the visceral transplant community, but it is likely due to inexperience and concerns relating to skin transplantation at that time.

While these 'skin grafts' (i.e. split-thickness skin transplanted without an intact blood supply) have previously been utilised as monitors in organ transplantation, the primary vascularisation of skin (i.e. a free flap) in the context of visceral organ transplantation may be important because the route of migration of antigen presenting cells (APCs) from skin grafts and vascularised flaps may differ[366,367]. Moreover, the significant ischaemic injury sustained by an avascular skin graft may increase its susceptibility to rejection over and above that of vascularised skin[368]. This increased sensitivity may not accurately reflect the immune status resulting in overtreatment of presumed rejection episodes. Furthermore, there is evidence that donor cell numbers diminish in transplanted non-vascularised skin grafts in the long term, thereby reducing their value in alloimmune surveillance.

Given the potential ability of the skin to predict rejection in tissues which are lower on the scale of rejection susceptibility, it is important that skin transplanted as part of a more

complex procedure does not result in induction of an immune response which damages the primary visceral transplant (i.e. the pancreas/kidney). While it has often been stated that skin presents a high level of 'immunogenicity'[369], and thus it may be inferred that an immune response of significant magnitude is produced from skin transplantation, this is not necessarily the case. In experimental mouse systems in particular, skin does not induce a greater immune response as measured by T cell proliferation and graft infiltration in comparison to solid organ transplants[345]. This provides some reassurance regarding the safety of transplanting additional tissue as part of a visceral transplant. This study lends significant support to this, with low rates of rejection and DSA formation (discussed further in Chapter 6).

In this study two patients (10%) developed acute rejection of the sentinel skin flap yet there was no evidence of acute rejection of the pancreas or kidney allografts during the first year after transplantation. There are several potential explanations for this discordant rejection. It is feasible that the SSFs are acting as originally hypothesised; that is, acting as a sentinel marker of an evolving alloimmune response that is visually apparent as an SSF rash far earlier than dysfunction or rejection of the visceral allografts. Hypothetically, without treatment of this response, the patients would go on to develop acute rejection of their pancreas or kidney transplants. This explanation is supported by the fact that only 10% of patients developed an episode of acute rejection - this is in line with that reported in the literature for SPK transplants. Reported rates of pancreas or kidney rejection range from 5 to 50%[167,171,370], although in the current era of lymphocyte depleting induction regimes with tacrolimus and mycophenolate maintenance it is more likely to be in the range of 10 to 20%. Gerlach et al. reported a similar finding in bowel transplant patients with VCA transplants[356] - the rate of intestinal rejection was much lower than expected but the overall rate was similar when the VCA rejection episodes were included. Anecdotal evidence from

the Oxford multivisceral program also supports the theory that there might be a time delay between VCA and visceral rejection: a patient with an intestinal transplant was non-compliant with immunosuppressive medication and subsequently developed acute rejection of their VCA followed by intestinal dysfunction and acute rejection 7 - 10 days later. It is notable that in this study all 4 episodes of SSF rejection corresponded with suboptimal levels of immunosuppression - tacrolimus levels of 4 - 7 ng/ml and mycophenolate hold due to neutropaenia. Furthermore, all of the SSF rejection episodes had Grade II/III rejection suggesting a substantial and established alloimmune response. This is in contrast to hand and face VCA transplants where the majority of rejection episodes have been Grade I [371].

It is certainly possible that the lack of pancreas or kidney rejection episodes reflects the small numbers in this study and a larger cohort may identify the true rate of rejection seen in pancreas and kidney transplantation irrespective of the sentinel skin flaps. This study was never powered to show a difference in either graft survival or rejection rates. Nevertheless, post-hoc power analysis suggests that an observed visceral rejection rate of 0% in 22 participants when it is assumed to be 15% in a known population provides 100% power (alpha 0.05) and only 29 participants would be required if a rejection rate of 12% was assumed[372]. Therefore, the 19 patients with intact SSFs in this study are not far from achieving statistical power: the study has subsequently been extended to accommodate this. Ultimately, it is graft survival that is most important; however, a study designed and powered to demonstrate a reduction in 1-year pancreas graft loss from 10% to 5% would need over 230 participants (alpha 0.05, 80% power). Given the slow rate of recruitment (18%) described in Section 5.4.2, this would not be feasible without significant logistical modifications.

It is also plausible that none of these patients would have gone on to develop any rejection - the SSF rejection was never going to proceed to rejection of the visceral organs, and

therefore these patients were treated with unnecessary additional immunosuppression. The only way to definitively clarify this would be to design a study where subjects who developed rejection of the SSF were not treated and observed to see what happened to both the SSF and visceral allografts. This would potentially expose patients to an unacceptably high risk of allosensitisation, graft failure and ulceration or necrosis of the SSF.

The last potential explanation for the absence of pancreas rejection episodes relates to the fact that rejection between pancreas, donor duodenum, and kidney can be discordant[172,190,216]. There may have been subclinical rejection of the pancreas allograft that was not evident biochemically and was discordant with the kidney. If this coincided with an episode of SSF rejection, then it would then have been treated by the steroid pulses given to treat the SSF rejection. Conversely, there may have been subclinical rejection of the pancreas that was discordant with both the SSF and kidney. This seems less plausible given substantial evidence to suggest that episodes of pancreas allograft rejection are associated with high rates of graft loss, even when treated[169,373,374]. In their study of 228 pancreas transplants with up to 10 years of follow-up, Dong et al. describe acute pancreas allograft rejection being strongly associated with graft loss (hazard ratio was 3.79 overall but rising to 6.25 if the rejection was between 12 and 24 months)[169]. The only way to remove this source of bias would have been to perform pancreas biopsies at the same time as SSF and kidney biopsies. This is a significant limitation of this study, however, as previously mentioned pancreas biopsies are not practiced at the Oxford Transplant Centre.

Overall outcomes in this study were comparable or better than other reported series in pancreas transplantation. A 1 year graft survival of 95% is notable given the current national average of 89%[101] and the single pancreas that was lost was due to thrombosis with pancreatectomy on day 1 post transplantation (i.e. a technical loss). The lack of any further graft loss is interesting but should not be over-interpreted in the context of small numbers.

Graft function was also good in this study with a mean GFR of 55 ml/min/1.73m^2, and mean HbA_{1c} of 35.4 mmol/mol at 1-year post-transplantation. The observational nature of the study does introduce further bias when looking at outcomes. Patients may be reviewed more frequently or investigated more extensively as part of the study. For example, one patient developed a sudden deterioration in glucose tolerance during OGTT and this was investigated as per the study protocol. In this case, CT angiography demonstrated significant stenosis of the pancreas 'Y' arterial graft which was successfully treated with angioplasty and resulted in return of good allograft function.

Three methods were used in this study to assess therapeutic monitoring of tacrolimus levels. Tacrolimus and warfarin share a high level of inter- and intra-patient variability confined within a narrow therapeutic window. It has been known for some time that time in therapeutic range for warfarin has a significant impact on the associated reduction in cerebrovascular events[375]. However, it has only been fairly recently that this has also been shown to be the case with tacrolimus levels and associated immunological events in transplantation[376,377]. Davis et al. demonstrated that a time in range of less than 60% was associated with DSA formation (HR 4.18) and acute rejection (HR 4.18) in the first year after kidney transplantation[376]. Therefore, a median time in range of 85.2% in this study is good and may also have contributed to the excellent graft survival. Nevertheless, this does not tell the whole story – the 2 patients who experienced SSF rejection had a tacrolimus time in range of 74% and 93%. Variability of tacrolimus levels has also been associated with immunological events[378,379] and the participant in this study with 3 SSF rejection episodes had a coefficient of variation of 51% which was by far the worst of all participants.

The cohort of patients in this study all received alemtuzumab induction therapy. This caused a profound lymphocytopaenia that did not start to recover until after 3 months and had still not returned to normal in most patients by 1 year after transplantation (this will be discussed

further in Chapter 6). Even when allowing for the lower rates of acute cellular rejection seen with alemtuzumab induction, we would still have expected to see episodes of visceral organ rejection in more than 10% of patients. There have been historical concerns regarding potentially higher rates of infections and malignancy in patients treated with alemtuzumab. These were largely refuted by the 3C study, a large randomised controlled trial which showed no significant difference in some of these secondary outcomes when compared with basiliximab induction (with the possible exception of a slightly higher BKV rate)[380]. The rates of CMV, BKV, and urinary tract infections were slightly higher in this study although confounded by small numbers.

One important aspect of this early-phase study was to assess safety. From an immunological perspective, the low rate of rejection episodes and lack of increased DSA formation (Chapter 6) provides cautious optimism for its safety and certainly supports ongoing research. With regard to surgical safety, four patients experienced morbidity that was entirely attributable to the sentinel skin flap (Table 5.4). The forearm radial free flap is often described as the workhorse of reconstruction by plastic and head and neck surgeons – the reported rate of flap failure when used in these settings is between 4 - 17% [381,382]. One patient (5%) in this study had technical flap failure with necrosis that required explantation and therefore negated any benefit the SSF may have provided. Overall, this study has shown that it is possible to perform simultaneous SSF and visceral transplantation with good outcomes, including the use of the first 5 DCD VCA transplants ever performed.

All patients were advised that the SSF did not need to be permanent and could be removed at any time at their request under local anaesthetic. It is interesting to note that not a single patient has asked for the SSF to be removed after the end of the study and several have stated that it has provided significant psychological benefit. This is both in regard to coming to terms with their new organs by having a visual reminder that a deceased person has

donated their organs and also by helping with the uncertainty surrounding rejection and organ longevity by providing something that they can monitor on a daily basis. The visibility of the transplanted skin makes it ideal as a sentinel marker of rejection. Patients can ‘keep an eye’ on their graft and if its appearance changes, they can send a digital photograph and get a prompt medical review. This has the advantage of giving patients more autonomy and enables self-management. It also allows remote monitoring that might save unnecessary commuting and use of clinical resources.

5.6 Conclusion

In conclusion, this is the first study to report the use of a donor-derived vascularised skin flap for the sole purpose of monitoring rejection in pancreas transplantation. The prompt diagnosis of rejection is both a challenge and a necessity; this is a particular problem in pancreas transplantation where current techniques do not generally provide a sufficient window of opportunity for treatment of rejection before irreversible sequelae have occurred. If sentinel skin flaps could provide us with a robust method for detecting early rejection, then this could significantly reduce rates of graft loss due to rejection. Conceivably the greatest advantage of having a sensitive and specific marker of visceral rejection would be the potential to tailor immunosuppressive regimes. Those identified as being at risk of rejection would receive increased immunosuppression before the onset of any damage to the allografts, whilst those with stable graft function might benefit from a cautious reduction of immunosuppression, thus minimising the damaging side effects of this medication. The results of this study are encouraging and provide a strong foundation on which to take this technique forward. Future studies should aim to further contribute to the safety and efficacy of this technique as well as investigate whether this can translate into improved graft outcomes over the long term.

