Metabolic outcomes of islet transplantation and correlation of graft function with hepatic imaging and immune response

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ABSTRACT

Introduction
Recipients of an islet transplant at all UK NHS-funded centres were invited to take part in an ethically approved experimental medicine follow-up study. Objectives were to determine metabolic outcomes post-intervention and identify potential surrogate markers for graft failure.

Methods
Assessment of islet recipients was undertaken pre-transplant, 1 month post-transplant and 3-6 monthly thereafter. Clinical review included HbA1c, hypoglycaemia frequency, insulin requirements, donor specific antibody (DSA) screening, magnetic resonance imaging (MRI) for hepatic fat assessment and continuous glucose monitoring (CGM) one week prior to standardised meal tolerance test (MTT).

Results
Twenty recipients received 35 islet infusions (single graft:n=7; two:n=11; three:n=2; transplant mass per recipient (median[IQR]) 8770(6536-13045) IEQ/kg), with all recipients receiving induction and maintenance immunosuppression. Graft function was maintained in 80% of recipients at 24(13.5-36) months. Frequency of severe hypoglycaemia reduced from 20(7-50) to 0.3(0-1.6) episodes per patient-year (p<0.001), with improved hypoglycaemia awareness (Gold score: pre-transplant 6(5-7); post-transplant 3(1.5-4.5); p<0.03) and HbA1c 6.2(5.7-8.4)% post-transplant, and cessation of exogenous insulin in 45% of recipients. In a single site sub-study, CGM demonstrated a continuous relationship between stimulated C-peptide and glucose variability, hyperglycaemia risk and hypoglycaemia risk. No significant difference in liver fat fraction was noted pre and post-transplant (p=0.94), and no direct correlation with 90 minute C-peptide on MTT at 12 months was identified. DSA was detected in 5/23(21.7%) grafts in 14 recipients and was associated with rapid graft failure.

Conclusions
The primary goals of the UK islet transplant program to prevent recurrent life-threatening hypoglycaemia and restore optimal glycaemic control in recipients have been attained up to 36 months post-transplant. Demonstration of a continuous relationship between endogenous C-peptide secretory capacity and parameters of glycaemic control has substantial implications for therapeutic interventions maintaining endogenous insulin production in individuals with type 1 diabetes. Therapeutic interventions preventing DSA formation post-transplant might improve graft outcomes.
DECLARATION

I declare that the work presented in this thesis is my own with the exception of the mixed Poisson regression analysis work on continuous glucose monitoring outcomes in islet recipients undertaken in Chapter Four. My initial work using non-parametric statistical tests suggested a continuous relationship between endogenous C-peptide production and continuous glucose monitoring outcomes in islet recipients but I wanted to explore the relationship in greater depth. I therefore requested assistance from the Newcastle University Statistics Department and Dr Nick Steen’s analysis produced a mixed Poisson regression model that confirmed a continuous relationship between endogenous C-peptide production and outcome measures of blood glucose control. This work was used to create Figure 4.5, and enabled the work to achieve publication in Diabetes Care.

All sources of information have been referenced. This work has not been presented for any other degree.

Signed: Augustin Brooks

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DEDICATIONS

I would like to dedicate this thesis to my wife Eileen, for her unfailing support throughout this work, and to my son Benedict who was born during my time undertaking research at Newcastle University – both have been a magnificent source of inspiration for the completion of this thesis.

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ABBREVIATIONS

ADRR - Average daily risk ratio
AIRE - Autoimmune regulator
Alk P – Alkaline phosphatase
ALT - Alanine transaminase
AR\textsubscript{max} - maximal acute secretory response
AST - Arginine stimulation test
ATG - Anti-thymocyte globulin
B cell - B cell receptor carrying lymphocyte of the humoral immune system
\( \beta \) cell - Pancreatic insulin-producing beta cell
BMI - Body Mass Index
CD - Cluster of differentiation glycoprotein
CD4\textsuperscript{+} T cell - Thymus matured lymphocyte expressing cluster of differentiation 4 glycoprotein (‘Helper’)
CD8\textsuperscript{+} T cell - Thymus matured lymphocyte expressing cluster of differentiation 8 glycoprotein (‘Cytotoxic’)
CDC - Complement Dependent Cytotoxic
CGM - Continuous glucose monitoring
CI - Confidence Interval
CITR - Clinical Islet Transplantation Registry
CSII - Continuous subcutaneous insulin infusion
CP/G index - C-peptide/glucose index
CT - Computed tomography
CTLA-4 - Cytotoxic T-lymphocyte antigen
DAFNE – Diabetes Adjustment For Normal Eating
DBD - Donors after brain death
DCCT - Diabetes Care and Complications Trial
DCD - Donors after circulatory death
DIR - Daily insulin requirement
DSA - Donor specific antibody
dsRNA - Double-stranded ribonucleic acid
DTT - Dithiothreitol
ELISA – Enzyme-linked immunosorbent assay
FITC - Fluorescein isothiocyanate
GAD - Glutamic acid decarboxylase
GCSF - Granulocyte colony stimulating factor
GFR - Glomerular filtration rate
GP AST - Glucose potentiated arginine stimulation tests
GST - Glucagon stimulation test
HBGI - High blood glucose index
HDL - High density lipoprotein
HLA - Human leukocyte antigen
HYPO Score - Composite hypoglycaemia score
IA-2A - Insulinoma-associated protein 2 antigen
IAA - Insulin autoantibody
IAK - Islet after kidney
IBMIR - Instant blood mediated inflammatory reaction
ICA - Islet cell antibody
IEF - Islet estimated function
IEQ - Islet equivalents
IF1H1 - Interferon-induced helicase 1
IFCC - International Federation of Clinical Chemistry
IFNα - Interferon alpha
IFNγ - Interferon gamma
Ig - Immunoglobulin
IL - Interleukin
IL2Rα - Interleukin-2 receptor α
IPEX - Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IQR - Interquartile range
ITA - Islet transplant alone
IU - International units
IV GTT - Intravenous glucose tolerance test
LBGI - Low blood glucose index
LDL - Low density lipoprotein
LYP - Lymphoid protein tyrosine phosphatase
MAD - Mean absolute difference
MCP-1 – Monocyte chemoattractant protein-1
MDI - Multiple daily insulin injections
MFI - Median Fluorescence Intensity
MHC - Major Histocompatibility Complex
MIF – Macrophage migration inhibitory factor
MODY - Maturity onset diabetes of the young
MR - Magnetic resonance
MRI - Magnetic resonance imaging
mRNA - Messenger ribonucleic acid
mTOR - Mammalian target of rapamycin
MTT - Meal tolerance test
MTT90 C-peptide - Stimulated C-peptide at 90 minutes into a meal tolerance test
NAFLD - Non-alcoholic fatty liver disease
NGSP - National glycohaemoglobin standardisation program
NHS - National Health Service
NICE - National Institute for Health and Clinical Excellence
NOD - Non-obese diabetic
ODT - Organ Donation and Transplantation
OGTT - Oral glucose tolerance test
PAK - Pancreas-after kidney
PCR - Polymerase chain reaction
PTA - Pancreas-transplant alone
ROI - Region of interest
S/C - Subcutaneous
SD - Standard deviation
SMBG - Self-monitoring blood glucose
SPK - Simultaneous pancreas-kidney transplant
SUITO - Secretory unit of islet transplant objects index
T1D - Type 1 diabetes
T cell - Thymus matured lymphocyte of the cellular immune system
TEF - Transplant estimated function
TF - Tissue Factor
TNFα - Tumour necrosis factor alpha
TRIGR - Trial to Reduce IDDM in the Genetically at Risk
UK - United Kingdom
UNOS - United Network for Organ Sharing
USA - United States of America
VNTR - Variable number of tandem repeat
ZnT8 - Zinc transporter 8
CHAPTER ONE

INTRODUCTION
Chapter 1 - Introduction

1.1 Diabetes: definition, classification and aetiology

1.1.1 Definition of diabetes
Diabetes mellitus is a heterogeneous group of disorders characterised by an increase in plasma glucose (Alberti and Zimmet, 1998). Its causation is complex and due to an interaction of genetic and environmental factors. Hyperglycaemia can occur as a consequence of a relative or absolute deficiency of endogenous insulin production. Persistent hyperglycaemia can cause serious health complications, including ketoacidosis in the short-term and increased risk of blindness, kidney failure, neuropathy (The Diabetes Control and Complications Trial Research Group (1993)), heart disease, stroke and peripheral vascular disease in the longer-term (Zoungas et al., 2012).

1.1.2 Classification of diabetes
The two most common forms of diabetes are type 1 and type 2 diabetes. Type 2 diabetes occurs as a consequence of insulin resistance, where the muscles and adipose cells do not respond adequately to insulin produced by intact β cells. By contrast, type 1 diabetes is a chronic autoimmune disorder that is triggered by environmental factors in genetically susceptible individuals, producing a state of insulin deficiency that results in hyperglycaemia and a greater predisposition to ketone formation.

Major recent advances in the aetiology of diabetes have allowed further sub-classification. Genetic analysis has unveiled monogenic forms of diabetes, and perhaps the most prominent example of this is the group of diabetes disorders classified as Maturity Onset Diabetes of the Young (MODY) (Hattersley, 1998). In addition, gestational diabetes can occur during pregnancy due to the tendency of the human body to become more insulin resistant whilst in the pregnant state (Catalano, 2010, International Association of et al., 2010).

1.1.3 Prevalence and incidence of diabetes
The American Centre for Disease Control states that 7.8% of the United States population (23.6 million people) have a diagnosis of diabetes. In the US population, 1.6 million new cases of diabetes were diagnosed in people aged 20 years or above in 2007.
The prevalence of type 1 diabetes for residents of the United States aged 19 years or less is 1.7/1000. The incidence of type 1 diabetes has been rising globally over recent decades. The number of new cases of type 1 diabetes in European children under the age of 5 years is expected to double between 2005 and 2020, whilst the prevalence of cases in individuals younger than 15 years will rise by 70% (Patterson et al., 2009).

1.1.4 Aetiology of type 1 diabetes

The aetiology of type 1 diabetes remains unclear. It is apparent that there is a genetic predisposition to the disorder (Todd and Farrall, 1996), although studies in identical twins demonstrate a concordance rate of less than 50% (Petersen et al., 1997), suggesting that environmental factors play a crucial role in its causation.

Genetic factors

Monogenic forms of type 1 diabetes

Autoimmune diabetes is only rarely caused by mutational defects in a single gene but examples illustrate how genetic constitution might predispose an individual to diabetes through defects in immune tolerance mechanisms.

Monogenic forms of type 1 diabetes are typically accompanied by other autoimmune conditions. The IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) occurs as a consequence of mutations in the Foxp3 transcription factor, which leads to dysfunction of regulatory T cells and multi-organ autoimmunity (Wildin et al., 2001); 80% of affected children develop diabetes. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy occurs as a result of mutations in an autoimmune regulator (AIRE). Deficiencies in AIRE inhibit expression of insulin in the thymus, allowing autoreactive T cells to enter the peripheral circulation: 20% of affected individuals develop autoimmune diabetes (Villasenor et al., 2005).

Human Leukocyte Antigen associations with type 1 diabetes

Type 1 diabetes has strong genetic links to the human leukocyte antigen (HLA) class II genes on chromosome 6p21 (IDDM1 locus) (Nerup et al., 1974). Molecular cloning and sequencing of HLA genes and class II proteins have made it possible to study genetic loci that may be associated with type 1 diabetes. HLA-DQB1*0302-A1*0301 has a greater association than any of the other HLA-DRB1*04 subtypes (Undlien et al.,
DRB1*0401 confers a further additive risk (Kockum et al., 1996). By contrast, the HLA-DRB1*1501-DQA1*0102-DQB1*0602 haplotype – found in 20% of the general population but only 1% of individuals with type 1 diabetes – confers dominant protection against type 1 diabetes (Erlich et al., 2008). The presence of HLA class II proteins associated with diabetes might promote antigen presentations that generate T-helper cells responsible for an immune response to specific islet cell autoantigens. This in turn could lead to the formation of cytotoxic T cells which destroy insulin-producing β cells in islets of Langerhans, and the formation of islet autoantibodies.

A less prominent association with type 1 diabetes has been found for HLA class I alleles (Fennessy et al., 1994). HLA-B*39 allele is a significant risk factor for type 1 diabetes, whilst HLA-A*02 increases the risk in individuals possessing the high-risk class II DR3/4-DQ8 haplotype. HLA-A*0201 is one of the most prevalent class I alleles, with a frequency of greater than 60% in individuals with type 1 diabetes.

Other candidate genes for type 1 diabetes

A recent Genome Wide Association Study focusing on type 1 diabetes found over 40 different loci that affect the risk for type 1 diabetes (Cooper et al., 2008).

A genetic predisposition to type 1 diabetes is conferred by the insulin gene region on chromosome 11 (IDDM2) (Bell et al., 1984). Susceptibility to diabetes lies in the variable number of tandem repeat (VNTR) polymorphisms in the promoter region of the insulin gene: VNTR type I alleles of the gene (26-63 repeats) predispose individuals to diabetes in a recessive type fashion; VNTR class III alleles (140 to more than 200 repeats) appear to be dominantly protective. The protective effect may be explained by regulation of proinsulin mRNA in the thymus of class III allele carriers, which may enhance immune tolerance to preproinsulin, a key autoantigen in diabetes pathogenesis (Pugliese et al., 1997).

A linkage to the cytotoxic T lymphocyte antigen (CTLA-4) gene on chromosome 2 (IDDM12) is not fully understood (Nistico et al., 1996). CTLA-4 is critical to T cell apoptosis, and it has been speculated that long AT repeats in the encoding of the gene may lead to the CTLA-4 protein not being properly formed; this might result in increased survival of T cells responsible for β cell destruction.
Interferon-induced helicase 1 (IFIH1) encodes a helicase that contributes to the recognition of dsRNA from picornaviruses; it therefore acts as a cytoplasmic sensor for viral infection. A genetic defect encoding the helicase might interfere with the detection and clearance of viral infections, and this in turn might predispose an individual to a diabetogenic response. The presence of type 1 diabetes-associated polymorphisms has been reported in individuals with susceptible genotypes (Liu et al., 2009), and rare protective IFIH1 variants in type 1 diabetes have also been identified (Nejentsev et al., 2009).

Allelic variation in the interleukin-2 receptor α gene (IL2Rα) region has been implicated in type 1 diabetes causation (Lowe et al., 2007), with lower levels of IL2Rα possibly accounting for functional defects in regulatory T cells.

The PTPN22 gene encodes lymphoid protein tyrosine phosphatase (LYP), and it is an important negative regulator of T cell receptor signalling. Functional variants of PTPN22 have been associated with type 1 diabetes (Bottini et al., 2004).

**Environmental factors**

**Viral associations with type 1 diabetes**

The clinical manifestation of type 1 diabetes is a consequence of an underlying, sustained autoimmune process. A combination between susceptibility genes and environmental factors may initiate an autoimmune response that affects insulin-producing β cells. The wide gap between the initiation of autoimmune damage and clinical onset of symptoms has posed a problem in looking for environmental triggers, of which several may be required before an autoimmune process against the β cells is invoked. Lymphocytic infiltrates in pancreatic islets are a hallmark feature of type 1 diabetes (Gepts, 1965), and abundant levels of HLA class I and IFN-α in the islets of children with recent-onset diabetes suggested that beta-cell tropic infection might upregulate both HLA class I and IFN-α to leave a molecular ‘viral signature’ in individuals with type 1 diabetes (Foulis et al., 1987).

The seasonal variation in diabetes was first documented soon after the discovery of insulin (Adams, 1926), suggesting that viral infections may underpin causation of diabetes. Cytomegalovirus (Pak et al., 1988), parvovirus (Kasuga et al., 1996) and
encephalomyocarditis virus (Craighead and McLane, 1968) have all been associated with type 1 diabetes causation, although no direct evidence to diabetes causation has been identified.

Extensive circumstantial evidence has suggested that enteroviruses – especially coxsackieviruses – may precipitate type 1 diabetes. Neutralising antibody titres in the serum of individuals recently diagnosed with type 1 diabetes were found to be higher than in healthy controls (Gamble et al., 1969), and this was confirmed in later studies using polymerase chain reaction (PCR) detection (Clements et al., 1995). Prospective studies within the Finnish population have shown an association between enterovirus infection and diabetes development (Lonnrot et al., 2000). Coxsackie virus has striking protein sequence similarities to GAD, and this may contribute to molecular mimicry that is associated with HLA-DR3 susceptibility (Kaufman et al., 1992).