Chapter 6

Exploring Immunobiology of Sentinel Skin Flaps

6.1 Introduction

Since Medawar's earliest investigations in transplantation, skin has always been believed to be one of the most susceptible tissues to rejection and one of the most resistant to immunosuppression or regulation when transplanted in isolation[345,369,383,384]. In clinical VCA transplantation, skin is often the first component to manifest signs of rejection and often the most difficult component to control rejection[343,344,346]. It is thought that tissues functioning as a barrier to the environment, such as skin, lung and intestine are at the top of the immunological hierarchy, whereas kidney, liver, and cornea are nearer the bottom. This enhanced susceptibility of skin to rejection may therefore provide benefit in visceral transplant monitoring.

Skin has many features that make it susceptible to an intense alloimmune response. A large proportion of cells in the skin are related to the immune system. There are a high number of antigen presenting cells (APCs) and endothelial cells capable of expressing HLA Class II, thus providing T cell activation signals through direct allorecognition. In a physiological setting and in response to antigen, these cells in the skin are more likely to produce an immune effector response whereas similar cells in the gut are more likely to induce tolerance[385,386]. This reflects the functional differences between these two tissues but has implications for pancreas and SSF transplantation where both tissue types are being transplanted. Skin also has more than twice as many T cells as peripheral blood, the majority of which are resident memory T cells[387,388]. There is considerable heterogeneity of the T cell receptors and cytokine activity of these cells and it is not clear what the implications of this

are in transplantation, although their high abundance in tissues known to be at high risk of rejection suggests a role.

There are skin specific antigens such as Skn-1, Skn-2, and Epa-1 that may contribute to the increased susceptibility of skin to rejection by increasing the potential for allorecognition by the recipient's T cells. These antigens appear to be confined to the epidermis but have been shown to play a role in rejection of the epidermis in chimeric models of tolerance in pigs[368,389-391]. The skin also contains alarmins which are damage-associated molecular patterns (DAMPs) that can trigger an immune response following injury[392]. This is particularly pertinent in the context of ischaemia-reperfusion and traumatic injury at the time of transplantation but also subsequent trauma in the long term. DAMPs may be important for the skin's ability to recruit circulating recipient T cells that result in rejection, whilst also explaining why even mild tissue damage after VCA transplantation can directly result in acute rejection[393,394].

The elevated stature of skin as a highly immunogenic tissue is likely to have suppressed research into its use in human transplantation. Whilst the immunological pathophysiology underpinning solid organ transplantation has become increasingly well characterised since the 1980s, the same cannot be said about VCA transplantation. Efforts have focused on the application of VCAs in reconstructive surgery, and although numbers have been slowly increasing over the past two decades, the scientific characterisation that underpins this work has not kept pace. Experience from solid organ transplantation has often been extrapolated into the VCA field due to disparity in the literature; however, as demonstrated by recent insights into antibody mediated rejection in VCAs[395], there are likely to be key differences between VCA and solid organ transplantation. When exploring the use of VCAs for monitoring visceral organs, there remain numerous unanswered questions regarding the efficacy, safety, and pathophysiological basis of this technique.

Given the novelty of VCAs transplanted alongside a solid organ, there is a relative blank canvas for immunological studies. Donor specific antibodies are clinically important in solid organ transplantation, contributing to antibody mediated rejection and graft loss; however, several authors have commented that DSA and antibody mediated rejection occur less frequently after VCA transplantation than might be expected[396-398]. In the context of SSFs performed alongside pancreas transplantation, any increase in rates of DSA formation could have a harmful effect on the simultaneously transplanted pancreas and kidney allografts. It is therefore essential that this is evaluated as part of any study investigating the simultaneous transplantation of VCAs and solid organs.

Under-immunosuppression leads to rejection whereas over-immunosuppression contributes to comorbidity via opportunistic infections and malignancy. As discussed in Chapter 5, the main hypothetical advantage of a sentinel skin flap is that it will act as a real-time visual indicator of the level of immune function and enable prompt changes to immunosuppressive regimens. Ideally, we would like a marker that can give this answer clinically without the need for the additional morbidity of a VCA transplant. Several biomarkers have been proposed including measures of T cell activation (Cylex ImmuKnow) and a stimulated interferon gamma assay (Qiagen QuantiFERON Monitor)[399]. The QuantiFERON Monitor (QFM) uses a fixed innate and adaptive immune stimulant so that the cell mediated immune response can then be measured. Its early use in liver transplant patients has been reported but there has been no subsequent description of its ability to correlate with clinical events[400].

6.2 Aims and Objectives of Chapter 6

The aim of this chapter was to explore some of the immunological characteristics of sentinel skin flap and pancreas transplantation. Several sub-studies are described that aimed to assess basic immunological safety and establish a foundation for future work.

The objectives of this chapter were:

- To investigate the formation of *de novo* donor specific antibodies in sentinel skin flap and pancreas transplantation.
- To investigate the use of a stimulated interferon-gamma assay in pancreas transplantation.
- To perform basic immunophenotyping of patients at different timepoints and states of immunological activation.

6.3 Methods

Blood samples were collected according to protocol visits and 'trigger events' during the sentinel skin flap study, as described in Chapter 5.3.4

6.3.1 Donor Specific Antibodies

All patients were genotyped for HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ, and HLA-DP using polymerase chain reaction sequence-specific primer methods[401]. Pancreas transplant recipients were categorised by HLA-A, HLA-B, and HLA-DR mismatch (0, 1, or 2).

Recipient sera were screened pre-transplant and at each post-transplant research visit for the presence of HLA antibodies by Luminex technology using LABScreen® Mixed kits and antibody specification was performed using LABScreenPRA® and Single Antigen beads as appropriate (One Lambda Inc., Canoga Park, CA, USA). Results were reported as mean fluorescence intensity (MFI); only values of >1000 are reported (although clinically relevant values are disputed and discussed further in Section 6.5). All HLA samples were processed by scientists at the Oxford Transplant Immunology Laboratory, Churchill Hospital, Oxford.

6.3.2 Stimulated interferon gamma

The QuantiFERON Monitor Assay was performed on 1 ml of heparinised whole blood as per the manufacturer's instructions. Blood was stimulated with the QFM lyosphere (containing anti-CD3 and R848) within 6 hours of phlebotomy. Stimulated blood was then incubated for 16 - 24 hours at 37 °C. Blood was then centrifuged for 15 minutes at 3000g and plasma collected. Plasma samples were stored at -80 °C and batch processed. An enzyme linked immunosorbent assay (ELISA) was used to measure the interferon gamma,

reported in International Units per ml (IU / ml). Samples were processed at 3 dilutions - undiluted, 1 in 10, and 1 in 100 to ensure accurate assessment. A full ELISA protocol can be found at https://www.quantiferon.com, an abbreviated version is appended as Appendix C. All QFM samples were processed by me at the TRIG laboratories, Nuffield Department of Surgical Sciences, Oxford.

6.3.3 Immunophenotyping

6.3.3.1 PBMC isolation and storage

40 ml of blood were taken in EDTA tubes and stored on ice for a maximum of 2 hours. Peripheral blood mononuclear cells (PBMC) were separated using an established ficoll separation technique and frozen to -80 °C according to protocol (Appendix D). They were transferred to liquid nitrogen within 4 weeks for long term storage at -180 °C.

6.3.3.2 PBMC thawing

Cells were transferred to a -80 °C freezer at least an hour prior to thawing. Each cryovial was placed in a 37 °C water bath until the last ice crystal was visible. Cells were transferred to a universal tube and RPMI 1640 solution pre-warmed to 37 °C was progressively added to dilute out the DMSO freezing solution. This was added at a rate of 0.5 ml over the first minute, 1 ml for the second minute, 2 ml over the third minute and so on until there was a total volume of 10 ml. The cell solution was centrifuged at 520g, the supernatant removed, and cell pellet resuspended in 1 ml of RPMI solution for processing. Retrieved cell numbers were counted using a haemocytometer.

6.3.3.3 PBMC flow cytometry

Beckman Coulter Duraclone IM panels were used with a Navios flow cytometer. These panels are made by Beckman Coulter according to established protocols and have been validated in detailed immunological studies such as the ONE Study[402]. Five panels were run on each sample where sufficient cell numbers allowed. These panels were: Basic Phenotyping, T cell subsets, T cell receptors, T regulatory cells, and B cells. The full details of these panels can be found in Appendix E. 750,000 cells were used for all panels except the T reg panels where 1 million cells were used. Samples were processed according to the manufacturer's instructions which can be found in references[403-407]. All samples were processed by me at the TRIG laboratories, Nuffield Department of Surgical Sciences, Oxford.

6.4 Results

6.4.1 Donor specific antibodies

Demographic and transplant data including HLA mismatch and cRF have been presented in Chapter 5.4 (Table 5.2). Figure 6.1 demonstrates DSA formation during the first year in this cohort of 20 patients who received an SSF alongside pancreas transplantation. Overall, 30% of patients developed *de novo* DSA (DSA) during the first year; however, only half of these persisted at 1 year.

Only 1 patient developed HLA Class II DSA, the rest were all Class I. Antibody levels were generally low with only one patient developing a high MFI at 3 months which then reduced to a low MFI at 6 months and had gone by 12 months. There was no association between rejection episodes and the development of DSA. Similarly, low tacrolimus levels and reduced mycophenolate doses did not correspond with development of DSA.

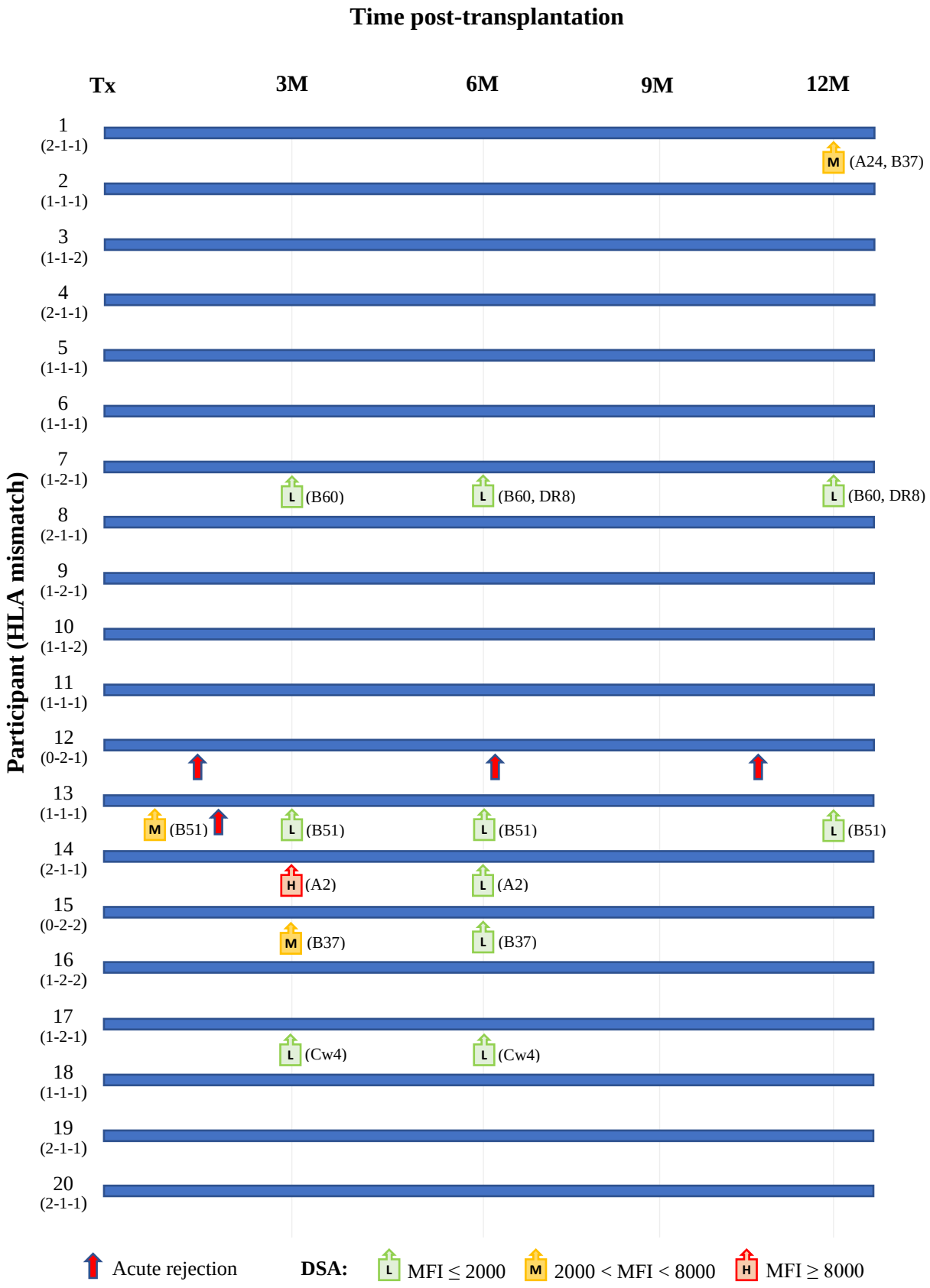


Figure 6.1: All SSF patients during the 12 month study period with immunological events and donor specific antibodies shown. Note DSAs were tested 3, 6, and 12 months in addition to any protocol events; negative DSAs are not illustrated. DSA levels reported as MFI.

6.4.2 Interferon gamma

Stimulated interferon gamma (IFN-γ) was significantly lower at all timepoints after transplantation compared with pre-transplant values, reflecting iatrogenic immunosuppression after transplantation (Figure 6.2). There was an incremental increase in stimulated IFN-γ at each timepoint. The small number of rejection events precluded meaningful statistical analysis of these episodes.

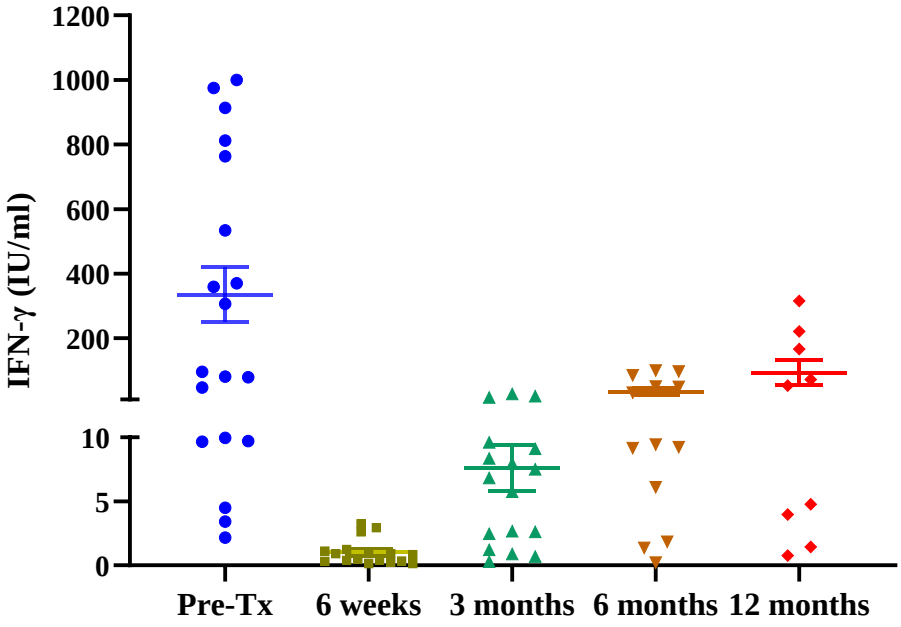


Figure 6.2: Stimulated IFN-γ levels before and after transplantation. Lines represent mean (± SEM). There was a significant difference in overall post-operative IFN-γ ($F = 8.10$, $p < 0.001$) and also between each timepoint (all $p < 0.01$, corrected for multiple comparisons).

6.4.3 Immunophenotyping

83 samples were available for immunophenotyping (19 pre-Tx; 17 at 6 weeks; 16 at 3 months; 16 at 6 months; 11 at 12 months; 4 at rejection events). Table 6.2 displays the results for the first stage of immunophenotyping analysis. T lymphocyte populations reduced immediately after transplantation and had a variable recovery over the 12 month period. Monocytes became the predominant cell type immediately after transplantation and reduced slowly thereafter. B lymphocytes formed a higher proportion of the lymphocyte population over the 12 months. T regulatory cells formed a much higher proportion of CD4+ cells after transplantation and this subsequently reduced over time.

Effector memory CD8+ T cells appeared to form a much higher proportion of CD8+ cells during an episode of rejection. ROC curve analysis demonstrated an AUC of 0.95 ($p = 0.002$; Figure 6.3). An effector memory CD8+ T cell proportion of more than 63.8% had a 100% sensitivity and 93.5% specificity for rejection.

Table 6.2: Immunophenotyping of the SSF cohort over 12 months. All values are mean percentages (± SD) unless otherwise stated.

Cell type	Pre-Tx	6 weeks	3 months	6 months	12 months	Rejection	P value (without rejection)
Number of samples	**19**	**17**	**16**	**16**	**11**	**4**	-
CD14+ monocytes †	28.6 (13.53)	64.5 (21.4)	51.5 (16.76)	45.8 (17.69)	28.8 (18.45)	36.6 (36.24)	< 0.001
CD14+ CD16+ *	7.7 (6.42)	10.5 (12.06)	14.7 (13.05)	8.9 (6.39)	10.1 (7.09)	15.8 (7.71)	0.281
CD14+ CD16- *	92.3 (6.41)	89.6 (11.93)	85.5 (12.82)	91.2 (6.36)	90.0 (7.04)	84.4 (7.55)	0.295
Lymphocytes †	53.3 (20.19)	11.7 (8.58)	19.6 (12.74)	29.4 (17.44)	18.8 (20.23)	9.2 (7.87)	< 0.001
CD19+ B cells ‡	7.1 (6.89)	9.2 (11.92)	27.5 (16.35)	23.6 (14.9)	34.6 (17.98)	8.4 (15.38)	< 0.001
CD56+ NK cells ‡	10.1 (8.4)	45.9 (18.9)	31.2 (13.57)	28.3 (18.85)	18.6 (17.8)	28.3 (29.03)	< 0.001
CD3+ T cells ‡	78.5 (11.04)	13.8 (12.66)	26.1 (16.85)	39.3 (24.23)	43.0 (19.97)	45.0 (38.34)	< 0.001
CD4+ T cells **	66.4 (10.96)	54.7 (24.18)	63.4 (25.81)	49.1 (24.71)	54.1 (27.85)	50.5 (2.7)	0.171
CD8+ T cells **	28.7 (9.57)	35.4 (21.37)	27.4 (22.15)	39.1 (23.54)	32.6 (26.42)	42.2 (2.44)	0.471
T cells (as a proportion of CD4+ and CD8+ respectively):							
Central memory CD4+	66.1 (16.97)	49.5 (12.32)	50.3 (9.48)	52.0 (14.04)	59.3 (14.35)	47.9 (16.79)	0.002
Naive CD4+	15.8 (16.73)	2.4 (2.6)	3.8 (4.38)	3.8 (3.95)	14.6 (15.54)	0.8 (0.16)	< 0.001
Effector memory CD4+	16.5 (12.81)	46.6 (12.4)	44.9 (8.82)	42.8 (15.7)	25.1 (15.1)	50.9 (16.53)	< 0.001
TemRA cells CD4+	1.6 (2.91)	1.4 (2.36)	1.0 (1.34)	1.4 (2.44)	1.0 (1.63)	0.4 (0.36)	0.930
Central memory CD8+	24.1 (14.84)	14.0 (9.53)	11.2 (7.79)	9.2 (7.22)	11.2 (8.45)	7.6 (4.72)	< 0.001
Naive CD8+	28.0 (20.61)	8.8 (11.1)	19.3 (21.37)	19.3 (23.1)	39.4 (31.39)	1.1 (0.58)	0.006
Effector memory CD8+	30.0 (15.47)	43.6 (15.86)	40.6 (19.81)	43.3 (22.78)	29.9 (23.33)	77.8 (11.43)	0.098
TemRA cells CD8+	17.9 (13.58)	33.5 (23.56)	28.9 (21.19)	28.3 (19.08)	19.5 (16.51)	13.5 (16.51)	0.108
T regulatory cells (as a proportion of CD4+ cells):							
FoxP3+	12.8 (13.23)	40.0 (20.68)	39.8 (26.9)	27.9 (24.72)	27.0 (20.43)	21.3 (7.48)	0.002
FoxP3+Helios+	6.9 (3.52)	36.1 (17.37)	27.6 (13.42)	14.7 (5.44)	10.1 (4.86)	18.3 (8.5)	< 0.001

† As a proportion of all PBMCs; ‡ As a proportion of gated lymphocytes; * As a proportion of gated monocytes; ** As a proportion of gated CD3+ cells

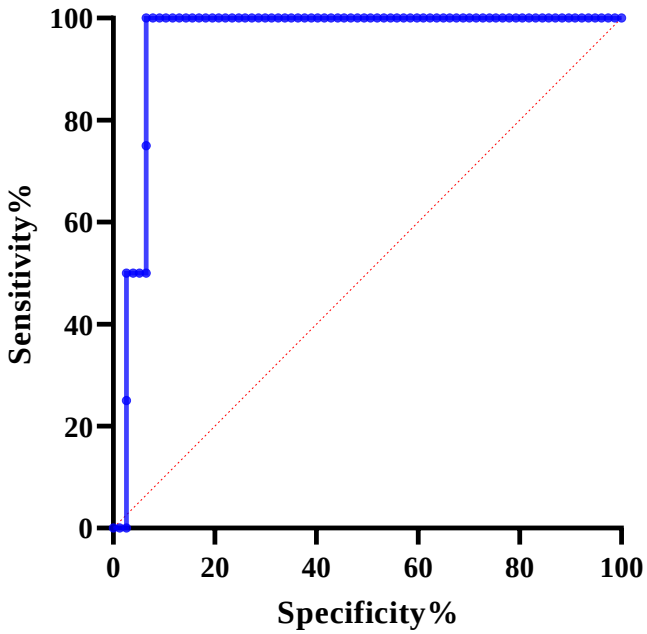


Figure 6.3: ROC analysis of CD8+ effector memory T cells for patients with and without acute rejection episodes (AUC 0.95, p = 0.002).