Congenital rubella has been strongly associated with type 1 diabetes onset in affected children (Forrest et al., 1971, McIntosh and Menser, 1992) but any direct evidence for autoimmunity is scarce, and it may be that the virus interferes with β cell mass development rather than initiating the autoimmune process. In addition, rubella has largely been eliminated from the developed world by vaccination programmes, and yet the incidence of type 1 diabetes continues to increase. Multiple large studies have found no evidence linking vaccination programmes to the increased incidence of type 1 diabetes (Hviid et al., 2004).

Despite extensive research, no direct evidence for a particular viral strain causing type 1 diabetes has been identified. Some indirect evidence warranting a focus on bacterial agents – most notably mycobacterium avium (Sechi et al., 2008) – has been proposed but no direct link to diabetes causation has been found.

**Other environmental associations with type 1 diabetes**

**Cow’s milk**

The albumin content of cow’s milk has been proposed as a trigger for islet autoimmunity, as a cross-reactivity was found between serum antibodies from individuals with type 1 diabetes and antibodies against the beta cell surface protein ICA-1 (Karjalainen et al., 1992). However, subsequent reports have been unable to demonstrate any causal evidence (Norris et al., 1996).
Wheat gluten
Increased peripheral blood T cell reactivity to wheat gluten was more frequently found in individuals with type 1 diabetes compared to controls (Klemetti et al., 1998) but no robust evidence to gluten initiating autoimmunity in diabetes has been found (Hummel et al., 2000).

Vitamin D
Vitamin D effectively inhibits dendritic cell differentiation and immune activation, and increased vitamin D intake has been reported to reduce the incidence of type 1 diabetes (Mathieu et al., 2005). Absence of vitamin D in childhood may contribute to poor expression of DRB1*0301 in the thymus, which has been associated with an increased risk of diabetes (Israni et al., 2009); vitamin D may therefore have an important role in inducing self-tolerance and protect against autoimmunity.
Figure 1.1 Aetiology and pathogenesis of type 1 diabetes

**Genetic factors**
- 40+ loci affecting risk (Genome Wide Association Study)
  - HLA associations (IDDM1)
  - VNTR insulin gene polymorphisms (IDDM2)
  - Monogenic disorders (IPEX, AIRE)
  - CTLA-4 gene long AT repeats

**Environmental triggers**
- **Viral associations**
  - Enteroviruses (esp coxsackie)
  - Congenital rubella
  - Cytomegalovirus
  - Parvovirus
  - Encephalomyocarditis virus
- **Bacterial agents**
  - Mycobacterium avium
- **Other associations**
  - Cow’s milk albumin
  - Wheat gluten
  - Vitamin D deficiency

**Autoimmune system disorder**
Consequence of a cytotoxic T cell response:
- Interferon-induced helicase 1 (IFIH1)
  - Genetic defect interferes with viral clearance
  - Predisposes to diabetogenic response
- Allelic variation in IL2Rα gene
  - Low levels of sIL2Rα
  - Functional defects in regulatory T cells
- PTPN22 gene encodes LTP tyrosine phosphatase
  - Negative regulator of T-cell receptor signalling
  - Functional variants assoc’ with T1DM

**Destruction of insulin-producing β cells:**
- β cell autoantibody formation (IAA, GAD65, IA-2, ICA, ZnT8)
- CD4 T helper cytokine production
- CD8 cytotoxic T cell destruction
β cell destruction leads to a linear decay in β cell mass

**Clinical onset of type 1 diabetes**
Reduction and eventual absence of endogenous insulin production results in:
- Hyperglycaemia
- Osmotic symptoms & dehydration
- Ketone formation & acidosis
1.1.5 Pathogenesis of type 1 diabetes

The β cell decline hypothesis remains a widely referenced benchmark model for type 1 diabetes (Eisenbarth, 1986). This model proposes that genetically susceptible individuals encounter an environmental trigger that initiates an autoimmune process, which subsequently leads to autoantibody formation and a linear decay in β cell mass. This is followed by hyperglycaemia and eventually a complete absence of endogenous C-peptide.

Antibodies formed against prominent antigens in the pancreatic β cell are important markers of β cell autoimmunity in type 1 diabetes. The most significant are autoantibodies to insulin (micro IAA), glutamic acid decarboxylase (GAD65), insulinoma-associated antigen-2 (IA-2A) (Verge et al., 1998) and zinc transporter 8 (ZnT8) (Wenzlau et al., 2007). Radioligand binding assays to these antibodies demonstrate high diagnostic sensitivity and specificity for type 1 diabetes, with many laboratories achieving a sensitivity of up to 80%.

Autoantibody formation often precedes onset of hyperglycaemia and osmotic symptoms in type 1 diabetes. The early presence of autoantibodies clearly implicates a role for antibody-producing plasma B cells in the causation of type 1 diabetes, and they are likely to be active participants in the immune response because of their capacity to present antigen to other cells of the immune system. However, T cells are considered to be the final executors of β cell destruction. Cytotoxic CD8+ T cells have been found to be prominent in areas of insulitis in non-obese diabetic (NOD) mice, and can undertake cell-mediated β cell destruction upon activation by MHC class I expression on β cells. A deficiency in MHC class I expression due to a lack of beta-2 microglobulin has been shown to be sufficient to arrest diabetes development in NOD mice (Hamilton-Williams et al., 2003). CD4+ T helper cells are also likely to play an important role in the autoimmune process through cytokine production that assists the CD8+ T cell destruction of β cells.

Studies have attempted to determine the predictive value of autoantibody presence in the later development of diabetes in healthy members of the population. The number of autoantibodies rather than the specificity of the autoantibody is thought to be most predictive of progression to overt diabetes. Whilst the presence of one autoantibody
alone may not be predictive of disease, almost all individuals expressing multiple diabetes associated autoantibodies progress to diabetes with long-term follow-up (Bingley et al., 1997, Achenbach et al., 2005). Relatives of patients with type 1 diabetes who are positive for two or more autoantibodies have a greater than 70% risk of developing diabetes over a seven year observation period (Sherr et al., 2008).
Figure 1.2 – Graphical illustration of the β cell decline hypothesis
1.2 Current treatments for type 1 diabetes

1.2.1 Insulin treatment for type 1 diabetes

The discovery of exogenous insulin therapy was a monumental achievement, transforming type 1 diabetes from a terminal disease to a manageable medical condition (Banting et al., 1922). However, although the previously fatal complication of diabetic ketoacidosis can now be readily treated, existing insulin formulations are unable to mimic the natural regulatory ability of the $\beta$ cells of the endocrine pancreas. The Diabetes Control and Complications Trial (DCCT) confirmed that tight blood glucose control can reduce the incidence of complications (The Diabetes Control and Complications Trial Research Group (1993)) but regulation of blood glucose levels within the normal physiological range remains a challenge in even the most motivated patients. Nearly all individuals with type 1 diabetes remain prone to microvascular and macrovascular complications in the longer term (Genuth, 2006, Aronson, 2008).

1.2.2 Burden of hypoglycaemia with insulin treatment in type 1 diabetes

Hypoglycaemia is a potentially lethal side-effect of insulin treatment in patients who have labile blood glucose control or hypoglycaemia associated autonomic failure (Cryer et al., 2003). Repeated hypoglycaemia can result in impaired awareness of low blood glucose levels, leading to individuals being unable to take appropriate action when their blood glucose levels are low (Figure 1.3). Hypoglycaemia unawareness has been reported to affect 20-30% of unselected individuals with type 1 diabetes, and it is associated with an increased risk of severe hypoglycaemia requiring third party assistance (Clarke et al., 1995, Frier, 2008). One episode has potential implications regarding lifestyle choices and driving. Ultimately it can be truly life-threatening, as it potentially causes an inability to awaken from sleep when blood glucose levels are low, increasing the risk of ‘dead-in-bed’ syndrome and sudden death (Gill et al., 2009). Up to 10% of the mortality in patients with type 1 diabetes may be as a consequence of hypoglycaemia unawareness (Cryer, 2008). Many patients with insulin-dependent type 1 diabetes have some degree of hypoglycaemia unawareness, and as a consequence the benefits of stringent glycaemic control may be outweighed by the risk of a potentially fatal insulin overdose (Cryer, 2005). The frequency of hypoglycaemia unawareness has not declined in the past two decades (McCrimmon and Sherwin)(2010), and despite major advances in conventional insulin therapy (including structured education, insulin analogues, continuous subcutaneous insulin infusion pump therapy, continuous glucose
monitoring and even steps towards the closed loop bioartificial pancreas), there remains a group of patients in whom recurrent severe hypoglycaemia cannot be prevented without relaxation of overall glycaemic control. All adults with type 1 diabetes should be assessed for impaired awareness of hypoglycaemia, and this should take place at the individual’s annual care review.
Figure 1.3 – Vicious cycle of recurrent hypoglycaemia

- Individual with type 1 diabetes experiences episodes of recurrent hypoglycaemia
- Blunting of autonomic response to hypoglycaemia makes individual less capable of responding to autonomic warning signs of hypoglycaemia
  - Autonomic warning signs start to be lost when hypoglycaemia starts to occur at <3.2 mmol/L
- Individual now more prone to episodes of severe hypoglycaemia, which can lead to loss of consciousness & seizures
  - Patient often dependent on 3rd party help to overcome hypoglycaemia episode
- Increased risk of confusion during hypoglycaemia episode, with risk that individual less able to respond to hypoglycaemia, & unable to self-manage hypoglycaemia
  - Individual prone to confusion when hypoglycaemia <2.8 mmol/L
**1.2.3 Alternative approaches to treatment of type 1 diabetes**

Increased knowledge of the genetic susceptibility and ability to predict who might be at risk of developing type 1 diabetes has allowed clinicians to target interventions for diabetes management at three different stages during the pathogenesis of diabetes. Primary prevention aims to administer treatment to high risk individuals prior to their development of autoimmunity; secondary interventions might be administered when the development of autoimmunity is recognised; whilst tertiary interventions might be used soon after the clinical manifestations of diabetes become apparent when a significant number of \(\beta\) cells remain (Wherrett and Daneman, 2009). Research is therefore currently looking at alternative approaches to insulin management, and efforts have recently focused on immunosuppression trials in diabetes at the time of or shortly after diagnosis.

**1.2.4 Primary prevention trials for type 1 diabetes**

The TRIGR study (Trial to Reduce IDDM in the Genetically at Risk) is testing whether substitute milk products given whenever breast milk is not available in the first 6-8 months of life decreases the risk of developing type 1 diabetes in genetically susceptible children (a first degree relative with type 1 diabetes and a high risk HLA allele) (Akerblom et al., 2005, Knip et al.). The trial is a double-blind, randomised control trial comparing hydrolysed infant formula to a cow’s milk-based formula. Early outcome results have shown that children receiving the extensively hydrolysed casein-based formula have less autoantibody positivity compared to those receiving the cow’s milk-based formula (17% versus 30%), although to date there has been no impact on the incidence of type 1 diabetes onset; the trial is ongoing and due for completion in February 2017.

The Diabetes Prevention Trial for type 1 diabetes investigated whether administration of oral or subcutaneous insulin prevented the onset of type 1 diabetes in genetically predisposed individuals (Pozzilli, 2002, Skyler et al., 2005, Barker et al., 2007). No impact on the incidence of type 1 diabetes was shown with either intervention by the trial. However, a post-hoc analysis of oral insulin administration demonstrated a 4-year delay in disease onset in subjects with higher titres of insulin autoantibodies. Further randomised trials in high-risk individuals are currently underway.
Intranasal insulin administration has also been tested as a preventative therapy in at risk individuals: a double-blind, placebo-controlled trial did not demonstrate a beneficial effect, even when intranasal insulin was administered soon after autoantibody seroconversion (Nanto-Salonen et al., 2008).

1.2.5 Secondary and tertiary intervention trials in type 1 diabetes

Secondary and tertiary intervention trials have the advantage over prevention trials in that potential trial entrants are more readily identified and efficacy can be evaluated within a much shorter timeframe. Secondary intervention involves screening for genetic, autoantibody and other markers associated with a risk of developing diabetes at birth, in school children or in adults. These interventions can be non-antigen specific - where the intervention used aims to modulate the immune response, preferably without negative effects on T regulatory cells - or aimed at a particular antigen that might be considered a target for halting the progression to the clinical onset of diabetes.

Non-antigen specific trials

The first non-antigen specific, double-blind, placebo-controlled clinical trial for type 1 diabetes was with the calcineurin inhibitor cyclosporine A, which prevents interleukin-2 (IL-2) transcription and reduces the function of effector T cells. During the trial, cyclosporine was administered to individuals with a clinical diagnosis of type 1 diabetes within 2 months of the initiation of insulin therapy. Cyclosporine induced remission of type 1 diabetes and insulin independence was achieved. However, its chronic use led to unacceptable side-effects – most notably nephrotoxicity – and its therapeutic effect vanished with cessation of the treatment (Stiller et al., 1983, Jenner et al., 1992). This demonstrated that cyclosporine did not therefore induce immune tolerance or immunoregulation, merely a state of non-specific immunosuppression.

Other non-antigen specific approaches have been attempted with ketotifen (Bohmer et al., 1994) and nicotinamide (Gale et al., 2004) but each intervention has failed to improve clinical outcomes in type 1 diabetes long-term.

Antigen specific trials

The ultimate aim of antigen specific therapies is to promote regulatory T cell responses (active tolerance) or anergy of pathogenic T cells (passive tolerance) in order to induce
tolerance against multiple autoreactivities, thereby preventing an autoimmune attack on β cells. The selected antigen should be able to induce ‘bystander suppression’ by activating T regulatory cells to secrete cytokines that can dampen down immune function and modulate the response of antigen presenting cells (Tang and Bluestone, 2008).

**Trials to induce tolerance against GAD 65**

Treatment of spontaneously non-obese diabetic (NOD) mice with GAD peptide reduced the incidence of diabetes onset (Tian et al., 1996). An aluminium hydroxide formulation of full length recombinant human GAD65 (GAD65-Alum) was subsequently developed and shown to preserve residual insulin secretion in subjects with late-onset autoimmune diabetes (Agardh et al., 2005). However, in phase III trials, GAD65-Alum failed to alter the natural history of C-peptide loss in individuals recently diagnosed with type 1 diabetes (Wherrett et al., 2011).

**Trials using anti-CD3 monoclonal antibodies**

Anti-CD3 monoclonal antibodies cause short-term internalisation of the T cell receptor-CD3 complex that makes cells ‘blind’ to antigens (Chatenoud et al., 1994). It alters T cell receptor-mediated signal transduction so that anergy or apoptosis is preferentially induced in activated T helper cells. Anti-CD3 treatment also results in regulatory T cell development (You et al., 2007), which protects against damage from effector T cells long after the drug has been eliminated from the body.

Oxelizumab (Europe) and Teplizumab (US) are humanised anti-CD3 monoclonal antibodies that were used in open-label trials and showed that C-peptide production could be maintained for a longer duration in comparison to placebo in patients with newly diagnosed type 1 diabetes, and drug-treated subjects used less insulin while maintaining good glycaemic control (Herold et al., 2002, Keymeulen et al., 2010). However, drug recipients also experienced the side-effects of ‘cytokine storms’ and reactivation of Epstein-Barr virus. In an effort to prevent these side-effects occurring, later trials used lower doses of the anti-CD3 agents, with insulin dose combined with HbA1c improvement selected as the primary end-point over C-peptide production. No significant effect from anti-CD3 monoclonal antibody therapy was achieved when compared to placebo in the subsequent trials (Sherry et al., 2011).
Other trials using antigen–specific immunosuppressant therapies

Rituximab is a CD20-specific monoclonal antibody that is used to deplete the antigen-presenting B cell population (Hu et al., 2007). In a double-blind, placebo-controlled phase II trial, individuals receiving rituximab soon after a clinical diagnosis of type 1 diabetes had a higher C-peptide at 1 year compared to placebo, with lower insulin requirements and better glycaemic control (Pescovitz et al., 2009). However, review of the risk-benefit ratio associated with the treatment meant that the clinical team leading the trial felt unable to recommend rituximab for clinical use in the management of type 1 diabetes.

Abatacept (a CTLA-4 Immunoglobulin) interferes with the stimulation of T cells, resulting in limited clonal expansion and induction of passive cell death (Salomon and Bluestone, 2001). In a phase II trial, subjects receiving monthly infusions of the drug had a significantly greater C-peptide level than those receiving placebo but reports also suggest that the effect is not sustained, and that a decline in C-peptide parallel to that of the placebo group occurs after 9 months (Orban et al., 2011).