6.5 Discussion

There have been over 200 VCA transplants performed since the first hand transplant in 1998. The small numbers performed has meant that we have translated the rejection principles learnt through solid organ transplantation into VCA transplantation. However, it is becoming apparent that the skin component of a VCA has differences that require special consideration in the context of both cellular and antibody mediated rejection. Transplantation of both a solid organ and a VCA, as in the SSF study described in Chapter 5, provides further challenges.

Donor specific antibodies play an important role in rejection of solid organ transplants and graft outcomes in heart, lung, kidney, and pancreas transplantation are poorer in the presence of DSA[177,408-410]. There are reports of early and late DSA formation in VCA transplantation, however, their liability in long term graft outcomes remains uncertain[396]. Kaufman et al. reported a series of 5 hand transplant patients that developed features of chronic rejection including with one graft loss secondary to ischaemia[411]. However, the first report of *de novo* DSA associated with antibody mediated rejection was from Weissenbacher et al. who described a patient that developed rejection 9 years after bilateral forearm transplantation[411]. This episode was successfully treated with rituximab after steroid failure.

DSA formation is associated with episodes of acute rejection and it is unclear why the rate of DSA formation in VCA transplantation remains relatively low given that acute rejection has been reported in up to 85% of VCA transplants at 1 year[412,413]. Such a high rate of acute rejection would be unacceptable in solid organ transplantation and one of the key concerns when planning the sentinel skin flap study described in Chapter 5 was the possibility of a high number of VCA rejection episodes. This could theoretically cause allosensitisation

with development of *de novo* DSA that contribute to poorer graft outcomes for the pancreas and kidney allografts.

The results from this study are encouraging - 30% of patients developed dnDSA overall and only 15% of these persisted at 1 year. The majority of these DSA (71%) were low level with an MFI of less than 2000. A level of less than 2000 is very unlikely to be associated with the development of clinically relevant DSA and some have suggested MFIs of 3000 or higher are required [414,415]. In this context, only a single patient in our study had a DSA level of 3000 at 1 year after transplantation. Our results are in keeping with reported studies of DSA formation in pancreas transplantation. Mittal et al. described a DSA rate of 36.9% in a cohort of 433 patients whilst Parajuli et al. reported DSA rate of 22% in 541 pancreas recipients[177,220]. Considerable insight also comes from studies by the Oxford intestinal transplant group who have described a series of intestinal and multivisceral transplant patients, some of whom had concurrent abdominal wall VCAs transplanted[356,416]. Intestinal transplants are highly immunogenic, and episodes of acute rejection have been reported as 35 - 60% during the first year. However, the Oxford group reported acute intestinal rejection rates of 7% in those with a VCA and 27% in those without a VCA. Furthermore, the dnDSA rate was 37.5% in the VCA group compared with 61.5% in the non-VCA group. These results are encouraging although we should be aware of significant limitations posed by small numbers and heterogeneity of the cohorts.

Interferon gamma is a cytokine that is critical for innate and adaptive immunity. It is produced predominantly by Natural Killer (NK) cells and NK T cells as part of the innate immune response and by CD4+Th1 and CD8+ cytotoxic effector T cells once antigen specific immunity develops[417]. Given its bridging role between the innate and adaptive immunity, it has been proposed as a potential biomarker for the overall level of immune system activation. During the QuantiFERON Monitor assay a precise amount of innate and

adaptive stimulant is applied to a fixed quantity of a patient's blood. The resulting interferon gamma production theoretically indicates the level of overall response from the combined components of the immune system. This is of particular interest in transplantation where immunosuppression can be difficult to monitor, and associated morbidity is common. Sood et al. have investigated its use in healthy controls, cirrhotic patients and liver transplant patients at differing time points[399,400,418]. The stimulated IFN-γ levels dropped significantly in the early post-operative period before recovering to pre-transplant levels in the long-term. When looking at patients with cirrhosis undergoing liver transplantation, a low stimulated IFN-γ was associated with a hazard ratio of 4.1 for subsequent development of infection and very low levels were associated with death (HR 56.6). However, they have not reported any association with rejection episodes nor its use in the monitoring of transplant patients. The results presented here corroborate the findings of Sood et al.; there is a dramatic reduction in immune function (stimulated IFN-γ) immediately after transplantation and this gradually recovers over the subsequent year although not to pretransplant levels. The number of rejection episodes was too small to be able to make any meaningful analyses regarding the diagnostic use of this test. However, it is notable that the values of IFN-γ ranged from 0.38 to 54.3 IU/ml at the time of rejection (in keeping with other values for that timepoint after transplantation), and therefore it is difficult to see how this test will achieve a high sensitivity and specificity for monitoring rejection. It is important to note that the immunosuppression protocols used in pancreas and liver transplantation are different. Unlike liver transplantation, lymphocyte depleting biological induction agents are widely used in pancreas transplantation alongside dual maintenance therapy. The data in this chapter and Chapter 5 demonstrates that this has lasting effects on both total lymphocyte numbers and sub-populations.

There has been significant progress in the development of cellular immunotherapies that aim to ameliorate the alloimmune response or induce tolerance[419]. The ONE study is a phase I / II multicentre trial investigating the safety of infusing ex-vivo expanded regulatory T cells as well as others[420]. There is a dose escalation component to the study to enable determination of the maximum-tolerated dose. The subsequent TWO study has just started recruiting and aims to assess the use of regulatory T cells in reducing immunosuppression and preventing rejection. Studies such as these have required the development of robust antibody staining and gating protocols that can be reliably applied to flow cytometry across multiple centres. The Duraclone IM panels were developed as part of these studies and were used in the study described in this chapter as a basic exercise in immunophenotyping in VCA and pancreas transplantation[402]. Large quantities of data have been generated and detailed analysis is beyond the scope of this thesis. Table 6.2 presents some of these results and it is clear that dramatic changes occur in some cell populations after transplantation. ROC analysis of CD8+ effector memory cells demonstrated a very high sensitivity and specificity for diagnosis of acute cellular rejection of the SSF. However, the numbers are small and need validating in a larger cohort with a higher number of rejection events. Nevertheless, future detailed analysis of permutations in subtle immunological characteristics may deliver a robust biomarker for acute rejection.

6.6 Conclusion

This chapter reports three immunological sub-studies from the sentinel skin flap study. The low level of *de novo* DSA formation provides additional reassurance that we did not increase the immunological risk by co-transplanting a VCA with the pancreas allograft. Stimulated IFN-γ may help identify patients at risk of over immunosuppression but seems unlikely to be able to accurately diagnose rejection. Detailed immunophenotyping in a larger cohort with more rejection episodes is needed to investigate whether a robust biomarker for rejection is yielded from this technique.

Chapter 7

Overall Conclusions and Future Directions

Pancreas transplantation has become an established treatment option for patients with severe complications of diabetes. It leads to insulin independence, stabilisation of complications, and improved survival. To realise these benefits, the pancreas allograft needs to function for many years. Despite significant improvements in graft survival, there is still a considerable rate of graft failure. It is widely believed that one major reason for this is that we have no accurate and reliable way of monitoring the pancreas allograft, such that by the time graft dysfunction is clinically evident, damage is usually irreversible. Whilst there are tests that are associated with poorer graft outcomes (e.g. OGTT, DSA, HbA_{1c}), there are none that robustly identify impending graft injury within timelines that allow treatment. Major improvements in pancreas allograft survival have now been accomplished with refined surgical techniques and improved immunosuppression. It is likely that a series of more marginal gains are now required to make further incremental progress.

This thesis describes a series of novel approaches to pancreas allograft surveillance - two endocrine techniques and one immunological. The two endocrine studies have produced a combined cohort of pancreas recipients that have undergone 100 frequently sampled glucose tolerance tests; this is far in excess of any other reported studies and has already yielded valuable information.

Key findings of the HOMAtx study:

- Fasting systemic hyperinsulinaemia was confirmed in this cohort (91% higher than matched controls, $p = 0.001$; this was despite similar beta cell function as defined by HOMAtx %B).
- Correlation between HOMAtx %B and insulin secretion during hyperglycaemic clamps was confirmed ($r = 0.84$, $p < 0.001$).
- Correlation between HOMAtx %S and insulin sensitivity during hyperglycaemic clamps was confirmed ($r = 0.60$, $p = 0.039$)
- A significant difference in HOMAtx %B was observed between those with normal and diabetic glucose tolerance tests ($p = 0.006$).
- ROC analysis confirmed that a HOMAtx %B of 64.25 had a 100% sensitivity and 84.1% specificity for the detection of diabetic glucose tolerance during OGTT ($p < 0.001$).
- HOMAtx does not demonstrate any relationship with HbA_{1c}.
- Pancreas transplant patients can have a normal HbA_{1c} yet have significant pancreas graft dysfunction as demonstrated by diabetic glucose tolerance during OGTT.

One of the most important findings is that the validated HOMAtx model has a highly sensitive (100%) threshold for diagnosing pancreas allograft dysfunction that was not otherwise evident on routine clinical testing. Moreover, this assessment only requires a starved glucose and insulin or C-peptide sample. With the potentially imminent arrival of point of care C-peptide testing[421], a result could be obtained before the end of a clinic consultation. Indeed, such a device could theoretically be used at home in a similar manner to the current capillary glucose meters and therefore %B and %S could be monitored remotely on a daily basis.

Key findings of the UCPCR study:

- Correlation between 120-minute UCPCR and serum C-peptide during OGTT was confirmed ($r = 0.62$, $p < 0.001$).
- Correlation between UCPCR and serum C-peptide was preserved in those with an eGFR as low as 30 ml/min/1.73m^2 (CKD 3b).
- A significant difference in UCPCR was observed between those with normal and diabetic glucose tolerance tests ($p = 0.009$).
- ROC analysis confirmed that a UCPCR of 1.165 nmol/mmol had a 100% sensitivity and 64.1% specificity for the detection of diabetic glucose tolerance during OGTT ($p = 0.002$).
- UCPCR did not demonstrate any relationship with HbA_{1c}.
- Correlation between home UCPCR and recent OGTT was confirmed ($r = 0.37$, $p < 0.001$).
- ROC analysis confirmed that a home UCPCR of 1.140 nmol/mmol had a 100% sensitivity and 58.9% specificity for the detection of diabetic glucose tolerance during OGTT ($p = 0.002$).

Similar to the HOMAtx study, the UCPCR study has established a value that has a high sensitivity for predicting acute graft dysfunction. The key advantage with this test is that it can be sent directly to the laboratory from the patient's home after a routine breakfast. The Oxford Transplant Centre has patients from as far as the South Coast and Cornwall - any technique that could increase graft surveillance efficacy whilst reducing unnecessary travel would be warmly welcomed.

The next steps for HOMAtx and UCPCR now need to be decided. Large scale studies, comparing groups of patients that are monitored with and without these methods and

powered to graft survival, would be ideal, although are likely to be costly to run given the time to recruit and need to involve multiple centres. When performed as part of a clinical protocol the tests themselves have a low cost; less than £10 for HOMAtx and UCPCR, particularly when considering that serum glucose, insulin, and C-peptide are already performed routinely at most clinic visits. Given the level to which these techniques have now been validated in this thesis, there is a good argument to incorporate UCPCR and HOMAtx assessments prospectively into pancreas surveillance, and to use retrospective reviews to gain further data in terms of the magnitude of any clinical benefit (e.g. by analysis of national registry data). Figure 7.1 depicts how this could happen. Further work is also planned to fully validate the insulin sensitivity side of HOMAtx with hyperinsulinaemic euglycaemic clamps. Following this, there is scope for non-steady state modelling in pancreas transplantation. This could potentially remove the need for patients to be starved (a common reason for non-compliance).

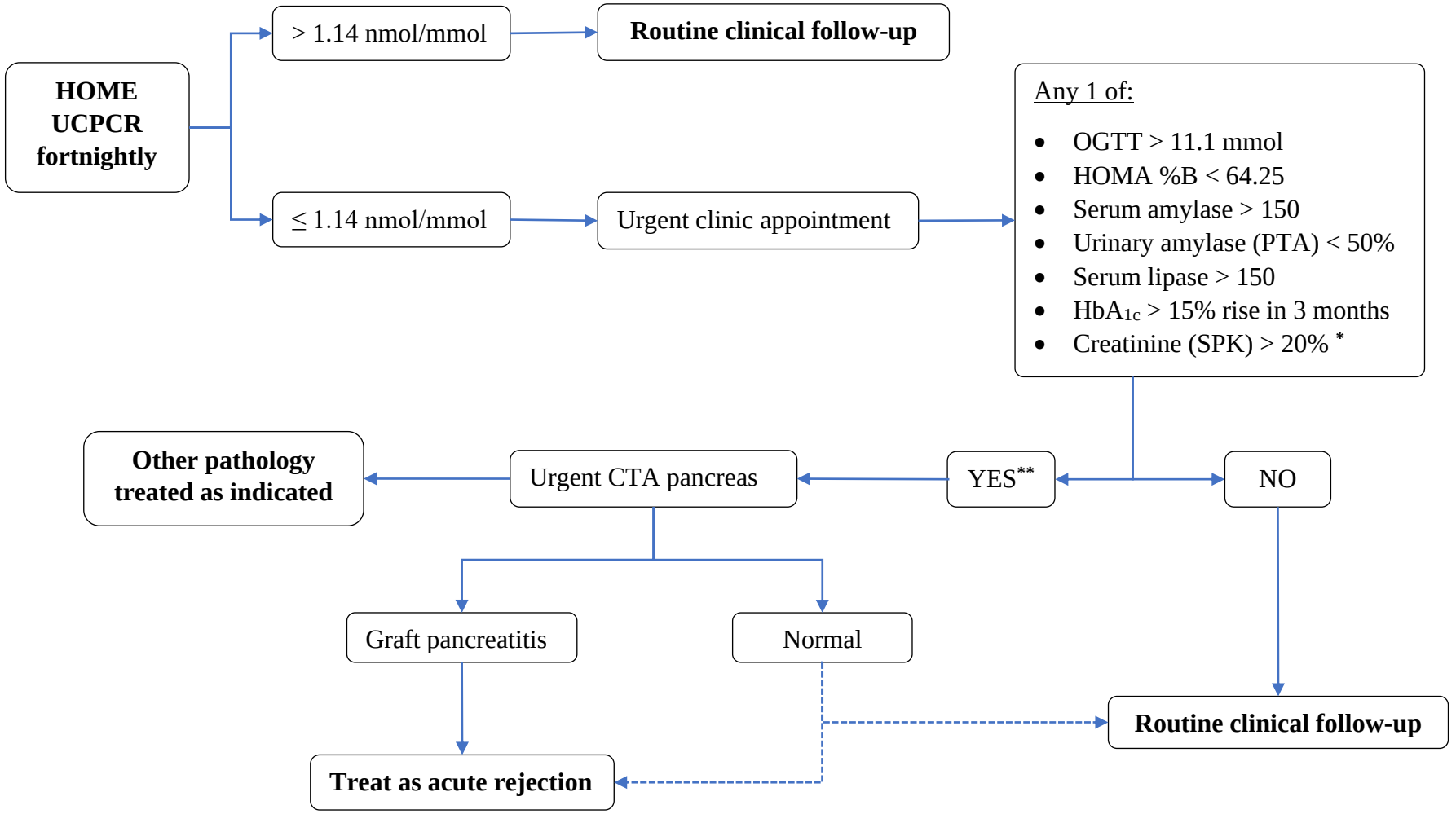


Figure 7.1: Theoretical algorithm for outpatient pancreas allograft surveillance during the first 12 months after transplantation.
* unexplained rise in creatinine would also prompt renal biopsy; ** pancreas biopsy also performed in some units

Key findings of the SSF Study:

- Pancreas and kidney allograft survival was 95% and 100% respectively.
- Excellent allograft function was observed (mean eGFR 55 ml/min/1.73m^2, mean HbA_{1c} 35.4 mmol/mol).
- There were no episodes of pancreas or kidney rejection in the first 12 months after transplantation.
- Two patients developed acute rejection of the sentinel skin flap, and 1 of these experienced more than one episode of acute rejection.
- There was no increased risk of DSA formation associated with SSF transplantation (30% of patients developed DSA during the first year after transplantation with 15% persisting at 1 year).
- Stimulated interferon gamma is unlikely to provide a valid tool for the detection of acute allograft rejection
- Effector memory CD8+ cells may be an accurate biomarker for allograft rejection. ROC analysis demonstrated that an effector memory CD8+ T cell proportion of more than 63.8% had a 100% sensitivity and 93.5% specificity for rejection ($p = 0.002$).

The sentinel skin flap study corroborates findings from the Oxford intestinal group and when combined this now forms a series of nearly 50 patients that have had concurrent visceral and VCA transplantation. The SSF's value in predicting an impending alloimmune response is still some way from certain. The lack of pancreas biopsies limits the sensitivity of study designs that would otherwise produce definitive evidence. Nevertheless, the fact remains that there have been no cases of discordant rejection involving the primary visceral allograft in either this pancreas study or the intestinal programme. The SSF study has now

been extended to 30 patients - if there are still no primary visceral rejection events, this would achieve statistical power in post-hoc analysis. Whilst not ideal statistical methodology, it allows for an outcome that was not predicted at the beginning of this early phase study.

A large powered controlled trial of this technique would need more than 250 patients and would therefore be unfeasible in the context of the UK pancreas transplant programme. If the current study extension confirms the hypothesis that the SSF is providing a real-time immunological assessment and pre-empting primary visceral rejection, then the question remains as to how to integrate this technique into clinical practice to improve outcomes. The main question is that of feasibility - there are substantial resource implications to this technique. There is a real issue of expertise: although a skilled organ retrieval surgeon would rapidly acquire the capability to retrieve the skin flap, implantation requires microvascular skills which are outside the skill-set of most abdominal transplant surgeons, and therefore necessitate the involvement of a different surgical specialty. In the context of clinical innovation, we have been fortunate in Oxford that a group of reconstructive surgeons has been willing to provide this service at short notice and on a 24/7 basis: the logistics would be much more demanding if the use of SSF became routine. The implication of this is clear: the level of evidence of benefit needs to be very high and the amount of benefit needs to be substantial.

The small size of the national pancreas transplant programme is, paradoxically, a compounding problem in this context. If the numbers were greater (e.g. 1000 cases per year rather than 200), then the justification to provide an SSF service would be easier. Given the current transplant numbers it is more likely that routine SSF monitoring would be feasible if it were shown to bring similar benefits to other organ transplant types. In this context, forthcoming trials in lung and heart transplantation are of great potential importance and

may help generate interest in spreading this approach to the much larger fields of kidney and liver transplantation.

Conversely there is an argument to restrict SSF to just those patients most likely to benefit – those that fare the most poorly at present. Patients receiving a pancreas transplant without a concomitant kidney from the same donor (PTA and PAK) have inferior graft survival in almost every reported analysis, and it is believed that the lack of immune monitoring is the primary reason for this. This is, logically, the right population in which to implement new methods such as SSF. However, the logistics of implementing SSF as a clinically routine procedure are aggravated by the fact that the requirement for this would be very infrequent. This creates problems at every level – retrieval, implantation and data analysis – because the frequency even in the largest units would be below that needed to justify the resources or to retain the skills.

If SSF were to prove a major advance in immune monitoring and, as a result, the long-term results of pancreas transplantation were significantly improved, then the risk-benefit of pancreas transplantation might be transformed. Patients with rapidly progressing secondary complications of diabetes (nephropathy, retinopathy, neuropathy), as well as those with very difficult to manage blood sugar control, would be assessed for transplantation at a much earlier stage, and at a point in the disease pathway when benefit from transplantation is much more likely to be beneficial (allowing that stabilisation is more likely than reversal of microvascular complications). Ideally such increased clinical benefits would be combined with a parallel reduction in the risks of pancreas transplantation (e.g. by improvement in early pancreatitis). Improvement on both sides of the risk-benefit balance would substantially alter the threshold for pancreas transplantation, such that many more patients would stand to benefit.