Combination immunotherapy

Cancer therapy has benefitted from the use of combination therapies to treat malignancies, and a similar approach has been adopted in some immunotherapy trials aimed at reversal of type 1 diabetes. However, due to the possible side-effects of the drugs used, substantial regulatory and logistical hurdles exist for this approach in clinical practice, and extensive preclinical toxicity studies are needed before such regimens can be implemented in clinical trials.

Basiliximab (chimeric mouse-human) and daclizumab (humanised IgG) are anti-CD25 monoclonal antibodies that do not cause cytokine release syndrome and have been used in combination therapy regimens aimed at maintaining endogenous C-peptide production in patients with newly diagnosed type 1 diabetes. However, in a double blind, placebo-controlled trial, daclizumab in combination with mycophenolate mofetil failed to preserve β cell function 1 year post-therapy (Gottlieb et al., 2010).
Re-evaluation of the immunotherapy approach

Immunotherapy used at the point of diagnosis of type 1 diabetes has not yet delivered convincing data to be considered for routine clinical use. Some research groups have suggested that better outcomes might be achieved if immune interventions are used as a secondary intervention in those at high risk of developing diabetes, in the hope that immunotherapy can be used to halt the pathogenesis of the autoimmune process that results in diabetes clinically (Greenbaum et al., 2012, Staeva et al., 2013). However, this is likely to be a high-risk approach and the risk-benefit ratio is such that immune interventions have not yet provided sufficient evidence for their routine use as a secondary intervention to be warranted at the current time. More information is required regarding the long-term effects of the immunosuppressants used: high-dose / high-risk immunosuppressive treatments may have untoward effects, whilst low-dose / low-risk therapies may simply be ineffective. Given that in ideal cases, life expectancy might be only shortened by 10 years with exogenous insulin therapy, a high safety bar has been set for immune-based interventions, and they must achieve long-lasting outcomes with minimal side-effects to replace insulin therapy.

1.2.6 The demand to know benefits of maintained endogenous C-peptide

What has arisen from many of the immunosuppression trials is that the potential clinical benefits of maintained endogenous insulin production - as reflected by measurements of C-peptide – are not yet fully known. The benefits of endogenous insulin production were confirmed in the DCCT, in which the intensively treated group could be subdivided according to residual C-peptide secretion at entry into the trial: the subgroup with the highest stimulated C-peptide (>0.2 pmol/ml) had a significantly lower HbA1c over the first four years of the study, 50% less risk of progression of retinopathy and 65% less risk for severe hypoglycaemia with seizure or coma (The Diabetes Control and Complications Trial Research Group (1998)).

Beyond outcomes from the DCCT, the evidence base for the advantages of maintained C-peptide production is scarce. It has been suggested that individuals with long-standing type 1 diabetes may be protected from vascular complications if endogenous insulin production is maintained (Sjoberg et al., 1987, Panero et al., 2009). These clinical findings have led to the development of a ‘legacy effect’ concept, that suggest that maintenance of endogenous insulin production might offer protection from vascular complications in the later life of a patient with type 1 diabetes.
Many authors have subsequently called on research to define these benefits in greater detail, as expressed in the following quotations selected from published literature:

“As yet there is no formal proof that long term maintenance of C-peptide responses will decrease morbidity and mortality but the indirect evidence makes this a justifiable inference. One current limitation is that we do not know what constitutes a ‘protective’ residual C-peptide response.”

(Kolb and Gale, 2001)

‘Does partial preservation of residual beta-cell function justify immune intervention in recent onset Type 1 diabetes?’

Diabetologia (2001);44:1349-1353.

“. . . the degree and duration of endogenous C-peptide likely to provide a minimum or maximum benefit is unknown. Thus, parallel with clinical trials to test the therapeutic effects on β cell function, studies evaluating the relationship of C-peptide secretion . . . whether the clinical course is easier to manage in those with residual β cell function are needed.”

(Greenbaum et al., 2012)

‘Through the Fog: Recent Clinical Trials to Preserve β-Cell Function in Type 1 Diabetes’

Diabetes (2012);61:1323-1330

“In the long-term, it will be important to correlate preservation of C-peptide with improved clinical outcomes, such as short-term and long-term insulin dose requirements, HbA1c level, glycaemic variability and time in range, frequency of hypoglycaemia, and decreased occurrence of microvascular and macrovascular complications…. A major challenge will be to demonstrate to regulators and payers that preservation of C-peptide in T1D [type 1 diabetes], even for a limited period of time, with immune interventions provides clinical benefit.”

(Staeva et al., 2013)

‘Recent Lessons Learned From Prevention and Recent-Onset Type 1 Diabetes Immunotherapy Trials.’

Diabetes (2013);62:9-17.
Whilst immunotherapy intervention at the time of a clinical diagnosis of type 1 diabetes appears to be at a crossroads, it is clear that all research groups would benefit from a greater knowledge of the short-term and long-term clinical benefits of maintaining endogenous C-peptide production, and the level of C-peptide production required to achieve these benefits.
Figure 1.4 – Treatment interventions for type 1 diabetes

**Primary Prevention**
- Treatment of individuals with genetic susceptibility to type 1 diabetes prior to development of autoimmunity
  - Substitute milk products in first 6-8 months of life
  - Hydrolysed casein based infant formula versus cow's milk-based formula (TRIGR study)
- Diabetes Prevention Trial for type 1 diabetes
- Oral & S/C insulin administration, intranasal insulin administration

**Secondary Prevention**
- Treatment of individuals who have autoantibody formation suggesting high risk for developing type 1 diabetes
  - Modulate immune response when autoimmunity has developed but before onset of diabetes
    - Cyclosporine A, ketotifen, nicotinamide
  - Promote regulatory T cell responses (active tolerance) or anergy of pathogenic T cells (passive tolerance) to prevent autoimmune attack
    - Induction of Tolerance versus GAD 65 with GAD peptides
    - Anti-CD3 monoclonal antibodies (Ozilizumab, teplizumab)
    - CD28-specific monoclonal antibody (rituximab)
    - CTLA-4 immunoglobulin (abatacept)
    - Anti-CD25 monoclonal antibodies (basiliximab, daclizumab)

**Tertiary Prevention**
- Used with clinical onset of diabetes when clinical manifestations apparent and insulin replacement required to reduce the incidence of microvascular complications from hyperglycaemia long-term
  - Structured education & carbohydrate counting
  - Insulin pump therapy
  - Continuous glucose monitoring
  - Closed loop bioartificial pancreas

**Transplant Interventions**
- Reserved for individuals who have developed complications from long-standing type 1 diabetes, including loss of hypoglycaemia awareness
  - Pancreas transplantation
  - Islet transplantation

**Complications including loss of hypoglycaemia awareness**
1.3 Transplantation therapies in type 1 diabetes

Transplantation interventions in the management of type 1 diabetes are currently indicated in patients who have recurrent severe hypoglycaemia with loss of hypoglycaemia awareness. The patient must feel that their quality of life is affected by the recurrent severe hypoglycaemia to such a degree that they are willing to take on the risks of a transplantation procedure and the subsequent risks of immunosuppression therapy that are required to maintain transplant function. Two transplant interventions are currently available in the management of type 1 diabetes: pancreas transplantation and islet transplantation.

1.3.1 Pancreas transplantation

The first successful pancreas transplant was a simultaneous kidney-pancreas transplant in two patients with end-stage diabetic nephropathy (Kelly et al., 1967). One of the patients achieved near-normal glycaemia for two months post-transplant. Success rates for simultaneous pancreas-kidney transplants (SPK), pancreas-after kidney (PAK) transplant and pancreas-transplant alone (PTA) have since improved considerably. Whole organ pancreas transplantation can deliver insulin independence without significant hypoglycaemia but a 3-5% first year mortality is unavoidable with this major intervention. Patient mortality has been reported to be greater at 4 years following a pancreas-after-kidney transplant than in a matched cohort receiving a kidney transplant and on the waiting list for a pancreas transplant (Venstrom et al., 2003). SPK pancreas graft survival rates are approximately 70% at 5 years post-transplant, but 5 year graft survival rates for PAK and PTA are considerably lower at approximately 50% (Gruessner, 2011). A recent United Network for Organ Sharing (UNOS) study reported that SPK pancreas graft survival has not improved since 1995 (Waki et al.).

Pancreas transplantation is a major surgical intervention with significant short-term morbidity and mortality, the risks of which are unacceptable to some patients and their clinicians for it to be considered as a routine intervention in the management of type 1 diabetes. This has encouraged the search for alternative interventions in the management of patients affected by recurrent severe hypoglycaemia.
1.3.2 Islet transplantation

Islets can be isolated from a donor pancreas and transplantation of allogeneic isolated pancreatic islets offers beta-cell replacement for type 1 diabetes through a minimally invasive percutaneous infusion into the hepatic portal vein under radiological guidance. Islet transplantation has the advantage over pancreatic transplantation that peri-procedural complications are estimated to have a 20-fold lower morbidity risk, with the commonest complication rates reported to be an 8% risk of bleeding, a 3% risk of portal venous thrombosis and a 1% risk of bile leakage (Ryan et al., 2005a).

Successful reversal of diabetes by isolated islet transplantation was achieved in animal models in 1972 (Ballinger and Lacy, 1972). Subsequent development of the procedure centred on the selection of the portal vein and the liver as the site of delivery for isolated islets (Kemp et al., 1973). The introduction of an automated method for the isolation of human pancreatic islets (Ricordi et al., 1988) and improvements in the collagenase enzymes used for islet isolation and purification has enabled islet transplantation to become a clinical intervention for the restoration of endogenous C-peptide production in patients with type 1 diabetes.
Figure 1.5 – Islet transplantation: from donor to recipient
Early outcomes in islet transplantation

In the landmark Edmonton study on islet transplantation (Shapiro et al., 2000), seven individuals with recurrent severe hypoglycaemia and a mean type 1 diabetes duration of 35 years all attained insulin independence and freedom from severe hypoglycaemia after multiple islet infusions at a median follow-up of 11.9 months. The trial utilised an induction immunosuppression regimen using daclizumab (monoclonal antibody to the IL-2 receptor) but its revolutionary breakthrough was to use a maintenance immunosuppression regimen that was glucocorticoid-free: steroid treatment as part of a maintenance immunosuppression regimen is common in transplant medicine but steroid therapy is believed to be particularly detrimental to transplanted islet function. Instead, the Edmonton team used the calcineurin inhibitor tacrolimus with the mTOR (mammalian target of rapamycin) inhibitor sirolimus for maintenance immunotherapy.

Following its tremendous initial success, later follow-up of the Edmonton cohort revealed progressive loss of β cell function and recurrence of diabetes. Islet transplantation in 65 patients conducted by the Edmonton group showed that only 10% of patients remained insulin-independent at 5 years, although partial graft function persisted in 80% (Ryan et al., 2005a).

A subsequent trial utilising the Edmonton protocol in 36 patients at nine international academic centres (six in North America, three in Europe) was unable to replicate the successes achieved by the Edmonton team (Shapiro et al., 2006). Twenty-one of the 36 islet recipients achieved insulin independence post-transplantation. At one year’s follow-up, 16 of 36 islet recipients achieved the study’s primary end-point and were insulin independent; 10 had partial graft function and 10 patients had complete graft loss. At three years follow-up, only one patient remained insulin independent. Nonetheless, patients with partial graft function had substantial clinical benefits compared to pre-transplant, including improved glycaemic control and freedom from severe hypoglycaemia.

In light of the successes achieved by the Edmonton team, clinical islet transplant centres have been set up across the world. A glucocorticoid-free, low tacrolimus, sirolimus-based immunosuppression regimen has been shown to be efficacious in a Swiss study reporting outcomes in nine recipients with type 1 diabetes and end-stage renal failure undergoing combined islet-kidney transplantation (Lehmann et al., 2004). Five patients
achieved insulin independence and none experienced severe hypoglycaemia following transplantation after a mean follow-up of over two years. The ongoing challenge for transplant centres has been to elucidate the mechanisms that might be responsible for islet graft failure, and to identify predictors of graft failure that might allow a clinical intervention to be made at a sufficiently early stage so that graft function might be maintained. In 2004, the Clinical Islet Transplantation Registry (CITR) was established under the guidance of the National Institutes of Health and the US Food and Drug Administration. The Consortium is collecting outcome data on islet transplantation from academic centres around the world, with the aim of identifying factors that might improve islet transplant outcomes.

**Developments in immunosuppression used with islet transplantation**

Since the initial Edmonton trials, improvements in induction and maintenance immunotherapy have been made. Anti-thymocyte globulin (ATG) is a polyclonal antibody used at induction with the capacity to delete and inhibit T cells that might be responsible for the alloimmune and autoimmune responses made against transplanted islets (Bonnefoy-Berard et al., 1998). An alternative induction agent is Campath (alemtuzumab); a recombinant humanised monoclonal antibody that binds to the CD52 antigen to cause cell mediated lysis of T and B lymphocytes. TNF-α antagonists such as etanercept are now being used alongside induction agents to reduce the inflammatory responses in the peri-transplant period (Farney et al., 1993, Froud et al., 2005).

Sirolimus has been associated with nephrotoxicity, and may inhibit β cell function; its use has therefore been replaced by mycophenolate mofetil in most maintenance immunosuppression regimens.

The risk-benefit ratio in having to take immunosuppression in patients undergoing islet transplantation differs from that substantially in comparison to patients with newly diagnosed type 1 diabetes. The majority of patients with type 1 diabetes are unlikely to develop hypoglycaemia unawareness and problematic recurrent severe hypoglycaemia. However, patients undergoing islet transplantation are often selected for the procedure on the grounds that they have developed hypoglycaemia unawareness and are experiencing recurrent severe hypoglycaemia that has a significant impact on their quality of life; they often meet the criteria to undergo an islet transplant because their pre-transplant clinical status carries with it a significant mortality risk due to hypoglycaemia. This group of patients therefore have much more to gain from an
intervention that necessarily requires the use of immunosuppressant therapy, whereas in individuals with a new diagnosis of type 1 diabetes, the risk-benefit ratio from immunosuppression therapies is less clear-cut.
1.4 Assessment of islet graft function

In order to make an assessment of the clinical success of an islet transplant in re-establishing endogenous insulin production in an individual recipient, and to be able to compare clinical outcomes from transplantation between centres, a method of assessing islet graft function must be selected.

1.4.1 Glycated haemoglobin (HbA1c)

HbA1c is a measure of the level of glycated haemoglobin in red blood cells, and gives a reflection of a patient’s overall blood glucose control over the previous 6 to 12 weeks preceding measurement, and achieving a near-normal HbA1c reflects excellent glycaemic control (Higgins, 2012). However, HbA1c is influenced by exogenous insulin doses and caution is required in using the result as a measure of islet transplant function. Even insulin independent islet recipients with a normal HbA1c post-transplantation have been shown to have impaired glucose tolerance that was only detected on additional metabolic testing (Shapiro and Lakey, 2000).

1.4.2 Insulin requirement

The initial goal of many islet transplant studies was for patients with diabetes to achieve insulin independence but this target is not always attained, especially in the longer term. Even if insulin independence is not reached, many patients achieve a significant reduction in exogenous insulin dose as their overall blood glucose control improves with endogenous insulin production by transplanted β cells. A reduction in the magnitude of exogenous insulin required is therefore a measure of insulin transplant function, and this has been recognised by many groups that develop scoring systems to assess transplanted β cell function.

1.4.3 Assessment of endogenous insulin secretion by C-peptide

Measurement of C-peptide is one of the simplest methods of assessing endogenous insulin production and therefore islet function. C-peptide is a breakdown product in the formation of insulin from proinsulin, secreted on an equimolar basis to insulin and unlike the latter – it is not removed in first-pass metabolism through the liver (Palmer et al., 2004). The high reproducibility and close relationship of C-peptide measurements to endogenously secreted insulin makes it a suitable monitoring tool for β-cell function.
Exogenous insulin does not contain C-peptide and therefore C-peptide measurement reflects endogenous insulin production only.

A barrier to the use of C-peptide in clinical care is a lack of available reference ranges for specific populations with diabetes. Data quoted in published research may relate to different assays or populations and is rarely presented as a reference range. Optimal standardization of C-peptide measurement between laboratories has yet to be achieved, meaning C-peptide results produced by different methods may not be comparable (Little et al., 2008, Wiedmeyer et al., 2007).

1.4.4 Stimulation tests for assessment of endogenous C-peptide production

Stimulation tests can be used to assess islet response to a standardised stimulus. They are more complicated to perform than assessments of random or basal levels of metabolic markers but often provide much more information than one-off blood tests. Correlations between fasting C-peptide and post-stimulation C-peptide are high in insulin-treated patients, although the use of stimulated C-peptide appears to offer modestly better clinical utility (Greenbaum et al., 2008b, Gjessing et al., 1987). Furthermore, the DCCT evidence used stimulation tests to report that increased C-peptide levels in patients with type 1 diabetes were directly associated with improved glycaemic control, reduced incidence of hypoglycaemia and decreased microvascular risk; the DCCT made no analysis of associations with fasting C-peptide (The Diabetes Control and Complications Trial Research Group (1998)). Stimulation tests are typically performed after a period of fasting and discontinuation of exogenous insulin for at least 12 hours, although individual groups have many variations on the preparation period prior to the test. Patients on insulin pump treatments have been permitted to continue their insulin infusion at a basal rate throughout the test, whilst some groups allow rapid acting insulin to be given subcutaneously up to 4 hours before commencing a stimulation test. Stimulation testing can be divided into two groups: those that use a glucose stimulus and those that use direct pharmacological stimulation.

The Meal tolerance test (MTT)

The MTT assesses endogenous insulin production in response to ingestion of a standardised meal. The ‘meal’ is typically a commercial caloric supplement that contains between 350-500 kcal and 50 to 65 g of carbohydrate. Blood samples are typically taken at 0, 15, 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 minutes post-
ingestion for glucose, C-peptide and insulin. The use of very different measurement units for reporting C-peptide values can lead to confusion in clinical care. C-peptide is commonly reported in nmol/l, pmol/l or ng/ml (1 nmol/l = 1000 pmol/l = 3 ng/ml). C-peptide response to the meal tolerance test in subjects without diabetes has been reported to be an increase from a fasting level of 0.80±0.36 to 2.63±0.78 nmol/l post-stimulus (Hovorka et al., 1998). Traditionally, the most important value from the test is the 90 minute C-peptide (often deemed to be the ‘peak’ C-peptide in healthy subjects), although time to peak C-peptide response has been shown to variable in subjects without diabetes (Pozzan et al., 1997).

The MTT has been widely used to study the response to therapeutic interventions in type 1 diabetes, and in a cross-sectional study of 259 patients aged 8-35 years with a mean diabetes duration of 1.5 years, C-peptide was shown to peak at 90 min during an MTT (Greenbaum et al., 2008b). Subsequently, 90 minute C-peptide has been shown to be a highly sensitive and specific measure of peak C-peptide in children and adolescents with type 1 diabetes (Besser et al., 2013).

The MTT has been used extensively to monitor the function of transplanted islets (Baidal et al., 2009, Berney and Toso, 2006, Rickels et al., 2005, Ryan et al., 2005a, Alejandro et al., 1997). The 90 minute glucose is considered to be a good measure of β cell reserve following islet transplantation, and it provides an assessment of the homeostatic ability of an individual to handle a secretagogue load; a level of <10.0 mmol/L (180 mg/dL) at 90 minutes indicates good graft function (Ryan et al., 2005a).

**Hyperglycaemic clamp studies**

Hyperglycaemic clamp studies have been used to assess insulin response in islet transplant patients (Luzi et al., 1996, Luzi, 1999). Concerns have been expressed that prolonged exposure to hyperglycaemia might cause β cell stress in transplanted islets, and the studies are not routinely used in the investigation of islet transplant subjects.

**Oral glucose tolerance test (OGTT)**

The OGTT is well known throughout the diabetes community, as it is still the only stimulation test listed in the American Diabetes Association’s criteria for the diagnosis of diabetes (American Diabetes Association (2011)). At the start of the test, subjects
consume 75 g of oral glucose and blood samples are taken at 0, 30, 60, 90 and 120 minutes for assessment of the blood glucose response.

**Intravenous glucose tolerance test (IV GTT)**

In this test, subjects are given 300 mg/kg of 50% dextrose. Blood samples are taken at -10, 0, 3, 4, 5, 7, 10, 15, 20, 25, 30 and 60 minutes for glucose, insulin and C-peptide. Acute response to glucose for C-peptide and insulin release can then be calculated by the mean of their values at 3, 4 and 5 minutes minus the mean of their baseline values at -10 and 0 minutes.

**The glucagon stimulation test (GST)**

In the GST, blood samples for insulin, C-peptide and glucose are collected after the intravenous administration of 1 mg of glucagon.

**Comparison of the MTT to the GST as a stimulation test for C-peptide**

The MTT has been compared to the GST in the stimulation of C-peptide production in 266 patients with a recent diagnosis (within 4 years) of type 1 diabetes (Greenbaum et al., 2008a). The MTT produced a significantly higher C-peptide response in comparison to the GST, and was therefore felt to be a more sensitive test of residual β-cell function. For this reason, it might be preferred as a test of graft function in islet transplant subjects. The greater sensitivity of the MTT might be due to the incretin effect produced by the oral food bolus during the test, whereby glucagon-like peptide 1 production will produce additional insulin release.

The GST does have the advantage that it takes less time to perform: time to peak C-peptide has been reported at 6 minutes in the GST, compared to 80 to 90 minutes in the MTT (Greenbaum et al., 2008a). However, although the peak C-peptide in both tests is positively associated with fasting C-peptide, only the MTT peak C-peptide has been shown to be inversely associated with fasting glucose. The GST is also associated with a higher frequency of adverse effects, with nausea reported in 75-81% of subjects. Most importantly, the results from an MTT have been reported to be significantly more reproducible than those of the GST. The MTT therefore appears to have considerable advantages over the GST to both the assessor and the test subject in the assessment of stimulated C-peptide production.
**The Arginine stimulation test (AST)**

In the AST, 0.07 mg/kg of arginine hydrochloride (up to a maximum of 5 g) is administered over 30 seconds and blood samples are taken at -10, 0, 2, 3, 4, 5, 7, and 10 minutes (Dupre et al., 1969). The AST has the advantage over the GST that it is clinically well tolerated and does not provoke nausea. Acute response to arginine for C-peptide and insulin release can then be calculated by the mean of their values at 2, 3, 4 and 5 minutes minus the mean of their baseline values at -10 and 0 minutes. The area under the curve for insulin above the mean of the baseline values is a reflection of islet mass.

**Glucose potentiated arginine stimulation tests (GP AST)**

The glucose potentiated arginine stimulation test allows determination of the maximal acute secretory response ($AR_{\text{max}}$) as a measure of β cell secretory capacity. The test involves the subject undertaking an arginine stimulation test whilst a hyperglycaemic clamp is used to achieve a raised plasma glucose, with the aim of increasing β cell insulin release during the AST at increased blood glucose levels.

**Comparison of the MTT to the AST as a stimulation test for C-peptide**

The AST has been compared to the MTT for the assessment of residual C-peptide production in 19 subjects with a recent diagnosis (1 to 42 months) of type 1 diabetes (Greenbaum et al., 2004). No difference in peak C-peptide value was observed for the MTT and AST, and there was a strong relationship between the obtained mean and peak C-peptide values from both the AST and MTT. A strong inverse relationship also existed between time to peak on the MTT and the amplitude of the arginine stimulated C-peptide response. No relationship between basal glucose and stimulated C-peptide was found in the arginine tests but a weak relationship between basal glucose and mean C-peptide was found in the MTT results. The MTT therefore has a reported sensitivity for stimulated C-peptide that is equivalent to the AST and provides a similar measure of β cell function when used in the assessment of patients with recent onset type 1 diabetes.

**Previous use of the MTT in islet transplant patients**

The MTT has previously been used in the metabolic assessment of islet graft function post-transplant (Ryan et al., 2005a, Barton et al., 2012). Marked metabolic
abnormalities during an MTT have been shown to be detectable early in the post-transplant period which can act as markers for islet graft dysfunction, and the MTT has emerged as a preferred test in the monitoring of islet graft function post-transplant.

1.4.5 Assessment of Glucose Variability - Continuous glucose monitoring

Continuous glucose monitoring (CGM) provides a continuous data stream to give information on the rate and direction of blood glucose fluctuations. The impact of therapeutic interventions on blood glucose variability - and the subsequent risk of hypo or hyperglycaemia – can accordingly be quantified in a way that cannot be provided by self-monitoring blood glucose devices (SMBG).
1.4.6 Scoring indices of islet graft function

Work with metabolic function tests in islet transplant recipients has led to the conclusion that no single metabolic measurement provides an exhaustive evaluation of the clinical success of a transplant. Research groups have therefore attempted to create scoring indices using the metabolic data that can be used clinically to provide a rapid assessment of current – and possibly future – islet graft function.

The β-score

The β-score is a composite scoring system designed to provide clinicians with more than just a statement about whether the graft is producing insulin or not (Ryan et al., 2005b). The score can be between 0 and 8, and its calculation is based on scoring each of the four factors of fasting plasma glucose, HbA1c, daily insulin requirement and 90 minute stimulated C-peptide MTT with 0, 1 or 2, based on their values.

Using stepwise regression analysis, it has been shown that the β-score could be predicted from linear combination of each of the four independent variables in islet transplant patients. In the original paper stating its use, only 16% of transplant patients achieved a score of 8/8, with most patients achieving a score of 6-7 once adequate islet mass and insulin independence was achieved. A prospective longitudinal study in 14 transplant patients has suggested that calculation of the β-score might have a role in predicting future graft survival (Vantyghem et al., 2009).

C-peptide/glucose (CP/G) index

Plasma C-peptide levels fluctuate according to the degree of glycaemia at the time they are measured. Any plasma C-peptide may indicate good graft function if glucose levels are normal but they may be inappropriately low if glucose levels are high. The CP/G therefore corrects for high glycaemic values, and it is calculated by:

\[
\text{C-peptide / glucose ratio} = \left(\frac{\text{Fasting C-peptide (ng/ml) \times 100}}{\text{Glucose (mg/dL)}} \right)
\]

A retrospective analysis on results from 22 islet transplant patients was used to determine whether C-peptide or the CP/G might best predicted a high 90 minute glucose during an MTT (Faradji et al., 2007). CP/G was found to be superior to C-peptide, and also had a significant negative correlation with 90 minute glucose during MTTs at 3, 6, 9, 12, 15 and 18 months post-transplant. Multiple logistic regression for all time points revealed that a CP/G of >1.0 was associated with having a lower 90 minute glucose
level (<10 mmol/L), a higher acute insulin response during an intravenous glucose
tolerance test and a higher β-score (>6). CP/G may therefore be more indicative of graft
dysfunction and clinical outcome than C-peptide alone.

**Transplant Estimated Function (TEF)**

TEF estimates daily insulin secretion by the transplant and is simpler to calculate than
the β-score (Caumo et al., 2008). Stepwise multiple regression analysis was carried out
on β-scores measured in islet transplant patients and daily insulin requirement was
found to be the best predictor of the β-score, whilst HbA1c and fasting glucose had a
lesser influence on β-score prediction. C-peptide values were reported to have only a
marginal significance on the β-score. ‘Transplant Estimated Function’ therefore only
uses the daily insulin requirement (DIR) and HbA1c for its calculation. It was reasoned
that a change in total daily insulin would influence glycaemic control, such that:

$$\Delta\text{HbA1c} = k.\Delta\text{DIR}$$

where: ‘k’ is the sensitivity of glycaemic control to insulin supply

As a transplant recipient has two sources of insulin supply (exogenous insulin and
endogenous β cell secretion from islets), the change in HbA1c can be calculated as:

$$\Delta\text{HbA1c} = k.[\Delta\text{DIR}+\text{TEF}]$$

where: TEF is insulin produced by the transplanted islets

TEF can therefore be calculated by:

$$\text{TEF} = a.\text{DIR} + b.\text{HbA1c} + c$$

where: ‘a’ = -1, ‘b’ =1/k and ‘c’ is a constant dependent on pre-transplant DIR &
HbA1c, calculated by $c = -a.\text{DIR}(\text{pre-Tx}) - b.\text{HbA1c}(\text{pre-Tx})$

These calculations are all dependent on having a value for ‘k’, which has been given as
a population value estimated from ΔHbA1c and ΔDIR recorded in a group of patients
with type 1 diabetes: ‘k’ is calculated to be -5.43. Once TEF is known, ‘Islet Estimated
Function’ (IEF) can be calculated by dividing TEF by the number of infused islets. An
advantage of the TEF calculation over the β score is that it does not require the results
of an MTT. However, a major limitation of the TEF is that by use of a population value
for parameter ‘k’, it assumes the same degree of insulin sensitivity in all patients. The
group proposing use of TEF state that a 50% variation in ‘k’ does not alter its calculated
correlations with insulin response during metabolic tests.
The SUITO index also estimates engrafted islet mass (Matsumoto et al., 2005). It can be calculated by:

\[
\text{Fasting C-peptide [ng/dL]} / \left[ \text{Fasting blood glucose (mg/dL)} - 63 \right] \times 1500
\]

A score of 100 is equal to 100% of pancreatic β cell function, and a score of greater than 25 is required for insulin independent status. It has been suggested that calculation of the SUITO index within 1 month of allogeneic (Matsumoto et al., 2009) or autologous (Matsumoto, 2011) islet transplantation is an excellent predictor of insulin independence.

**Which scoring index should be used to assess islet graft function?**

There is no consensus on which test is best to monitor β cell mass and function in islet transplant patients. A comparative evaluation of each of the simple indices (β-score, C-peptide/glucose ratio, TEF and SUITO) has been attempted (Caumo et al., 2011) but concluded that no index outperformed the others.
1.5 Assessment of hypoglycaemia in islet recipients

At present, none of the scoring systems assessing islet graft function comment on the outcome that is used to determine whether a patient should have an islet transplant in the first place; that of a reduction in the frequency of severe hypoglycaemia. This is a major shortfall and separate assessments for hypoglycaemia frequency and quality of life are required post-transplantation. Current scoring systems for the assessment of hypoglycaemia awareness aim to predict impaired awareness and increased risk of severe hypoglycaemia.

1.5.1 The Gold Score

This scoring system is based on the response to a single question: ‘Do you know when your hypos are commencing?’. Results are expressed by a 7-point Likert scale, where 1 = ‘always aware’ and 7 = ‘never aware’. Impaired awareness of hypoglycaemia is suggested by a value of ≥4. This score is based on results from a prospective case-control study with 60 participants and 12 months follow-up (Gold et al., 1994); 29 participants were noted to have impaired awareness and 31 participants had normal awareness of hypoglycaemia. Participants with impaired awareness of hypoglycaemia had an increased frequency of severe hypoglycaemia episodes (≥1 severe hypoglycaemia episodes in 66% with impaired awareness versus 26% with normal awareness; higher incidence of severe hypoglycaemia episodes per patient per year: 2.8 with impaired awareness versus 0.5 with normal awareness).

1.5.2 The Clarke Score

This score is made up of eight questions characterising an individual’s exposure to episodes of moderate and severe hypoglycaemia to assess the glycaemic threshold for and symptomatic response to hypoglycaemia. The assessment gives a score where a value of ≥4 indicates impaired awareness of hypoglycaemia. The scoring system is derived from a prospective case series study in 78 subjects with 9-12 months follow-up (Clarke et al., 1995). Thirty-eight of the subjects were classified as having impaired awareness of hypoglycaemia, defined as being less able to detect biochemical hypoglycaemia (<3.9 mmol/L), with less autonomic and neuroglycopenic symptoms in response to hypoglycaemia. Frequency of severe hypoglycaemia events was increased in subjects with impaired awareness.
1.5.3 The HYPO Score

This is a composite hypoglycaemia score based on the frequency, severity and degree of impaired awareness of hypoglycaemia that provides a measure of the extent of problems with hypoglycaemia to complement an assessment of problems with glucose control (Ryan et al., 2004). The score is developed from an assessment of glucose readings collected from patients over a 4 week period (minimum of two capillary glucose readings a day), noting details of each hypoglycaemic event (glucose <3.0 mmol/L), the number of occurrences of hypoglycaemia, and a completed questionnaire about the frequency and severity of hypoglycaemia episodes over the previous year. In particular, emphasis is placed on which symptoms occur and whether outside help from a third party was obtained to either recognise or treat a hypoglycaemic event. A score of ≥433 is representative of problematic hypoglycaemia, ≥1,047 is indicative of very serious unawareness of hypoglycaemia.