Pancreas transplantation is in need of clinical innovation, not just because there has been little development since the 1990s, but mainly because there are real clinical barriers that prevent the rolling out of this therapy to a wider population of patients who would stand to benefit. Part of the challenge in testing and introducing new techniques in pancreas transplantation is the very low volume of activity, both overall (e.g. 200 cases per year in the UK) and in each unit (e.g. in the US there are 156 centres performing under 1000 pancreas transplants[422]). This is not an insuperable problem, but one that requires carefully designed and managed collaborations. The prize is considerable: collegiate working, leading to improved outcomes and expanded selection criteria, so that more people can benefit from this life-changing therapy.

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Appendix A - Pancreas Allocation

UK National allocation system for solid organ pancreas transplantation. Islets have a slightly different scoring system; the pancreas goes to the recipient with the most points whether for islet or solid organ transplantation. Full policy available from NHSBT.

Factor	Points allocation
Total HLA Mismatch	0 to 4 HLA mismatches = 730 points 5 to 6 HLA mismatches = 0 points
Waiting time	Waiting time (days)^2 / 365
Sensitisation	Sensitisation (%)^3 / 1000
Travel time	**DBD:** Closest three centres = 365 points Outside closest three = 0 points **DCD:** Closest centre = 10,000 points Closest three centres = 5,000 points Outside closest three = 0 points
Donor body mass index	22 or less +730 23 to 25 +365 26 to 28 0 29 to 31 -365 32 or over -730
Dialysis status	On dialysis = 180 points Not on dialysis = 0 points
Donor to recipient age matching	Age difference (years)^2 / 13.9

Appendix B - SSF Retrieval SOP

Sentinel Skin Flap Retrieval - SOP

Pancreas Offers

Pancreas offers will be reviewed by the on call transplant recipient coordinator and consultant as per current guidelines and practice. If a pancreas is accepted for a recipient that has consented to a sentinel skin flap then the skin flap retrieval process will be coordinated by James Barnes and Henk Giele (i.e. liaising with the donor SNOD, NORS teams, and recipient on call team).

Sentinel Skin Flap Retrieval Team

The sentinel skin flap (SSF) will be retrieved by a team from the Oxford Transplant Centre that is separate to the abdominal and cardiothoracic NORS teams. The SSF retrieval team will consist of at least 1 retrieval surgeon with appropriate training to specifically retrieve sentinel skin flaps. The SSF retrieval team will make their own way from Oxford to the donor hospital and back by private transport.

Retrieval Procedure

A longitudinally orientated radial forearm flap comprising the forearm skin and underlying fat and fascia is retrieved. This overlies the radial artery and its venae comitantes, together with the cephalic vein and the lateral cutaneous nerve of the forearm.

A tourniquet using an Esmarch bandage is applied to the upper arm. A 12 cm long 3-4 cm wide ellipse of skin is marked over the course of the radial artery (this follows the ulnar edge of brachioradialis), with the centre of the flap 2 cm more distal than the mid-forearm level. The origin of the radial artery lies at the centre of the ante-cubital fossa just medial to the biceps tendon.

The flap is raised by incising the distal half of the skin markings through skin and fascia and dividing the distal end of the artery and its venae comitantes at the wrist after ligating it. The pedicle and flap are then dissected from distal to proximal dividing the fascial tissue and branches between the pedicle and the muscles lying on both sides and deep to the radial artery. The flap and vascular pedicle are raised away from the forearm facilitating dissection. The remainder of the skin is incised through skin, fat and fascia. On incision of the proximal skin the cephalic vein and the adjacent lateral cutaneous nerve of forearm should be ligated and tagged for later coaptation/anastomosis. The proximal radial venae comitantes are ligated and divided but the radial artery is just divided.

The donor site is closed directly. The width of skin retrieved is planned to allow direct closure of the defect; a 4 cm defect is usually compatible with direct closure of the skin. If there is difficulty in closing the skin, the forearm muscle bulk can be reduced by resecting some muscle, thus facilitating closure.

Equipment

All instruments and consumables for SSF retrieval are stored in the Oxford Transplant Centre perfusion laboratory. There are 2 dedicated instrument sets for SSF retrieval. These sets will be processed by Oxford University Hospitals NHS Foundation Trust (OUH) Sterile Services Department. To avoid any potential errors, instrument sets will be collected directly from sterile services during normal working hours.

Organ Storage and Transport

Once raised, the flap is perfused with 40 ml of cold heparinised saline and then UW solution through the radial artery. The flap is then stored in a sterile Aldon bag containing cold UW solution that is placed in another Aldon bag containing cold saline, which is then placed in a further bag. The SSF will be transported back to Oxford in a separate organ box in the same ambulance as the pancreas. Upon arrival at Wytham Ward the SSF will be checked into the secure organ fridge with the pancreas and/or kidney as per current practice with all organs for transplantation at OUH. If there is insufficient room for all organs to be stored in this fridge then organs will be stored in the cold storage facility in the transplant tissue typing laboratory. The SSF will be signed out of the fridge as per current practice at OUH.

Appendix C - QFM abbreviated ELISA protocol

Stage 2 — IFN-γ ELISA

1. Equilibrate ELISA components, with the exception of the Conjugate 100x Concentrate, to room temperature for at least 60 minutes.



2. Reconstitute the kit standard to 8.0 IU/ml with distilled or deionized water. Prepare 4 standard dilutions.
3. Reconstitute freeze-dried Conjugate 100x Concentrate with distilled or deionized water



4. Prepare working strength conjugate in Green Diluent and add 50 µl to all wells.



5. Add 50 µl test plasma samples (undiluted, 1:10 and 1:100 dilutions as appropriate) and 50 µl standards to the appropriate wells. Mix using shaker.



6. Incubate for 120 ± 5 minutes at room temperature.



7. Wash wells at least 6 times with 400 µl/well of wash buffer.



8. Add 100 µl Enzyme Substrate Solution to wells. Mix using shaker.



9. Incubate for 30 minutes at room temperature.



10. Add 50 µl Enzyme Stopping Solution to all wells. Mix using shaker.



11. Read results at 450 nm with a 620 to 650 nm reference filter.

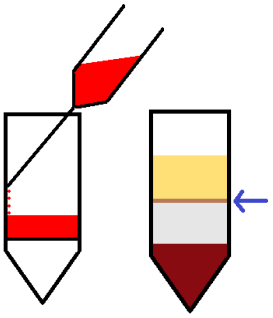


12. Analyze results.



Appendix D - PBMC separation SOP

1. Collect blood into 4x 10ml EDTA tubes, 1x 4ml serum tube, 2x Tempus tubes, 1x 2ml heparin tube
2. Spin serum tubes at 3000rpm for 5 mins (store 2x 1ml aliquot)
3. Put 15mls ficoll into each 50ml falcon
4. Add blood to mixing falcon and dilute 50:50 with rinsing buffer (rinse out EDTA tubes with 4mls x2)
5. Carefully overlay the dilute blood on top of the ficoll (with 20mls syringe and blunt needle)
6. Centrifuge 1800-1900rpm, brake off, room temp, for 20mins
7. Remove PBMC layer with Pasteur pipette into fresh 50ml falcon
8. Store plasma if required
9. Add rinsing buffer to make up to 50mls
10. Centrifuge 2000rpm, brake on high, 4 degrees, for 5mins
11. Remove supernatant, agitate pellet, add rinsing buffer to 50mls
12. Centrifuge 1000rpm, brake on, 4 degrees for 5 mins
13. Remove supernatant, agitate pellet, resuspend in 5mls running buffer
14. Remove 10µl and add to 96 well plate with 10µl trypan blue
15. Remove 10µl of the mix and add to counting slide
16. Count cells in 5 large squares, multiply x5 (=25 squares)
17. Multiply x2 (because diluted 50% with trypan blue) = no. of cells per ml
18. Then multiply x5 to make total no. of cells in the sample (aim to freeze in 1.3-2 x 10^7 aliquots)
19. i.e. total = 50n x10^4
20. Top up to 50mls with rinsing buffer
21. Centrifuge 1200rpm, brake on, 4 degrees, 5 mins
22. Decant supernatant and remove last few µl with p1000
23. Resuspend in FCS. Use 900µl per aliquot (i.e. if intend to freeze 3 aliquots then add 2.7mls FCS)
24. Prepare cryovials by adding 100µl DMSO into each. Add 900µl of FCS/cell suspension into each
25. Freeze in Mr Frosty in -80 freezer



<u>Equipment:</u>

Biohazard waste bag
Haemocytometer
Cryovials, Mr Frosty
20ml syringes and blunt needles
96 well plate
50ml Falcon tubes (each pt requires 2x 50ml falcon tubes + 1 for mixing)
5/25ml pipettes, pasteur pipettes, pipette tips (100/1000µl) + pipette boy
70% alcohol
Rinsing buffer (500mls PBS + 2mls 0.5M EDTA)
Running buffer (PBS/EDTA +2% protein: 10mls FCS in 500mls or 2.5g BSA in 500mls)
Ficoll-Paque/Histopaque/Lymphoprep (protect from sunlight)
DMSO
Trypan blue
FCS (fetal calf serum) and BSA (bovine serum albumin)

Appendix E - Duraclone IM Panels

DuraClone IM Phenotyping Basic Tube, 25 tests, RUO

REF B53309 – 25 tests

IFU- B53309-2.0

BECKMAN COULTER

	Specifications of Constituent 1	Specifications of Constituent 2	Specifications of Constituent 3	Specifications of Constituent 4	Specifications of Constituent 5	Specifications of Constituent 6	Specifications of Constituent 7	Specifications of Constituent 8
Specificity	CD16	CD56	CD19	CD14	CD4	CD8	CD3	CD45
Clone	3G8	N901	J3_119	RMO52	13B8.2	B9.11	UCHT-1	J33
Immunogen	Human neutrophils	Human chronic myeloid leukemia cells	SK LY18 Lymphoma Hybridoma	Human monocytes	Human thymocytes	Cytotoxic human T clone HLA A2	T cell line + IL2	Laz 221 cell line
Isotype	IgG1	IgG1	IgG1	IgG2a	IgG1	IgG1	IgG1	IgG1
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Source	Ascites fluid or supernatant of in vitro cultured hybridoma cells							
Purification	Affinity chromatography							
Fluorochrome	Fluorescein isothiocyanate (FITC)	R Phycoerythrin (PE)	R Phycoerythrin-Texas Red-X(ECD)	R Phycoerythrin-Cyanine 7 (PC7)	Allophycocyanin(APC)	Alexa Fluor 700 (A700)	Allophycocyanin Alexa Fluor 750 (APC-A750)	Krome Orange
λ Excitation	488 nm	488 nm	488 nm	488 nm	633 nm	633nm	633 nm	405 nm
Emission peak	525 nm	575 nm	613 nm	720nm	660 nm	720nm	783 nm	528 nm