1.5.4 The DAFNE Hypoglycaemia awareness rating

This score is a three question hypoglycaemia assessment that is used in the national DAFNE database. It asks patients to rate their awareness of hypoglycaemia by stating whether they (1) usually recognised that they were hypoglycaemic at a blood glucose concentration ≥3 mmol/L; (2) <3 mmol/L or (3) not at all. Patients rating themselves in categories 2 and 3 were defined as having impaired awareness and had reported a mean of 3.6 severe hypoglycaemia episodes during the preceding year; compared to 0.87 in patients rating themselves as aware at blood glucose concentrations of 3 mmol/L or higher (Hopkins et al.).

1.5.5 The Pedersen-Bjergaard Score

Impaired awareness of hypoglycaemia is assessed by the question ‘Can you feel when you are low?’, with the respondent selecting one answer of ‘always’, ‘usually’, ‘sometimes/occasionally’ or ‘never’. A response of ‘usually’ implies impaired awareness of hypoglycaemia, whilst the responses of ‘sometimes/occasionally’ or ‘never’ imply severely impaired awareness of hypoglycaemia. This scoring system was developed from a questionnaire assessing the occurrence of hypoglycaemia and aspects of hypoglycaemia unawareness in 230 participants, 47% of whom were classified as having impaired awareness (Pedersen-Bjergaard et al., 2001). Groups with impaired awareness
awareness were found to have a 5.1 to 9.6 increased risk of severe hypoglycaemia compared to groups defined as having normal awareness.

### 1.5.6 Correlation between scoring systems for assessment of impaired awareness of hypoglycaemia

Two studies have undertaken comparison of scoring systems for impaired awareness of hypoglycaemia (Geddes et al., 2007, Hoi-Hansen et al.). The studies undertook direct comparison of the Clarke, Gold and Pedersen-Bjergaard scores with focus given to their correlation and ability to predict severe hypoglycaemia. Gold and Clarke scores defined 24-28% of study participants as having impaired awareness of hypoglycaemia and both scores correlated well in both studies. The Pedersen-Bjergaard score defined 13-15% of subjects as having impaired awareness of hypoglycaemia. However, correlation with Clarke and Gold score assessments was poor, suggesting the possibility that the Pedersen-Bjergaard score might not necessarily be picking up cases of impaired hypoglycaemia awareness. The studies concluded that Clarke and Gold questionnaires might be better discriminators for impaired awareness of hypoglycaemia in comparison to the Pedersen-Bjergaard questionnaire.

### 1.5.7 Scores as predictors of hypoglycaemia and severe hypoglycaemia

One study has reported that more episodes of biochemical hypoglycaemia are predicted with use of Clarke and Gold scoring, whilst no significant difference in hypoglycaemia frequency was noted in subjects assessed by the Pedersen-Bjergaard score (Geddes et al., 2007). Gold and Clarke scores predicted reduced autonomic warning symptoms during biochemical hypoglycaemia but again no significant difference was noted with Pedersen-Bjergaard scoring. All three testing methods did predict an increased rate of severe hypoglycaemia episodes.

In a second study in 518 study participants (Geddes et al., 2008), the Gold score classified 101 participants as having impaired hypoglycaemia awareness: rate of severe hypoglycaemia per person was six-fold in this group, with lower intensity of autonomic symptoms reported. A further study reported that Gold scoring (Schopman et al.) predicted an increased rate of severe hypoglycaemia in study participants.
Clarke and Gold scores have been used to assess impaired awareness of hypoglycaemia during continuous glucose monitoring (CGM) (Gimenez et al., 2009, Choudhary et al.). More episodes of hypoglycaemia and an increased incidence of severe hypoglycaemia were recorded with use of CGM in patients defined as having impaired awareness according to their Clarke and Gold scores in comparison to controls. Clarke scoring has also been found to correlate with clamp study outcomes investigating impaired hypoglycaemia awareness in 19 study participants (Janssen et al., 2000).

1.5.8 Choice of scoring system for assessment of impaired awareness of hypoglycaemia

Gold and Clarke scores correlate well in studies and have a high concordance with increased frequency of severe hypoglycaemia episodes. The Pedersen-Bjergaard score has less correlation with Gold and Clarke scores and not such a good concordance with prediction of severe hypoglycaemia episodes. The HYPO score has a good concordance with frequency of severe hypoglycaemia. However, the baseline data required and the method of its interpretation for the score’s calculation is complex, favouring the use of Gold and Clarke scores for assessment of impaired awareness of hypoglycaemia.

1.5.9 Impact of islet transplantation on hypoglycaemia awareness

Although islet transplantation prevents severe hypoglycaemia (Froud et al., 2005), metabolic studies using hypoglycaemic clamp data for the assessment of restoration of hypoglycaemia awareness are not conclusive (Rickels et al., 2007, Paty et al., 2002).

A retrospective study on 31 islet transplant patients (Leitao et al., 2008) used the Clarke score pre and post-transplant to assess hypoglycaemia awareness, with a score of ≥4 indicating hypoglycaemia unawareness. The proportion of patients with hypoglycaemia unawareness decreased from 87% to 13%, and the threshold for glycaemic awareness was increased from 41.4 +/- 17.6 mg/dL to 58.4 +/- 10.3 mg/dL. Such improvements in hypoglycaemia awareness were sustained regardless of classification of graft function post-transplant, although an increase in post-transplant hypoglycaemia score was observed in patients with loss of graft function. It was also noted that a lower hypoglycaemia score correlated with better β cell function according to stimulated C-peptide levels in an MTT. Improvements in hypoglycaemia score were therefore obtained post-transplant even if the graft was considered to have ‘failed’ on an MTT.
post-transplant. The study demonstrated that metabolic data may not be able to account for all of the clinical improvements observed post islet transplantation. It may be that avoidance of hypoglycaemia during the early post-transplant period produces increased hypoglycaemia awareness, which is maintained even if graft function is lost. This conclusion highlights the need to utilise alternatives to metabolic testing - such as the Clarke and Gold hypoglycaemia scores - to assess some of the clinical benefits of islet transplantation.
1.6 The need to develop novel markers of islet transplant attrition

The reasons for attrition in islet transplant function are currently unknown. It has been suggested that innate immunity, autoimmune responses, alloimmune responses and choice of immunosuppression (Lacotte et al., 2011, Van Belle and von Herrath, 2008) may all affect outcome. There may be unidentified mechanisms that result in islet graft dysfunction, and it may be that inflammatory changes occur at the transplantation site in the liver, leading to a reduction in functioning islet cell mass.

Donor pancreas and isolated islet characteristics have been previously analysed to assess whether they have any impact on clinical outcome (Nano et al., 2005). Islet yield has been significantly associated with donor age and body mass index (BMI). A high BMI is a marker of insulin resistance and high metabolic demand, both of which are a stimulus for islet neogenesis and a consequent increase in overall β cell mass. The presence of fatty tissue or of a thick capsule around the islets during digestion further improves isolation outcomes, as it limits the exposure of free islets to collagenase in solution, preserving islet integrity and survival. Good pancreas condition and weight are also associated with improved islet yield.

Multivariate analysis of single islet preparations used for transplantation has shown that reduction in insulin requirement was significantly associated with morphological aspects of islets (Nano et al., 2005). ‘Round-shaped’ islets were shown to have maintained morphology and a large portion of their peripheral capsule, whilst ‘fragmented’ islets had architectural disorganisation with irregular borders and free chains of cells protruding from the periphery. One month C-peptide post-transplant correlated positively with islet purity, higher transplanted islet equivalents per kilogram (IEQ/kg), and preparations with predominantly round-shaped islets.

After transplantation, early losses of graft function are primarily non-specifically immunological, thought to be the result of damage sustained during the isolation procedure or in the graft micro-environment within the liver. The principal problem of current metabolic tests is that they are late markers of islet graft dysfunction and reflect changes when it might no longer be possible to salvage a failing graft. Assessment of alternative markers post-transplantation might provide a novel marker of rejection that identifies islet stress at an early stage and allows rescue therapies to prevent complete graft failure.
Techniques for monitoring graft function must address four aims:

1. Understanding when grafts are damaged
2. Understanding by which mechanisms grafts are damaged
3. Detection of damage early enough to allow intervention for graft salvage
4. Allow immunosuppression to be tailored according to risk of immune events

Ultimately, one of the requirements of islet monitoring tests should be that they provide prognostic information if there is concern that the graft might be failing, and allow tailoring of strategies to overcome graft dysfunction.
1.6.1 Development of hepatic steatosis following islet transplantation

Physiological consequences of localised insulin production by transplanted islets might result in hepatic steatosis

Hepatic intraportal islet transplantation for patients with type 1 diabetes has the theoretical advantage of more closely mimicking natural insulin release in subjects without diabetes by promoting insulin delivery to a site close to the hepatic portal vein. This avoids the systemic hyperinsulinaemia associated with exogenous insulin delivery but localised insulin delivery in the liver has a powerful lipogenic response in hepatocytes, and may cause them to become overloaded with triacylglycerol. Lipohypertrophy has long been recognised to be a consequence of the promotion of local accumulation of triglyceride when insulin is delivered subcutaneously (Hu and Kan, 2013). In the liver, insulin upregulates the expression of lipogenic enzymes (Kakuma et al., 2000) and local lipoprotein lipase hydrolyses triacylglycerol to create an excess of fatty acids.

Hepatic steatosis occurs in recipients post-islet transplantation

Periportal steatosis following islet transplantation has been previously reported (Eckhard et al., 2004, Markmann et al., 2003, Bhargava et al., 2004). The Edmonton team have been at the forefront of developments in islet transplantation for many years and have examined the liver by ultrasound and magnetic resonance imaging (MRI) post-transplantation. Post-transplant scans showed fatty changes on MRI in 20% of subjects (median follow-up time 402 days, range 117 to 1190 days) (Bhargava et al., 2004).

Potential impact of steatosis on graft function

Hepatic steatosis is an abnormal and excessive intracellular accumulation of fat in hepatocytes (Basaranoglu et al.). Free fatty acids can trigger cell death by induction of oxidative stress and activation of apoptosis, which can potentially result in progressive hepatic disease. Steatosis is the histological hallmark of non-alcoholic fatty liver disease (NAFLD) (Matteoni et al., 1999), and its presence has a causative role in many hepatic and systemic disorders. A significant proportion of individuals with steatosis subsequently develop type 2 diabetes and its presence may interfere with insulin signalling (Semple et al., 2009). Lipotoxicity has been shown to have an acute effect on islet function and a chronic effect on islet cell mass in patients with type 2 diabetes.
(Poitout and Robertson, 2002). It is possible that exposure to high local concentration of fatty acids may damage transplanted islets.

1.6.2 Role of the humoral immune response in transplant graft rejection

Accumulated evidence from the field of transplantation suggests that the humoral immune response may have an integral role in graft rejection, with the development of donor specific antibodies associated with graft failure (Cai and Terasaki, 2005). The humoral immune response may cause damage to graft endothelium by activation of the complement system, recruitment of inflammatory cells and direct antibody-mediated cell cytotoxicity. Following endothelial damage, platelet activation may lead to thrombosis, whilst humoral and cellular infiltrates mediate ongoing graft tissue damage. These events produce irreversible ischaemic changes that could result in graft loss.

The finding of preformed antibodies in recipient serum to donor graft tissue underpins the concept of hyperacute rejection (Kissmeyer-Nielsen et al., 1966, Terasaki et al., 1971). Pre-formed Human Leukocyte Antigen (HLA) antibodies should be characterised pre-transplant to aid selection of the optimal donor for transplantation. HLA typing and screening for HLA antibodies is systematically undertaken in all candidates awaiting organ or cellular transplantation, with T and B cell cross-matches between recipient and donor tissue routinely performed pre-transplant.

Association of de novo DSA with graft rejection in renal transplantation

*De novo* HLA antibody and Donor Specific Antibody (DSA) development post-transplant are associated with reduced graft survival in renal transplants (Morris et al., 1969), with graft loss greatest in recipients with DSA (Lachmann et al., 2009, Everly et al., 2013). The central diagnostic criterion of antibody-mediated rejection of renal transplants is the demonstration of complement activation marker C4d in inflamed peritubular capillaries (Feucht, 2003). Renal transplant studies in cynomolgus monkeys have shown that DSA precedes C4d deposition and subsequent transplant glomerulopathy, suggesting that DSA might be the trigger for graft failure (Smith et al., 2008).

A cross-sectional prospective international trial assessing the impact of HLA antibodies and DSA on graft outcome in 1014 deceased kidney transplant recipients (Lachmann et al., 2009) found that 30% of recipients had HLA antibodies at a mean time of 6 years
post-transplant, of which 31% were DSA positive. In the presence of antibodies, median time to graft failure was 989 days (435-1591) post-assessment, compared to 1294 days (791-1952) in recipients without antibodies. Kaplan-Meier survival probability at 5.5 years post-assessment was 49% with DSA and 70% with non-DSA, versus 83% in recipients with no antibodies.

In a second study, 189 non-HLA identical primary kidney recipients known to be DSA negative pre-transplant were screened annually for DSA. DSA developed in 24.8% of recipients, with the greatest incidence in the first year post-transplant (11.2%) (Everly et al., 2013). DSA positive recipients experienced an acute rejection episode in 33% of cases, versus 23% of DSA negative recipients. In recipients with graft loss, 56% had DSA and all DSA negative chronic rejections were HLA antibody positive. From the onset of DSA, 24% of recipients lost graft function within 3 years, compared to a 96% five year survival rate for those without DSA. The relative risk of allograft loss was six to nine times higher for DSA positive recipients compared to DSA negative recipients.

**Association of de novo DSA with graft rejection in other fields of organ transplantation**

HLA antibody and DSA have been associated with poor outcome in other fields of organ transplantation. Cardiac antibody mediated rejection was first described in 1989 (Hammond et al., 1989), and diagnosis is based on the triad of clinical evidence of allograft dysfunction, serological evidence of DSA and endomysial antibody features (Kobashigawa et al., 2011). Both symptomatic and asymptomatic antibody mediated rejection has been shown to be associated with poor prognosis (Kfoury et al., 2009, Irving et al., 2011). In lung transplant recipients, the presence of DSA has been associated with a reduced survival rate (Hachem et al., 2010). Liver transplants are generally regarded as not being susceptible to antibody mediated rejection. However, CDC positive cross-match recipients were reported to have a one year graft survival rate of 29%, compared to 72% of cross-match negative recipients (Muro et al., 2005b). Preformed DSA has been associated with a 1.58 increased risk hazard ratio of early rejection after liver transplantation (O'Leary et al., 2013). Acute rejection rate in simultaneous pancreas-kidney recipients has been reported to be 45% among patients with anti-HLA antibodies versus 8% in recipients without HLA antibodies (Cantarovich et al., 2008). In a nine year follow-up of 167 consecutive pancreas transplant recipients (152 simultaneous pancreas-kidney, 15 pancreas-transplant alone) (Cantarovich et al.),
40 recipients developed anti-HLA antibodies, with 26 developing DSA. More rejection episodes were observed in recipients with positive anti-HLA antibodies than in those without (42.5% versus 11%), with the highest incidence of rejection reported in recipients developing DSA (53.8% incidence of rejection). Graft survival was significantly lower in DSA positive recipients (75% for DSA positive kidney and 75% for DSA positive pancreas grafts versus 100% for pancreas non-DSA and 95% for kidney non-DSA grafts).

The accumulated evidence suggests that DSA development is associated with reduced graft survival, and its detection may therefore be a useful prognostic marker in islet recipients.
1.7 Project Aims

1.7.1 Aim 1: Assess clinical outcomes in the UK islet transplant program

An integrated UK islet transplant network was commissioned in 2008 (Aldibbiat A, 2012) and fully-funded by the National Health Service (NHS) as a clinical intervention with proven efficacy for life-threatening recurrent severe hypoglycaemia freely available at the point-of-care. The service was endorsed by the National Institute for Health and Clinical Excellence (NICE), with the stated aims of preventing recurrent severe hypoglycaemia (Workgroup on Hypoglycaemia, 2005), attaining HbA1c <7.0% (<58 mmol/mol), reducing exogenous insulin requirement and also quantifying graft function by C-peptide secretion (NICE, 2008).

From the inception of the National Health Service (NHS) funded UK program, all recipients have been invited to participate in a prospective follow-up study to determine biomedical and psychosocial outcomes. The objectives of the current analysis were to:

1. Determine metabolic outcomes post-intervention over the first 3 years of the NHS-funded UK islet transplant program. The following clinical outcomes were assessed:
   a. Rate of severe hypoglycaemia
   b. Glycaemic control
   c. Insulin dose
   d. Duration of graft function

2. Assess whether NICE treatment targets for islet transplantation were achieved at 12 months post-intervention over the first 3 years of the UK program.

3. Establish whether equity of access to transplantation could be realised by the achievement of equivalent outcomes with both locally isolated and transported preparations.
1.7.2 Aim 2: Use continuous glucose monitoring to relate measures of blood glucose control to endogenous C-peptide production

Mainstream diabetes therapies have aimed at exogenous insulin replacement for blood glucose control in individuals with type 1 diabetes (The Diabetes Control and Complications Trial Research Group (1993)). However, preserved endogenous insulin secretion soon after diagnosis of type 1 diabetes is a therapeutic goal for new disease modifying therapies (Michels and Eisenbarth, 2011, Greenbaum et al., 2012, Staeva et al., 2013). Immunotherapy used at the point of diagnosis of type 1 diabetes has delivered prolongation of endogenous insulin production but long-term consequences on clinical outcomes are unclear; little data exists regarding what improvements in blood glucose control might be anticipated if a level of endogenous insulin production can be maintained beyond the anticipated honeymoon period. There is a need to define the short-term and long-term clinical benefits of maintained endogenous C-peptide production in greater detail (Kolb and Gale, 2001, Greenbaum et al., 2012), particularly the relationship between C-peptide preservation and glycaemic control and variability (Staeva et al., 2013). This study therefore aimed to:

1. Define the duration of time spent within defined blood glucose ranges following islet transplantation, using continuous glucose monitoring to assess blood glucose control.

2. Correlate blood glucose control with level of endogenous insulin production achieved post-transplantation, using stimulated C-peptide during meal tolerance testing as a measure of graft function.

3. Give a detailed assessment of blood glucose variability, hyperglycaemia risk and hypoglycaemia risk and investigate for any possible relationship with the level of endogenous insulin production in islet recipients.

Detailed glucose time series obtained by CGM have been previously used to assess blood glucose control pre- and post-islet transplantation (Kessler et al., 2002, Vantyghem et al., 2012, Geiger et al., 2005, Paty et al., 2006, Faradji et al., 2006). However, these studies are limited by a basic assessment of CGM outcomes that does not utilise CGM analysis to its full potential: CGM analysis can provide a number of
measures of blood glucose variability (Hill et al., 2011), as well as an assessment of hyperglycaemia and hypoglycaemia risk (Clarke and Kovatchev, 2009).

The aim of this work was to use CGM in islet recipients to determine whether restoration of beta-cell function at a given level is intrinsically associated with benefits in glycaemic control and prevention of hypoglycaemia. Defining the level of C-peptide at which clinical outcomes are achieved could have substantial implications for present and future therapeutic interventions aimed at maintaining or restoring endogenous insulin production.
1.7.3 **Aim 3: Assessment of hepatic fat in UK islet recipients**

Whether fatty changes in the liver are merely an incidental consequence of a successfully functioning islet transplant in a minority of patients, or whether the changes might predict future transplant failure is currently unclear. No consensus has yet been reached on whether the development of steatosis impacts on graft function.

This project aimed to use Magnetic Resonance Imaging (MRI) to make the first quantitative assessment of liver fat in islet recipients. The specific aims were:

1) To identify whether intra-hepatic islet transplantation results in morphological fatty change within the liver, using chemical shift based water-fat separation MRI.

2) To accurately quantify the distribution of fatty liver changes on magnetic resonance images obtained in islet transplant patients.

3) To compare images of the liver obtained in islet recipients with two control groups: patients with a known diagnosis of NAFLD and patients with type 1 diabetes on continuous subcutaneous insulin infusions.

4) To assess the impact of any fatty change in islet recipients on graft function.
1.7.4 Aim 4: Determination of *de novo* Donor Specific Antibody development in islet transplant recipients

The alloimmune response and anti-HLA antibody formation have been associated with reduced islet graft functional outcomes (Mohanakumar et al., 2006). Islet transplantation is associated with a relatively high rate of *de novo* DSA development (Campbell et al., 2007b), and association with immunosuppression withdrawal after graft failure has been previously confirmed. Prospective studies are required to elucidate the temporal relationship between DSA development and graft dysfunction, and this study aimed to:

1) Screen for *de novo* DSA development at regular intervals during the first 12 months following an islet transplant.

2) Assess the impact of *de novo* DSA development on graft function.

3) Assess whether available donor information (age, body mass index, HLA match, pancreas condition, harvesting and cold ischaemia time) has any association with DSA formation or graft outcome.

Islet cell transplantation also provides a unique opportunity to monitor recurrent autoimmune mediated islet destruction, and this study aimed to measure fluctuations in autoimmune antibody formation alongside alloimmune testing to determine the temporal relationships of the immune response with any graft dysfunction. The aim of the work was to identify potential surrogate markers that might predict islet graft failure.
CHAPTER TWO

METHODS
Chapter 2 - Methods

2.1 Assessment of clinical outcomes in the UK islet transplant program

2.1.1 The UK islet recipient population – indications for transplantation

Recipients of a first islet transplant between April 2008 and March 2011 at all NHS-funded centres (Edinburgh, King’s College London, Royal Free London, Manchester, Newcastle and Oxford (including patients assessed and followed up in Bristol)) were invited to take part in an ethically-approved experimental medicine follow-up study following informed written consent. Inclusion criteria included C-peptide-negative type 1 diabetes complicated by recurrent severe hypoglycaemia (≥1 event over the preceding 12 months requiring assistance to actively administer carbohydrate, glucagon, or other resuscitative actions (Workgroup on Hypoglycaemia, 2005)) despite optimised conventional management. Contraindications included insulin resistance (insulin requirement >0.7 units/kg to achieve HbA1c <9.0% (<75 mmol/mol)), BMI >28 kg/m^2 and any contraindications to immunosuppression therapy (including impaired renal function with isotopic GFR <60 mls/min/1.73m² or albumin excretion rate >300 mg/24hr (unless previous renal transplant)) (See Appendix One).

2.1.2 Donor tissue for transplantation

Pancreases from donors without diabetes were allocated to the best-matched ABO-compatible recipient at the top of the national waiting list following negative cross-match. Following standardised procurement, the pancreas was transported in ice-cold University of Wisconsin solution to the designated isolation facility according to geographical proximity / on-call rota. The service was initially commissioned for England alone with central isolation sites in Oxford and London (King’s College and Royal Free Hospitals sharing the London commitment). A separately commissioned service for Scotland was launched in 2009 with isolation and transplantation in Edinburgh. Isolation for recipients throughout the UK is now shared between Oxford, King’s College London and Edinburgh, with Royal Free Hospital continuing as a non-isolating satellite transplant centre.

2.1.3 Islet isolation from a donor pancreas

Following digestion with Collagenase NB 6 GMP Grade (Serva, Heidelberg, Germany), islets were purified, cultured (0-24 hours), quality-assessed and product-released
(Aldibbiat A, 2012, Huang et al., 2004). Islet preparations allocated to a recipient at a distant transplant centre were transported by road (maximum journey time 8 hours) in cooled standard 500 ml blood transfusion bags (Huang et al., 2004). Following confirmation of satisfactory temperature maintenance and negative Gram stain, maintained integrity and viability were confirmed from a side-arm sample prior to infusion directly from the transport bag. Confirmation of a minimum isolate mass of 3,000 IEQ/kg with islet viability >70% and purity >30% was required for transplantation.

2.1.4 Peri-operative management of islet transplant recipients

Islets were transplanted by percutaneous transhepatic delivery into the portal vein under radiological visualisation. Recipients received adjuvant intravenous insulin and heparin infusions perioperatively, followed by self-administered low molecular weight heparin for 7 days according to published protocols (Koh et al.)(2010). Intensified insulin regimens were continued in all post-discharge, with the goal being maintenance of optimal glycaemic control. Islet recipients were defined as having received ‘transported’ islets if ≥1 of their islet preparations had been isolated at a separate geographical location in the UK and required transport to a second site prior to transplantation.

2.1.5 Study protocol for the follow-up of islet transplant recipients

Severe hypoglycaemia over the preceding 12 months was recorded pre-transplant with careful prospective collection of all severe hypoglycaemic events post-transplant. Formal metabolic assessment was undertaken pre-transplant, at 1 month after each transplant and 3-6 monthly thereafter up to 36 months post-transplant (see Appendix Two). Clinical review included hypoglycaemia awareness assessment (by Clarke/ Gold questionnaires (score ≥4 = impaired awareness of hypoglycaemia)) (Clarke et al., 1995, Gold et al., 1994), HbA1c (TOSOH G7/G8 analyser, Reddich, UK with NGSP (DCCT) standardization until June 2009 and transition to IFCC standardization thereafter), total daily insulin dose and weight, and a standardised Meal Tolerance Test (Greenbaum et al., 2008a) with centralised glucose and C-peptide assay (Perkin Elmer AutoDELFIA, Massachusetts, USA until December 2011; Siemens Immulite 2000, Erlangen, Germany from December 2011; equivalence confirmed including excellent correlation (Pearson’s R² = 0.98)). Ongoing graft function was defined as stimulated serum C-peptide >50 pmol/L. Participants attended fasted and, if on exogenous insulin therapy, were advised
to withhold their pre-breakfast short-acting insulin dose on the day of assessment, with all tests organised to commence at 9 am. The decision to stop insulin therapy post-transplantation was made following review of blood glucose levels by the clinical team, and insulin independence was defined as cessation of insulin for >14 days.

2.1.6 Statistics for the assessment of clinical outcomes in recipients receiving transported and non-transported islet preparations

Data are reported as median and interquartile range. The Wilcoxon Signed Rank test was used to compare pre- versus post-transplant clinical outcomes. Outcomes between groups of recipients receiving transported versus non-transported islet preparations were compared using the Mann-Whitney U-test.
2.2 Continuous glucose monitoring in islet recipients to assess relationship between glycaemic control and endogenous insulin secretion

2.2.1 Continuous glucose monitoring in islet recipients

Recipients of an islet transplant between October 2008 and December 2012 at Newcastle-upon-Tyne Hospitals NHS Foundation Trust were invited to take part in an ethically-approved experimental medicine follow-up study following informed written consent.

In the week preceding metabolic assessments, an iPro continuous glucose monitor (iPro1; Medtronic, Minneapolis, USA) was placed on the anterior abdominal wall of islet recipients. The system registers glucose concentration every 10s and stores an average value every five minutes, within a range of 2.2-22.2 mmol/L (40-400 mg/dL). Participants were blinded to CGM data but were provided with a One Touch blood glucose meter (Lifescan, High Wycombe, UK) with standardized instructions on checking blood glucose at least once every 12 hours to enable standardized CGM calibration. A 3 to 5 day continuous blood glucose monitoring record was obtained in recipients prior to each metabolic assessment, with device removal prior to each meal-tolerance test.

2.2.2 Analysis of CGM records

Data from the sensor and calibration blood glucose meter were uploaded to a computer using Medtronic Solutions software (Medtronic, Minneapolis, USA). Records where mean absolute difference (MAD) between sensor glucose and capillary blood glucose readings exceeded 28% over a 24 hour period of CGM were considered to be at high risk of reflecting inaccurate blood glucose recordings, and were excluded from analysis. Periods of time where the sensor failed to record blood glucose values due to study participants not undertaking sensor calibration were also excluded from analysis.

Duration of normoglycaemia (3-10 mmol/L), hypoglycaemia (<3 mmol/L) and hyperglycaemia (>10 mmol/L) were calculated from the blood glucose sensor record and expressed as percentages of total blood glucose recording time.

Measures of blood glucose variability and estimates of hypo and hyperglycaemia risk were calculated by inputting blood glucose records in the Xcel format into the Easy
GV© computer program (available free for non-commercial use at www.easygv.co.uk) (Hill et al., 2011). Standard deviation of blood glucose (SD), average daily risk ratio (ADRR) (Kovatchev et al., 2006), low blood glucose index (LBGI) and high blood glucose index (HBGI) (Kovatchev et al., 2003) were calculated using this program.

2.2.3 Statistical Analysis of CGM records and assessment of relationship with endogenous insulin secretion in islet recipients

All statistical analysis was undertaken in collaboration with the Newcastle University Department of Statistics.

Relationship of stimulated C-peptide, metabolic status and CGM glucose profile was explored by cross-sectional analysis of all post-transplant assessments. Graft function was determined by the level of stimulated C-peptide achieved at 90 minutes post-stimulus during the meal tolerance test. Beta-cell function was categorised into four pre-defined groups: ‘low function’ C-peptide <200 pmol/L; ‘moderate function’ 200-500 pmol/L; ‘good function’ 500-1000 pmol/L; ‘excellent function’ >1000 pmol/L. C-peptide <200 pmol/L is a historically important cut-off point given previous clinical outcomes reported by DCCT (The Diabetes Control and Complications Trial Research Group (1997)); other cut-off points were selected to allow comparison of CGM outputs in groups of approximately equal size at increasing levels of endogenous C-peptide.

Results for individual recipients are reported as median (minimum to maximum range), results for group comparisons as median (interquartile range). Outcomes obtained from CGM records when graft function was ‘moderate’, ‘good’ or ‘excellent’ were compared to CGM outcomes when graft function was ‘low’ using the Mann-Whitney U-test; the non-parametric Jonckheere-Terpstra test was used to compare CGM outcomes across all groups.

The relationship between C-peptide and blood glucose control was investigated using a mixed Poisson regression model (Stata 12 data analysis and statistical software, Texas, USA). The dependent variable in each analysis was the measure of blood glucose control, either fitted directly or after an appropriate transformation. Variation between patients and variation between observations within patients were included as a random effects; C-peptide was included as a fixed effect. These models were used to generate plots of predicted values of the indicator against a range of C-peptide values.
Proportion of time spent within normoglycaemic range (3-10 mmol/L) was modelled by considering the proportion of time spent outside this range. The dependent variable was the number of 5 minute blocks of time where blood glucose levels were either <3.0 mmol/L or >10.0 mmol/L. A Poisson error structure was assumed for the variation between observations within patients; variation between subjects was assumed to follow a gamma distribution. The total number of blocks observed during a particular observation interval was included as an exposure variable (logₑ [total number of blocks] was included as a covariate with the regression coefficient constrained to 1).

Plots of blood glucose variability against C-peptide suggested a non-linear relationship between the two variables: standard deviation cannot be less than zero and therefore a linear relationship was not plausible. Taking a log transformation resulted in a plausible model and plots that were consistent with a linear trend on the log scale. For the log transformed observations, normal distributions were assumed for both variation between patients and variation between observations within patients.

The analyses of average daily risk ratio, high blood glucose index and low blood glucose index were based on log transformed values. For the low blood glucose index, one was added to all values prior to taking the log transformation.
2.3 Hepatic steatosis assessment in islet recipients

2.3.1 Magnetic resonance imaging in islet recipients

All recipients of an islet transplant between April 2008 and March 2013 at all NHS-funded centres were invited to take part in an ethically approved experimental medicine follow-up study following informed written consent.

All scans were undertaken at the Magnetic Resonance Research Centre, Newcastle University. Primary aims of the study were to invite all participants for post-transplant magnetic resonance imaging to assess hepatic fat content, and to determine the incidence of steatosis post-islet transplantation. Secondary aims were to investigate whether quantity of hepatic fat content had any impact on future graft function. A subset of patients from Newcastle and Manchester were invited for an additional pre-transplant scan for baseline assessment of pre-transplant liver fat content, and for further scans at multiple time-points post-transplant to assess the evolution of any variations in hepatic fat content post-transplantation.

As part of their routine clinical care, islet recipients underwent a liver ultrasound pre-transplant (to assess liver architecture for any abnormalities prior to islet transplantation), one day post-transplant (to assess for thrombosis or haemorrhage) and prior to any further islet transplant (to reassess liver architecture prior to re-transplantation).