DuraClone IM T cell subsets Tube, 25 tests, RUO

REF B53328 – 25 tests

IFU- B53328-1.0

BECKMAN COULTER

	Specifications of Constituent 1	Specifications of Constituent 2	Specifications of Constituent 3	Specifications of Constituent 4	Specifications of Constituent 5	Specifications of Constituent 6	Specifications of Constituent 7	Specifications of Constituent 8	Specifications of Constituent 9	Specification of Constituent 10
Specificity	CD45RA	CD197 (CCR7)	CD28	CD279 (PD1)	CD27	CD4	CD8	CD3	CD57	CD45
Clone	2H4	G043H7	CD28.2	PD1.3.5	1A4.CD27	13B8.2	B9.11	UCHT-1	NC1	J33
Immunogen	T lymphocyte line from Aotus trivirgatus	CCR7-transfected cells	Transfected murine cell line	PD1-Fclg	PHA-stimulated human T cells	Human thymocytes	Cytotoxic human T clone HLA A2	T cell line + IL2	Cells from quail ciliary ganglia	Laz 221 cell line
Isotype	IgG1	IgG2a	IgG1	IgG2b	IgG1	IgG1	IgG1	IgG1	IgM	IgG1
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Source	Conditioned Media	Ascites fluid								
Purification	Affinity chromatography									
Fluorochrome	Fluorescein isothiocyanate (FITC)	R Phycoerythrin (PE)	R Phycoerythrin-Texas Red-X(ECD)	R Phycoerythrin-Cyanine 5.5 (PC5.5)	R Phycoerythrin-Cyanine 7 (PC7)	Allophycocyanin(APC)	Alexa Fluor 700 (A700)	Allophycocyanin Alexa Fluor 750 (APC-A750)	Pacific Blue	Krome Orange
λ Excitation	488 nm	488 nm	488 nm	488 nm	488 nm	633 nm	633nm	633 nm	405 nm	405 nm
Emission peak	523 nm	575 nm	613 nm	692 nm	760 nm	650 nm	720nm	767 nm	455 nm	528 nm

DuraClone IM TCRs Tube, 25 tests, RUO

REF B53340 – 25 tests

IFU- B53340-1.0

BECKMAN COULTER

	Specifications of Constituent 1	Specifications of Constituent 2	Specifications of Constituent 3	Specifications of Constituent 4	Specifications of Constituent 5	Specifications of Constituent 6	Specifications of Constituent 7	Specifications of Constituent 8	Specifications of Constituent 9
Specificity	TCRγδ (i.e.TCR gd)	TCRαβ (i.e. TCRab)	HLA-DR	TCR Vδ1 (i.e. TCR Vd1)	CD4	CD8	CD3	TCR Vδ2 (i.e. TCR Vd2)	CD45
Clone	IMMU510	IP26A	Immu-357	R9.12	13B8.2	B9.11	UCHT-1	IMMU 389	J.33
Immunogen	Soluble gamma/delta T-cell receptor	T cell clone	EBV-transformed cell line	Soluble Vγ9 Cγ/Vδ1C	Human thymocytes	Cytotoxic human T clone HLA A2	T cell line + IL2	Soluble γ/ δ T-c receptor	Laz 221 cell line
Isotype	IgG1	IgG1 kappa	IgG1	IgG1	IgG1	IgG1	IgG1 kappa	IgG1	IgG1 kappa
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Source	Ascites fluid or supernatant of in vitro cultured hybridoma cells						Ascites fluid	Ascites fluid or supernatant of in vitro cultured hybridoma cells	Ascites fluid
Purification	Ion exchange or affinity chromatography	Affinity chromatography	Ion exchange or affinity chromatography	Affinity chromatography	Ion exchange or affinity chromatography	Affinity chromatography	Ion exchange or affinity chromatography	Affinity chromatography	Affinity chromatography
Fluorochrome	Fluorescein isothiocyanate (FITC)	R Phycoerythrin (PE)	R Phycoerythrin-Texas Red-X (ECD)	R Phycoerythrin-Cyanine 7 (PC7)	Allophycocyanin(APC)	Alexa Fluor 700 (A700)	Allophycocyanin Alexa Fluor 750 (APC-A750 or AA750)	Pacific Blue (PB)	Krome Orange (KRO)
Excitation peak	488 nm	488 nm	488 nm	488 nm	633 nm	633 nm	633 nm	405 nm	405 nm
Emission peak	525 nm	575 nm	613 nm	770 nm	660 nm	720 nm	783 nm	455 nm	528 nm

DuraClone IM Treg Tube, 25 Tests, RUO

REF B53346 – 25 tests

IFU- B53346-1.0

	Specifications of Constituent 1	Specifications of Constituent 2	Specifications of Constituent 3	Specifications of Constituent 4	Specifications of Constituent 5	Specifications of Constituent 6	Specifications of Constituent 7	Specifications of Constituent 8
Specificity	CD45RA	CD25	CD39	CD4	FoxP3	CD3	Helios	CD45
Clone	2H4LDH11LDB9(2H4)	B1.49.9	BA54	SFCI12T4D11(T4)	259D	UCHT-1	22F6	J33
Immunogen	T lymphocyte derived from Aotus trivirgatus	Human alloactivated T lymphocytes (FC2)	CD4+ CD8+ thymic clone B12	Peripheral blood lymphocytes	human FOXP3 recombinant protein	T cell line + IL2	Helios peptide (aa51-107)	Lazz 221 cell line
Isotype	IgG1	IgG2a	IgG1 kappa	IgG1	IgG1 kappa	IgG1 kappa	IgG	IgG1 kappa
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Hamster	Mouse
Source	Conditioned media	Ascites fluid or supernatant of in vitro cultured hybridoma cells	Supernatant	Conditioned media	Ascites fluid or supernatant of in vitro cultured hybridoma cells	Ascites fluid	Purified	Ascites fluid
Purification	Affinity chromatography	Affinity chromatography	Affinity chromatography	Affinity chromatography	Affinity chromatography	Ion exchange or affinity chromatography	Affinty chromatography	Affinity chromatography
Fluorochrome	Fluorescein isothiocyanate (FITC)	R Phycoerythrin (PE)	R Phycoerythrin-Cyanine 5.5 (PC5.5)	R Phycoerythrin-Cyanine 7 (PC7)	Alexa Fluor 647 (A647)	Allophycocyanin Alexa Fluor 750 (APC-A750)	Pacific Blue (PBE)	Krome Orange (KrO)
λ Excitation	468-509 nm	488 nm	488 nm	486-580 nm	633/638 nm	633 nm	405 nm	405 nm
Emission peak	504-541 nm	575 nm	692 nm	710-800 nm	665 nm	783 nm	455 nm	528 nm

DuraClone IM B cells Tube, 25 tests, RUO

REF B53318 – 25 tests

IFU- B53318-1.0

	Specifications of Constituent 1	Specifications of Constituent 2	Specifications of Constituent 3	Specifications of Constituent 4	Specifications of Constituent 5	Specifications of Constituent 6	Specifications of Constituent 7	Specifications of Constituent 8
Specificity	IgD	CD21	CD19	CD27	CD24	CD38	IgM	CD45
Clone	IA6-2	BL13	J3-119	1A4CD27	ALB9	LS198-4-3	SA-DA4	J33
Immunogen	Human IgD	Human B-CLL lymphocytes	SK LY18 Lymphoma Hybridoma	PHA stimulated human T cells	Bone marrow malignant cells	Human T cell line HUT 78	Human Ig heavy-chain from myeloma cells	Laz 221 cell line
Isotype	IgG2a	IgG1	IgG1	IgG1	IgG1	IgG1	IgG1	IgG1
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Source	Ascites fluid or supernatant of in vitro cultured hybridoma cells							
Purification	Affinity chromatography							
Fluorochrome	Fluorescein isothiocyanate (FITC)	R Phycoerythrin (PE)	R Phycoerythrin-Texas Red-X (ECD)	R Phycoerythrin-Cyanine 7 (PC7)	Allophycocyanin(APC)	Allophycocyanin-Alexa Fluor 750 (APC-A750)	Pacific Blue	Krome Orange
λ Excitation	488 nm	488 nm	488 nm	488 nm	633 nm	633 nm	405 nm	405 nm
Emission peak	523 nm	575 nm	613 nm	767 nm	650 nm	720 nm	455 nm	528 nm

Appendix F - Study Approvals

NHS
Health Research Authority

NRES Committee South Central - Oxford B
Whitefriars
Level 3, Block B
Lewin's Mead
Bristol
BS1 2NT

Telephone: 0117 342 1333

26 January 2015

Prof Stephen Gough
OCDEM, Churchill Hospital
Old Road
OX3 7LE

Dear Prof Gough

Study title:	**Validation Study of Homeostasis Model Assessment for Pancreas Transplantation**
REC reference:	**14/SC/1406**
IRAS project ID:	**164865**

Thank you for your letter of , 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 favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager, Mrs Siobhan Bawn, nrescommittee.southcentral-oxfordb@nhs.net . Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

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

The Sub-Committee were satisfied with the response to provisional opinion and gave the application a favourable opinion subject to the following condition:

Additional Condition

Permission to contact the transplant patients' GPs to be reinstated on the consent form.

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

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

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System 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 approvals 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. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

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 NRES. Guidance on where to register is provided on the HRA website.

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 taking part in the study, 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

Approved documents

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

Document	*Version*	*Date*
GP/consultant information sheets or letters [GP letter]	1	10 October 2014
IRAS Checklist XML [Checklist_16012015]		16 January 2015
Letter from funder [funding letter]	1	28 October 2014
Letter from sponsor [sponsor letter]	1	10 November 2014
Letter from statistician [statisticians letter]	1	15 January 2015
Letters of invitation to participant [patient invite]		22 December 2014
Other [OBB invite]		
Other [consent volunteers]	1.1	22 December 2014
Participant consent form [consent form]	1.1	22 December 2014
Participant information sheet (PIS) [PIL]	1.1	22 December 2014
Participant information sheet (PIS) [PIL 2]	1.1	22 December 2014
REC Application Form [REC_Form_12112014]		12 November 2014
Referee's report or other scientific critique report [Independent peer review]		
Research protocol or project proposal [Protocol]	1.1	22 December 2014
Summary CV for Chief Investigator (CI)		
Summary CV for student [cv Mittal]		

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/

14/SC/1406	**Please quote this number on all correspondence**

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

Yours sincerely



Mr Chris Foy
Chair

Email: nrescommittee.southcentral-oxfordb@nhs.net

NHS
Health Research Authority
NRES Committee South Central - Oxford B
Whitefriars
Level 3, Block B
Lewin's Mead
Bristol
BS1 2NT

03 August 2015

Professor Stephen Gough
OCDEM
Churchill Hospital
Oxford
OX3 7LE

Dear Professor Gough

Study title:	**Urinary C-peptide:creatinine ratio as a marker of graft function in pancreas transplantation**
REC reference:	**15/SC/0413**
Protocol number:	**1.0**
IRAS project ID:	**183156**

Thank you for your letter of 30 July 2015, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

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 favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Assistant , Miss Lidia Gonzalez, and nrescommittee.southcentral-oxfordb@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

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.