2.3.2 Control groups for hepatic steatosis assessment

Liver fat assessment in islet recipients was compared to two recruited control groups: subjects with a previous clinical diagnosis of NAFLD and adults with type 1 diabetes on continuous subcutaneous insulin infusion (CSII). Both control groups provided informed written consent and underwent a single magnetic resonance imaging scan for hepatic fat assessment. A history of hepatic disease, substance abuse or daily consumption of more than one alcoholic drink daily (>20 g/L) were exclusion criteria for recruitment into control groups. At the time of scanning, BMI and waist circumference were recorded and blood was taken for DCCT-aligned HbA1c, lipid profile and liver function tests. Participants with type 1 diabetes had an additional blood sample taken for endogenous C-peptide assessment (Siemens Immulite 2000 assay).
2.3.3 Magnetic resonance imaging protocol

Imaging was performed using a 3.0 T Philips Achieva (Philips, Best, NL) with a six-channel phased array cardiac coil. Multi-slice, two-dimensional spoiled gradient echo sequences were acquired from the liver, with three echoes in separate repetition times. TR= 50 ms, TE = 3.45/4.60/5.75 ms, flip angle = 5°, bandwidth = 435 Hz/pixel, field-of-view 400 x 280 mm, matrix 160 x 156, zero-filled to 1.4 x 1.0 mm resolution, slice thickness = 10 mm. To image the whole liver, three acquisitions were made in separate 17 second expiration breath-holds, acquiring slices per breath-hold. The use of a low flip angle minimised T₁ weighting in the images and hence bias, since the T₁ of hepatic fat is lower than that of water.

Separate fat and water images were computed using the hierarchical IDEAL algorithm of Tsao and Jiang on the complex data acquired (Tsao and Jiang), using a fixed fat model containing 6 resonances to model the spectral complexity of fat at chemical shift (Hamilton et al., 2011). The proton density fat fraction was computed from the separate fat and water images using a magnitude discrimination method (Liu et al., 2007) to preclude noise-related bias. All computations were performed in MATLAB (Mathworks, UK).

2.3.4 Fat map analysis

Proton density fat fraction maps were viewed utilising ImageJ software (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA) for subsequent quantitative region-of-interest (ROI) analysis. Each magnetic resonance scan typically provided 18 axial fat maps of the liver. The three fat maps providing the largest cross-sectional area of liver available for analysis were selected from each set, and a ROI was created for each fat map. The ROI for analysis was selected manually and aimed to cover the largest possible area of liver whilst avoiding visible vessels, hepatic ducts, liver capsule and falciform ligament (which have a higher fat content than neighbouring hepatocytes). ImageJ software was used to generate a histogram of hepatic fat fractions according to pixel shade intensity within the ROI. Mean, minimum, maximum and standard deviation of fat fraction were calculated for each MRI scan following analysis of three ROIs from separate fat maps to provide a quantitative assessment of hepatic fat content (See Appendix Three).
Fat map analysis was undertaken by two assessors (AA and AB) independently to check for reproducibility of quantitative hepatic fat assessment using the method described. Bland-Altman analysis (Bland and Altman, 1986) was used to assess inter-user variability. AB’s measurements were used for all other outcome analyses.

Hepatic fat outcomes are reported as median (interquartile range). Comparisons of hepatic fat between groups were assessed using the Mann-Whitney U-test, whilst comparisons across groups were made using the Kruskal-Wallis test.

2.3.5 Calculation of a heterogeneity index in islet recipients with focal steatosis changes

Two islet recipients were noted to have focal steatosis changes on their post-transplant scans. A method of quantifying this pattern of steatosis was sought, with the aim of producing a measure of heterogeneity for hepatic fat that would differentiate the islet recipient scans with focal steatosis changes from scans with an equivalent hepatic fat content where hepatic fat distribution was more homogeneous. Two areas of approximately 1 cm radius were subjectively selected on three fat maps for each scan: one area was selected to obtain the highest fat concentration possible on the fat map, whilst the second was selected to obtain the lowest possible fat concentration. The mean high fat fraction result for three fat maps was divided by the mean low fat fraction to give a ‘heterogeneity index’. Outcomes were compared to MRI scans from two control subjects with type 2 diabetes identified to have homogeneous hepatic fat distributions and matched for total hepatic fat content to the two islet recipients with focal steatosis changes.
2.4 De novo donor specific antibody assessment in islet recipients and assessment of impact on graft function

Recipients of an islet transplant between October 2008 and March 2013 at Newcastle-upon-Tyne Hospitals NHS Foundation Trust were invited to take part in an ethically-approved experimental medicine follow-up study following informed written consent.

Pancreases from donors without diabetes were allocated to best-matched ABO-compatible recipients according to a national waiting list following a negative cross-match. All previously detected HLA antibodies had been listed as unacceptable mismatches preventing transplantation from donors who expressed antigens to which antibodies had previously been detected. Islet recipients received induction immunotherapy with alemtuzumab, basiliximab or daclizumab pre-transplant; tacrolimus (Prograf: trough 8-12 ng/ml) and mycophenolate (500 mg - 2 g daily) were used for maintenance immunosuppression post-transplant.

2.4.1 Pre-transplantation cross-matching

Crossmatching and HLA antibody screening were performed at the Histocompatibility and Immunogenetics laboratory at the National Health Service Blood and Transplant facility in Newcastle (NHSBT, Newcastle, UK). A T and B cell complement dependent cytotoxicity (CDC) and flow cytometric cross-match was performed pre-transplant.

In the CDC crossmatch, lymphocytes extracted from donor spleen were incubated with patient serum; Dithiothreitol (DTT) was used to determine differential reactivity between IgG and IgM antibodies. A selection of pre-transplant serum samples reflecting the recipient’s immunological profile over this time was used for crossmatching, with a positive IgG T cell cross-match a contraindication to transplantation.

Flow cytometric crossmatching using lymphocytes extracted from donor spleen was performed using a fluorescein isothiocyanate (FITC) anti-human IgG conjugate (Sigma-Aldrich, Dorset, UK). T and B cell populations were separated using Phycoerythrin conjugated CD3 or CD20 (Beckman Coulter, CA, USA) respectively. The Flow cytometry cross-match was considered positive if the mean value of the test sera was greater than the mean plus two standard deviations of six negative controls.
In keeping with UK kidney transplant data, the total number of HLA mismatches was calculated by measuring the mismatches to HLA A, B and DR antigens. Some islet recipients received islets from two donors and therefore recipients could be exposed to up to twelve mismatches at HLA-A, B and DR (2 HLA-A, 2 HLA-B, 2 HLA-DR per transplant). If an HLA-antigen was a repeat mismatch, this was only counted as one mismatch. HLA-C, HLA-DQ and HLA-DP mismatches were also assessed but not included in the conventional total mismatch count.

2.4.2 HLA antibody and DSA assessment

Sera were drawn prospectively pre-transplant, one month after each received islet infusion and at 1, 3, 6, 9 and 12 months post-first transplant for antibody assessment.

Tests were performed to determine the presence or absence of IgG HLA antibodies to HLA-A, B, C, DR, DQ and DP using a Luminex 200 flow cytometer (Luminex Inc., Austin, TX, USA). HLA binding to microparticles coated with HLA molecules was determined using a two-stage screening and identification protocol. All samples were initially screened for the presence or absence of HLA antibodies with the Luminex cytometer using Labscreen mixed HLA antibody screening kits (One Lambda Inc., Canoga Park, CA, USA). A positive result for this mixed screen assay was determined using the manufacturer’s recommended guidelines of more than 1.5 times the negative control bead as positive and 1.2-1.5 times as reactive. HLA antibody specificities were then further determined in any tests that were positive or reactive using Labscreen single antigen kits (One Lambda Inc.). Standard manufacturer’s guidelines were followed to determine a positive reaction, with median fluorescence intensity (MFI) >1000 used as a cut-off value for a positive result. De novo antibodies were defined as a new HLA specificity post-transplant that was not detectable prior to the first islet infusion.

2.4.3 Autoantibody measurement

All available sera drawn for antibody sampling were tested for antibodies to Glutamic acid decarboxylase (GAD), Insulinoma-associated protein 2 antigen (IA-2A) and Islet cell antibody (ICA).
The threshold for GAD positivity was the 99th percentile of control subjects, equivalent to >9.9 arbitrary units (Triturus analyser, ELISA assay supplied by Euroimmun UK, London).

The threshold for IA-2A positivity was the 99th percentile of control subjects, equivalent to >10.0 arbitrary units (ELISA assay, Euroimmun UK, London).

ICA was recorded as ‘negative’, ‘weakly positive’ or ‘positive’ (Indirect Immunofluorescence, monkey pancreas substrate, conjugate used FITC IgG, INOVA diagnostics, San Diego, CA, US).

2.4.4 Statistical analysis of DSA outcome data

Descriptive statistics are presented as median (interquartile range). Clinical outcomes are reported for each individual graft post-transplantation, comparing outcomes for graft with a DSA positive response versus a DSA negative response. Comparisons were performed using the Mann-Whitney U-test, with p<0.05 considered to be significant. Graft failure was defined as stimulated serum C-peptide <100 pmol/L at 90 minutes during MTT assessment. Subsequent investigation of factors that might underlie DSA development in grafts with a DSA positive response versus those without a DSA response was also undertaken using the Mann-Whitney U-test.
CHAPTER THREE

Clinical outcomes in the integrated UK islet transplant program
Chapter 3 - Clinical outcomes in the integrated UK islet transplant program with locally isolated and transported preparations

3.1 Introduction

3.1.1 Islet transplantation restores endogenous insulin production

Restoration of endogenous insulin secretion in C-peptide-negative type 1 diabetes by deceased donor pancreatic islet allograft transplantation has been confirmed (Shapiro et al., 2000, Ryan et al., 2005a). Success has been replicated in centres internationally and clinical outcomes have continued to improve (Barton et al., 2012). Nevertheless, islet transplantation remains an experimental procedure in the majority of programs with widespread availability limited by the expense and complexity of establishing validated cell isolation facilities accessible to all potential recipients (Khan and Harlan, 2009).

3.1.2 The UK islet transplant network

An integrated UK islet transplant network fully funded by the NHS was commissioned in 2008 (Aldibbiat A, 2012) as a clinical intervention for life-threatening recurrent severe hypoglycaemia (See Appendix One). Islet transplantation has been approved by NICE, with the stated treatment aims of prevention of recurrent severe hypoglycaemia (Workgroup on Hypoglycaemia, 2005), achievement of HbA1c <7.0% (<53 mmol/mol), reduction in exogenous insulin requirement and quantification of graft function by assessment of C-peptide secretion (NICE, 2008). In contrast to other islet transplant programs (Ryan et al., 2005a, Vantyghem et al., 2009) achievement of insulin independence is not a primary goal.

Equity of access is a cornerstone of the NHS islet transplant program, with all potential recipients joining a single national waiting list following completion of assessment and offered organs that are optimally procured throughout the UK by the National Organ Retrieval Service. Islets are purified at a designated specialist isolation centre with local transplantation or transport to a distant centre according to patient location.

The islet program has been integrated with the vascularised pancreas transplant program from the outset, with a national agreement for deceased donor organ sharing. Organs procured from donors with a body mass index (BMI) of >30 and <40 kg/m² were used in preference for islet isolation, although organs from donors with a BMI of <30kg/m² not placed for whole organ pancreas transplantation were also offered for clinical islet
isolation. The goal was transplantation of a total transplant mass of >10,000 islet equivalents (IEQ)/kg recipient weight within 12 months of the first transplant, with no further grafts once this mass had been exceeded.

3.1.3 Assessment of clinical outcomes in the UK islet transplant program

From the inception of the NHS-funded UK program, islet recipients have been invited to participate in a prospective follow-up study to determine clinical outcomes following transplantation. The objectives of the current analysis were to determine whether metabolic outcome goals were achieved post-intervention over the first 3 years of the UK program, and whether equity of access to transplantation might be realised if equivalent outcomes were achieved with both locally isolated and transported preparations.

3.2 Methods

See section 2.1 entitled ‘Assessment of clinical outcomes in the UK islet transplant program’ for a description of study methods (See also Appendix Two).
3.3 Results

3.3.1 Islet recipients in the first three years of the UK islet transplant program

A total of 24 individuals received islet transplants over the first three years of the integrated UK islet transplant program. Data are reported in 20 recipients, as two had received initial islet transplants prior to April 2008, one had post-pancreatectomy diabetes and one did not provide research consent.

There were 16 islet transplant alone and 4 islet after kidney transplant recipients; 15 females and 5 males. Age was 49(44-54) years and diabetes duration 30(17-39) years with weight 61.0(55.5-76.0) kg. 12 month metabolic outcomes were obtained in all recipients, and at a median follow-up 24(13.5-36) months (see Table 3.1).

Participants received a total of 35 islet infusions (single graft: n=7; two: n=11; three: n=2). For recipients of a second graft, time to second transplant was 5(3-8.5) months. Donor age was 45(38-53) years with BMI 30.7(26.6-33.4) kg/m². There were 33 donors after brain death (DBD) and 2 donors after circulatory death (DCD) with cause of death intracranial haemorrhage in 67%. Total transplant mass per recipient was 8770(6536-13045) IEQ/kg.

For first transplants, induction was with alemtuzumab (n=13); daclizumab/ basiliximab (n=6); or anti-thymocyte globulin (n=1). For second transplants, alemtuzumab (n=6), daclizumab/ basiliximab (n=4) or no induction agent (n=3) was used. For third transplants, basiliximab was used at induction in one recipient and alemtuzumab in the other. Initial maintenance immunosuppression was tacrolimus (Prograf: trough 8-12 ng/ml)/ mycophenolate mofetil (500 mg - 2 g daily) (n=18); tacrolimus/ sirolimus (n=1); or ciclosporin/ mycophenolate mofetil (n=1)
Table 3.1 – UK islet recipient metabolic outcomes 12 months from time of first islet transplant and median 24 (13.5-36) months post-transplant

<table>
<thead>
<tr>
<th></th>
<th>Pre-transplant</th>
<th>12 months</th>
<th>24 (13.5-36) months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (Female / Male)</td>
<td>15 / 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recipient Age (years)</td>
<td>49 (44-54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>30 (16.5-38.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recipient Weight (kg)</td>
<td>61.0 (55.5-76.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITA / IAK</td>
<td>16 / 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total islet infusions / recipient</td>
<td>2 (1-2)</td>
<td>35</td>
<td>2 (1-2)</td>
</tr>
<tr>
<td>Transplant mass (IEQ/kg) / recipient</td>
<td>8305 (6536-11975)</td>
<td>8770 (6536-13045)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Donor Age (years)</td>
<td>45 (38-52)</td>
<td>45 (38-53)</td>
<td></td>
</tr>
<tr>
<td>Donor BMI (kg/m²)</td>
<td>30.7 (27.2-33.4)</td>
<td>30.7 (26.6-33.4)</td>
<td></td>
</tr>
<tr>
<td>Severe hypoglycaemia</td>
<td>20 (7-50)</td>
<td>0 (0-1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(episodes per patient-year)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>8.0 (7.0-9.6)</td>
<td>6.3 (5.8-7.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(mmol/mol)</td>
<td>64 (53-81)</td>
<td>44.5 (40-54)</td>
<td></td>
</tr>
<tr>
<td>Insulin (units/kg)</td>
<td>0.51 (0.41-0.62)</td>
<td>0.10 (0-0.36)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Incidence of graft survival</td>
<td>16 / 20</td>
<td>16 / 20</td>
<td></td>
</tr>
<tr>
<td>MTT90 C-peptide in recipients with ongoing graft function (pmol/L)</td>
<td>788 (407-1058)</td>
<td>450 (245-949)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table values given as median (interquartile range).

*Comparison of pre-transplant versus outcome at 12 months post-transplant, Wilcoxon Signed Rank test used for statistical analysis

Comparison of pre-transplant versus outcome at median 24 (13.5-36) months post-transplant, Wilcoxon Signed Rank test used for statistical analysis

ITA, islet transplant alone. IAK, islet after kidney transplant. IEQ/kg, islet equivalents/kilogram recipient weight. MTT90 C-peptide, stimulated C-peptide at 90 minutes in meal tolerance test.
3.3.2 Graft function survival and clinical outcomes

Graft function was maintained in 80% of recipients at 24 (13.5-36) months (Figures 3.1 and 3.2).

Severe hypoglycaemia was reduced post-transplant from 20 (7-50) to 0.3 (0-1.6) episodes per patient-year (p<0.001) (Table 3.1 and Figure 3.3A). This includes recipients who had lost graft function, who experienced 0.7 (0.4-1.5) episodes per patient during follow-up. Whereas all participants had experienced severe hypoglycaemia in the 12 months prior to transplantation, 60% of all recipients experienced no recurrent severe hypoglycaemia over 24 (13.5-36) months post-transplant. Hypoglycaemia awareness improved (pre-transplant: Gold score 6 (5-7); 24 (13.5-36) months: Gold score 3 (1.5-4.5); p<0.03).