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

Management permission ("R&D approval") should be sought from all NHS organisations

involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

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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).

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The favourable opinion applies to all NHS sites taking part in the study, 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

Approved documents

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

Document	*Version*	*Date*
Covering letter on headed paper [REC response letter]	1.0	30 July 2015
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [UCPCR Signed Sponsor Letter V1.0 19Jun 2015]	1.0	19 June 2015
GP/consultant information sheets or letters [UCPCR GP letter V1.0 19Jun15]	1.0	19 June 2015
Interview schedules or topic guides for participants [UCPCR Home Urine Instructions V1.0 19Jun2015]	1.0	19 June 2015
IRAS Checklist XML [Checklist_31072015]		31 July 2015
Letter from funder [UCPCR Research Services letter of funding V1.0 19Jun2015]	1.0	19 June 2015
Letter from sponsor [UCPCR Signed Sponsor Letter V1.0 19Jun 2015]	1.0	19 June 2015
Letters of invitation to participant [UCPCR Patient Invite Letter]	2.0	30 July 2015
Participant consent form [UCPCR Participant Consent Form]	2.0	30 July 2015
Participant information sheet (PIS) [UCPCR PIL]	2.0	30 July 2015
REC Application Form [REC_Form_25062015]		25 June 2015
REC Application Form [REC_Form_31072015]		31 July 2015
Referee's report or other scientific critique report [UCPCR Independent peer review form V1.0 19Jun2015]	1.0	19 June 2015
Research protocol or project proposal [UCPCR Protocol V1.0 19Jun2015]	2.0	30 July 2015
Summary CV for Chief Investigator (CI) [Stephen Gough Short CV V1.0 19Jun2015]	1.0	19 June 2015
Summary CV for student [Jame Barnes Short CV V1.0 19Jun2015]	1.0	19 June 2015
Summary CV for supervisor (student research) [Peter Friend Short CV V1.0 19Jun2015]	1.0	19 June 2015

Statement of compliance

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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/

15/SC/0413	**Please quote this number on all correspondence**

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

Yours sincerely

p.p.

Mr Chris Foy
Chair
Email:nrescommittee.southcentral-oxfordb@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Ms Heather House*

NHS
Health Research Authority
South West - Central Bristol Research Ethics Committee
Whitefriars
Level 3, Block B
Lewin's Mead
Bristol BS1 2NT
Email: nrescommittee.southwest-bristol@nhs.net

18 December 2015

Professor Peter Friend
Oxford Transplant Centre
Churchill Hospital
Oxford
OX3 7LE

Dear Professor Friend

Study title:	**Vascularised sentinel skin flaps to detect rejection in pancreas transplantation**
REC reference:	**15/SW/0333**
IRAS project ID:	**185583**

Thank you for your letter of 15th December 2015, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the alternate Vice-Chair.

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 the REC Manager, Mrs Nazneen Nathoo at nrescommittee.southwest-bristol@nhs.net.

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 NHS permission for research is available in the Integrated Research Application System, 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 contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

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 taking part in the study, 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).

Approved documents

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

Document	*Version*	*Date*
Covering letter on headed paper [REC Response Letter]	1	11 December 2015
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [SSF Sponsor Letter]		26 October 2015
GP/consultant information sheets or letters [SSF GP Letter]	1	26 October 2015
IRAS Checklist XML [Checklist_02112015]		02 November 2015
IRAS Checklist XML [Checklist_11122015]		11 December 2015
Letter from sponsor [SSF Sponsor Letter]		26 October 2015
Letters of invitation to participant [SSF Patient Invite Letter]	1	26 October 2015
Participant consent form [SSF Participant Consent Form]	2	11 December 2015
Participant information sheet (PIS) [SSF Patient Information Leaflet]	2	11 December 2015
REC Application Form [REC_Form_02112015]		02 November 2015
Referee's report or other scientific critique report [SSF Independent Peer Review Form]		08 October 2015
Research protocol or project proposal [SSF Protocol]	1.0	26 October 2015
Summary CV for Chief Investigator (CI) [Peter Friend Short CV]		08 September 2015
Summary CV for student [James Barnes Short CV]		08 September 2015
Summary CV for supervisor (student research) [Peter Friend Short CV]		08 September 2015

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/

15/SW/0333	**Please quote this number on all correspondence**

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

Yours sincerely

Mr Brian Pixton
Chair

Enclosures: "After ethical review – guidance for researchers" *[SL-AR2]*

Copy to: *Ms Heather House, R&D Department, Oxford University Hospitals NHS Trust, Churchill Hospital*

NHS
Blood and Transplant

West End Donor Centre
26 Margaret Street
London
W1W 8NB

Tel: 07525299064
Claire.Williment@nhsbt.nhs.uk

James Barnes
Nuffield Department of Surgical Sciences
Oxford Transplant Centre
Churchill Hospital
Oxford
OX3 7LE

Sent via e-mail to: james.barnes@nds.ox.ac.uk

29 January 2016

Dear Dr Barnes,

RE Study: Vascularised Sentinel Skin Flaps to Detect Rejection in Pancreas Transplantation

I am writing to thank you for contacting the Organ Donation and Transplantation Directorate of NHS Blood and Transplant regarding your above research proposal, wherein you request 20 skin flaps over a 12-month period. I would like to apologise the time it has taken for me to respond to this request and any inconvenience caused. This is due to a resource issue within our team, which has taken some time to address. I would like to reassure you that any further requests will be considered in a much more timely manner.

The Research, Innovation and Novel Technologies Executive Group have thoroughly reviewed your proposal and I am pleased to confirm that they have approved your application.

In order for us to enable your project to go live, we need to receive copies of the following documents:

- Research Ethics Committee approval
- Local Research and Development approval
- Local licence for recovering tissue for research purposes
- Signed copy of the attached Memorandum of Understanding

Please forward these, together with confirmation regarding the study's go live and completion dates, to the ODT Research mail box at LEESMA121@nhsbt.nhs.uk. Please note that if your go live date is beyond 6 months of receiving this letter, we will ask you to re-submit your application.

The Group also advised that you will need to liaise with Maggie Stevens (Specialist Nurse for Research and Service Delivery) to discuss the logistical elements of implementing your proposal, in particular the elements relating to:

- Transportation of tissues and reassurance that the study will not require additional or larger transport if travelling with NORS teams
- When you could be contacted regarding tissue for your study
- Liaison with SN-ODs for consent for tissue requested.
- Liaison with NORS Teams for tissue retrieval

Maggie will be in touch with you to discuss this in more detail. If in the meantime you wish to contact her, please e-mail her at margaret.stevens@nhsbt.nhs.uk.

If you have any queries then please feel free to contact me. I wish you well with your study.

Yours sincerely,



Claire Williment
Head of Transplant Development
RINTAG Secretariat

Copy to: Maggie Stevens (Specialist Nurse for Research and Service Delivery, NHS Blood and Transplant)

Appendix G - Clavien-Dindo classification of surgical complications

Grade I	Any deviation from the normal postoperative course without the need for pharmacological treatment or surgical, endoscopic, and radiological interventions. Allowed therapeutic regimens are: drugs such as antiemetics, antipyretics, analgetics, diuretics, electrolytes, and physiotherapy. This grade also includes wound infections opened at the bedside.
Grade II	Requiring pharmacological treatment with drugs other than allowed for grade I complications. Blood transfusions and total parenteral nutrition are also included.
Grade IIIa	Surgical, endoscopic, or radiological intervention that is not under general anesthesia
Grade IIIb	Surgical, endoscopic, or radiological intervention that is under general anesthesia
Grade IVa	Life-threatening complication requiring intermediate care or intensive care unit management, single organ dysfunction (including dialysis, brain hemorrhage, ischemic stroke, and subarrachnoidal bleeding)
Grade IVb	Life-threatening complication requiring intermediate care or intensive care unit management, multi-organ dysfunction (including dialysis)
Grade V	Death of a patient
Suffix "d"	If the patient suffers from a complication at the time of discharge, the suffix "d" (for "disability") is added to the respective grade of complication. This label indicates the need for a follow-up to fully evaluate the complication

Appendix H - Copyrights

Page	Type and name of work	Source of work	Copyright holder	Permission note
6 **7**	Fig 1.1: Pathophysiology of T1DM Fig 1.2: Staging of T1DM	Katsarou A. et al. Type 1 diabetes mellitus. Nature Reviews Disease Primers. 2017;3:17016	Springer Nature	License # 4560220707997 Granted on 01/04/19.
16	Fig 1.3: Long term survival on renal replacement therapy.	Byrne C. et al. K Renal Registry 20th Annual Report. Nephron. 2018;139(suppl1).	S. Karger AG, Berlin	Permission granted via email from Karger publishers on 10/04/19.
24	Fig 1.4: Yearly pancreas transplants over 20 years	Benjamens S. et al A steady decline in pancreas transplantation rates. Pancreatology. 2019;19(1):31-38.	Elsevier	License # 4565391269077 Granted 10/04/19.
24	Fig 1.5: Pancreas and islet transplants performed in the UK	Bunnett J, Counter C. Annual report on pancreas and islet transplantation. NHSBT;2018.		NHSBT Registry data. No copyright.
27	Fig 1.6: Schematics of pancreas transplantation.	Tolat P. et al. Pancreas Transplant Imaging: How I Do It. Radiology. 2015;275(1):14-27.	Medical College of Wisconsin	Permission granted via email by Prof Tolat on behalf of MCW on 19/04/19.
29	Fig 1.7: Types of beta cell replacement therapy.	Dean PG et al. Pancreas transplantation. BMJ. 2017;357:j1321.	BMJ Publishing Group	License # 4565990469967 Granted on 11/04/19
39	Fig 1.8: Pancreas allograft survival in the UK.	Hopkinson C. PAG - Pancreas Transplant Outcomes. NHSBT;2016		NHSBT Registry data. No copyright.
44	Fig 1.9: Temporal relationship between raised amylase…	Redfield R. et al. Diagnosis and Treatment of Pancreas Rejection. Current Transplantation Reports. 2015;2(2):169-175.	Springer Nature	Creative Commons CC BY license
69	Fig 3.1: The Homeostatic Model Assessment…	Hill N. et al. Expansion of the homeostasis model assessment of beta-cell function… Diabetes Care. 2013;36(8):2324-2330.	American Diabetes Association	License # 201474665 Granted 01/04/19