Significant improvement in overall glycaemic control was achieved with post-transplant HbA1c 6.2 (5.7-8.4)% (Figure 3.3B). HbA1c target of <7.0% was attained in 70% of recipients.

There was a reduction in insulin dose of >50% at 24 (13.5-36) months (pre-transplant: 0.5 (0.4-0.6) units/kg; 24 (13.5-36) months: 0.2 (0-0.4); p<0.001), with cessation of exogenous insulin in 45% of recipients. Rate of graft survival exceeded 70% in both insulin independent and insulin requiring islet recipients (see Figure 3.3A), and of the 9 recipients achieving insulin independence, 3 remained off exogenous insulin at 24 (13.5-36) months (see Figure 3.1).
Figure 3.1 – Bar chart showing graft function up to 36 months post-transplant, indicating timing of subsequent transplants, duration of insulin independence, prevention of recurrent severe hypoglycaemia and maintenance of HbA1c <7.0%.
Figure 3.2 - Kaplan-Meier plots of graft survival up to 36 months post-transplant (n=20).
Figure 3.3 - Box plots of severe hypoglycaemia incidence (A) and HbA1c (B) in all recipients pre- and 24 (13.5-36) months post-first islet transplant (n=20). Box indicates median, upper quartile and lower quartile ranges. Whiskers plotted with the lowest datum within 1.5 times the interquartile range of the lower quartile and the highest datum within 1.5 times the interquartile range of the upper quartile. *P < 0.001 vs. pre-transplant, Wilcoxon Signed Rank test.
3.3.3 Comparison of clinical outcomes in recipients receiving transported versus non-transported islet preparations

Nine recipients (45%) received ≥1 transported islet preparation (Table 3.2). Pre-transplant, recipients of solely locally isolated islets were well-matched with those who received transported preparations, with the exception of significantly higher HbA1c in the group receiving transported islets. Donor age, number of islet infusions and islet transplant mass were not significantly different between groups, although donor BMI was noted to be higher in recipients receiving transported islets. Islets were transported prior to transplantation from both DCD donors. Islet viability was high in both groups (median ≥90%), although higher in recipients of local islets. No difference in islet purity was noted.

No significant difference in metabolic outcomes was noted in recipients of transported and only locally isolated islets at 12 months follow-up (Table 3.2), with graft function well maintained in both groups up to 36 months post-transplant (Figure 3.4B).

Three of the 9 recipients who received transported islet preparations also received a locally isolated preparation. To further investigate whether islet transport had any influence on metabolic outcome, a reanalysis of outcomes from the six individuals (30%) who had received only transported islets was undertaken. These patients achieved a reduction in severe hypoglycaemia (35(4-50) events/year pre-transplant vs 0(0-4) events/year post-transplant); and HbA1c (9.6(8.8-10.3)% pre-transplant vs 6.4(5.9-9.1%) post-transplant). There were no significant differences in metabolic outcomes compared to the 11 recipients receiving solely locally isolated preparations (p-value for between-group differences: 12-month severe hypoglycaemia rate: p=1.00; HbA1c: p=0.65).
<table>
<thead>
<tr>
<th></th>
<th>Recipients of local isolations only</th>
<th>Recipients of ≥1 Transplanted preparation</th>
<th>*P</th>
<th>All Local vs ≥1 Transplanted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-transplant</td>
<td>12 months</td>
<td>*p</td>
<td>Pre-transplant</td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Sex (Female / Male)</td>
<td>8 / 3</td>
<td></td>
<td></td>
<td>7 / 2</td>
</tr>
<tr>
<td>Recipient Age (years)</td>
<td>48 (39-51)</td>
<td></td>
<td></td>
<td>54 (45-58)</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>31 (23-40)</td>
<td></td>
<td>0.51</td>
<td>27 (15-39)</td>
</tr>
<tr>
<td>Recipient Weight (kg)</td>
<td>61.0 (57.0-76.0)</td>
<td></td>
<td></td>
<td>59.0 (54.0-76.0)</td>
</tr>
<tr>
<td>Donor Age (years)</td>
<td>45 (38-53.5)</td>
<td></td>
<td></td>
<td>43 (37-50)</td>
</tr>
<tr>
<td>Donor BMI (kg/m²)</td>
<td>28.6 (24.0-32.7)</td>
<td></td>
<td></td>
<td>33.0 (29.1-34.3)</td>
</tr>
<tr>
<td>ITA / IAK</td>
<td>8 / 3</td>
<td></td>
<td></td>
<td>8 / 1</td>
</tr>
<tr>
<td>Number of infusions</td>
<td>2 (1-2)</td>
<td></td>
<td></td>
<td>2 (1-2)</td>
</tr>
<tr>
<td>Transplant mass (IEQ/kg)</td>
<td>8250 (6950-13393)</td>
<td></td>
<td>0.02</td>
<td>10919 (5799-11950)</td>
</tr>
<tr>
<td>Islet isolate viability (%)</td>
<td>95 (90-95)</td>
<td></td>
<td></td>
<td>90 (89-90)</td>
</tr>
<tr>
<td>Islet isolate purity (%)</td>
<td>70 (45-73)</td>
<td></td>
<td>0.43</td>
<td>70 (58-75)</td>
</tr>
<tr>
<td>Severe hypoglycaemia (episodes per patient-year)</td>
<td>15 (10-50)</td>
<td>0 (0-1)</td>
<td>&lt;0.01</td>
<td>21 (3.5-50)</td>
</tr>
<tr>
<td>HbA1c (%) (mmol/mol)</td>
<td>7.4 (7.0-8.2)</td>
<td>6.4 (5.8-7.1)</td>
<td>&lt;0.01</td>
<td>7.6 (7.6-10.0)</td>
</tr>
<tr>
<td>Insulin (units/kg)</td>
<td>0.49 (0.40-0.61)</td>
<td>0.10 (0.0-0.30)</td>
<td>&lt;0.02</td>
<td>0.51 (0.38-0.63)</td>
</tr>
<tr>
<td>Fasting C-peptide (pmol/L)</td>
<td>214 (70-293)</td>
<td></td>
<td></td>
<td>403 (52-434)</td>
</tr>
<tr>
<td>MTT90 C-peptide (pmol/L)</td>
<td>407 (126-830)</td>
<td></td>
<td></td>
<td>788 (114-1764)</td>
</tr>
</tbody>
</table>

Table 3.2 – Recipient metabolic outcomes 12 months following first transplant: Locally isolated islet preparations versus ≥1 transported islet preparation.
Figure 3.4 - Kaplan-Meier plots of graft survival comparisons up to first 36 months post-transplant. Figure 3.4A is a comparison of recipients achieving insulin independence (interrupted line) (n=9) versus those requiring insulin throughout follow-up (solid line) (n=11); Figure 3.4B is a comparison of those receiving only locally isolated (interrupted line) (n=11) versus transported (solid line) (n=9) islet preparations.
3.3.4 Complications and renal function post-transplant

There were no episodes of significant haemorrhage, thrombosis or other major peri-operative adverse events. Following discharge from hospital, one participant received granulocyte colony stimulating factor (GCSF) for asymptomatic neutropenia. One participant was treated for shingles and a second for folliculitis but none developed systemic sepsis over the first 12 months post-transplant. No new neoplastic disease was detected. Renal function was not altered significantly at 24 (13.5-36) months post-transplant (creatinine: pre-transplant 85 (75-104) μmol/l, post-transplant 103(85-133) μmol/l; p=0.91).
3.4 Discussion

3.4.1 Primary goals of the UK islet transplant program achieved: prevention of recurrent severe hypoglycaemia with improvement in glycaemic control

The primary goals of the UK islet transplantation program are to prevent recurrent life-threatening hypoglycaemia and restore optimal overall glycaemic control in recipients, regardless of geographical proximity to an isolation facility. This study has shown that these goals have been attained up to 36 months post-transplant.

Since definitive confirmation of the potential for at least short term insulin independence following islet transplantation by the Edmonton group, the debate has continued around whether the primary goal should be freedom from exogenous insulin replacement (Hering et al., 2005) or prevention of debilitating severe hypoglycaemia without sacrificing overall glycaemic control (Lehmann et al., 2008).

The UK health service made a decision to make this therapy available to patients with type 1 diabetes who have life-threatening hypoglycaemia and loss of hypoglycaemia awareness, with the primary goal being elimination of severe hypoglycaemia requiring third-party intervention. To underpin equitable sharing of the restricted potential donor pool between solid pancreas and isolated islet transplant recipients, it was agreed that no more than two transplants per recipient would be undertaken, in the absence of exceptional circumstances.

Further agreement has been reached nationally on congruent indications for pancreas and islet transplantation alone, with the primary listing criterion being recurrent severe hypoglycaemia despite optimised conventional self-management. Individuals are assessed for both procedures, with the majority rapidly reaching a definitive decision about whether they wish the more invasive procedure of pancreatic transplantation with increased mortality risk but prospect of long-term insulin independence (White et al., 2009), or the lower risk procedure of islet transplantation with the prospect of optimal metabolic control despite maintained insulin therapy. With the focus on freedom from severe hypoglycaemia, transplant physicians in the UK tend to intensify diabetes self-management post-islet transplantation rather than to attempt insulin withdrawal in all recipients, as we believe that maintained exogenous insulin may preserve islet transplant function and provide longer term metabolic benefits.
The UK program has realised its commitment to limit number of islet infusions per recipient, with 35% receiving single grafts. Given this and overall transplant mass of <10,000 IEQ/kg, insulin independence rate of <50% is not unexpected (Ryan et al., 2005a). In one recent single centre study, insulin independence was achieved in 14 consecutive islet recipients (Vantyghem et al., 2009). This necessitated a mean of 2.7 grafts per recipient with overall transplant mass 12,479 IEQ/kg. After median follow-up of 3.3 years, insulin independence had fallen to 58% of recipients. Notably, in that study all transplants for a single recipient were completed within less than 3.5 months. In contrast, median time to second transplant in the UK cohort was 5 months, with a range of 1-13 months.

The alternative paradigm with primary goal of optimal glycaemic control and avoidance of severe hypoglycaemia as opposed to insulin independence has been adopted by the Zurich group (Lehmann et al., 2008). This group reported equivalent metabolic outcomes to solid organ transplantation after simultaneous islet kidney transplantation (Gerber et al., 2008). Although one year insulin independence rate was lower in islet recipients (31% versus 96% in simultaneous pancreas kidney recipients), incidence of serious adverse events was also much lower. In that study, 38% of islet recipients received three or more infusions with a maximum of five and mean of 2.2±1.3 transplants per recipient.

3.4.2 Equivalent clinical outcomes achieved with transported and non-transported islet preparations, allowing equity of access to islet transplantation across the UK

Clinical success has previously been reported with transported islets (Kessler et al., 2004, Barshes et al., 2004) but a direct comparison of local and distant recipients who have been transplanted according to nationally agreed congruent protocols has not been published before.

Existing programs have been configured around a single isolation facility providing islets for both local and distant recipients. The UK program is unique in being fully integrated, dependent on the isolation facilities transplanting both locally isolated islets and preparations transported from another facility. This has enabled equitable allocation of donor pancreas directly to the most appropriate recipient nationally, regardless of which isolation facility is on-call. Establishment of a successful integrated islet and
vascularised pancreas transplant program has facilitated implementation of a unique organ pancreas allocation scheme, with equitable provision of organs to both whole organ and isolated islet recipients on a common national waiting list. This was launched in December 2010, and it is hoped the updated scheme will lead to further improvements in UK islet transplant outcomes through the provision of higher quality donor organs than those rejected for whole organ transplantation. Islet recipients awaiting a second transplant will be given enhanced priority under the new scheme, reducing time between transplants. Alongside changes to the allocation scheme, the impact of adjuvant peri-transplant anti-tumour necrosis factor-alpha therapy - associated with higher insulin independence rates in leading centres internationally (Bellin et al., 2012) - is now being evaluated in UK recipients.

3.4.3 Limitations in the clinical outcomes study

Limitations of the study include absence of an intensified conventional therapy control group. Nevertheless, listing for islet transplantation can only be considered following specialist diabetologist confirmation that fully optimised medical management has not been successful in preventing recurrent severe hypoglycaemia. Evidence of the severity of risk in UK prospective islet transplant recipients has been underlined by death of three patients on the national waiting list – all due to hypoglycaemic crisis. Numbers remain relatively low for definitive comparison of transported versus non-transported preparations. However, the current study provides sufficient evidence of metabolic success for UK recipients of both locally isolated and transported islets to justify ongoing central funding.
CHAPTER FOUR

Endogenous C-peptide determines blood glucose control outcomes in islet recipients
Chapter 4 - Endogenous C-peptide determines blood glucose control outcomes in islet recipients assessed by continuous blood glucose monitoring

4.1 Introduction

4.1.1 Residual endogenous insulin secretion is associated with a reduced incidence of microvascular complications in individuals with type 1 diabetes

Type 1 diabetes is widely considered to be a disease of absolute insulin deficiency after the honeymoon period but it has long been recognised that small amounts of retained endogenous insulin production are associated with reduced risk of complications and hypoglycaemia (The Diabetes Control and Complications Trial Research Group (1997), (1998), (Steffes et al., 2003). The Diabetes Control and Complications Trial noted that individuals with ongoing endogenous insulin production had a reduced rate of microvascular complications and were less prone to experiencing hypoglycaemia (The Diabetes Control and Complications Trial (1997)). Complication rates remained reduced if endogenous insulin production was maintained by achievement of tight glycaemic control in the years following diagnosis of type 1 diabetes (The Diabetes Control and Complications Trial Research Group (1998)). These clinical findings suggest a legacy effect, where individuals with type 1 diabetes may have improved blood glucose control and be protected from vascular complications in later life if endogenous insulin production is maintained (Sjoberg et al., 1987, Panero et al., 2009).

4.1.2 Islet recipients are a unique population in which to explore the relationship between endogenous insulin production and glycaemic control

Transplantation of isolated pancreatic islets offers beta-cell replacement for individuals with type 1 diabetes through a minimally invasive percutaneous infusion into the hepatic portal vein. Stimulated C-peptide levels are frequently measured in islet recipients and can be assessed alongside intensive glucose monitoring within an individual. Graft function in islet recipients can vary according to the quality and number of islets received, and therefore stimulated endogenous insulin production varies between individuals. In addition, insulin production within an individual varies with time from transplantation, and can decline in the years following transplantation (Ryan et al., 2005a). Previous studies have shown that increased insulin production is associated with improved clinical outcomes in islet transplant recipients (Vantyghem et
al., 2012, Ryan et al., 2005a), with evidence suggesting that even low levels of endogenous C-peptide restoration may be associated with a reduction in hypoglycaemia risk and awareness (Leitao et al., 2008, Vantyghem et al., 2009). The relationship between a measured level of endogenous insulin production and its impact on day-to-day glycaemic control has yet to be defined.

4.1.3 Continuous glucose monitoring for the assessment of blood glucose control and the risk of hyperglycaemia or hypoglycaemia

Continuous glucose monitoring (CGM) is used to give a measure of glycaemic control by providing a detailed glucose time series (288 readings / 24hrs). Scores developed to assess hyperglycaemia and hypoglycaemia risk (Kovatchev et al., 2006, Hill et al., 2007, Kovatchev et al., 1998, Kovatchev et al., 2002) can be calculated from CGM records (Hill et al., 2011). They provide further information about the impact an intervention can have on clinical outcomes than merely the duration of time spent hyperglycaemic and hypoglycaemic: they are especially relevant to our islet recipients, given that we are undertaking an intervention to reduce the risk of severe hypoglycaemia.

Risk indices - like the low blood glucose index (LBGI) and high blood glucose index (HBGI) (Clarke and Kovatchev, 2009) - try to overcome the dilemmas of hyperglycaemia and hypoglycaemia influence on measures of blood glucose variability. The LBGI and HBGI split the overall glucose variation into two independent sections related to excursions into hypo and hyperglycaemia, equalising the amplitude of the excursions with respect to the risk they carry. In a patient with diabetes, a drop in glucose levels does not have to be large to increase the risk of hypoglycaemia substantially, whilst increases in blood glucose have to be large prior to the patient being put at risk of a clinical event. LBGI is a summary statistic obtained from blood glucose readings that increases as frequency and extent of hypoglycaemia episodes increase. It has been used to predict future severe hypoglycaemia (Kovatchev et al., 1998) but its use is specifically designed to assess hypoglycaemia risk. Similarly, the HBGI is designed specifically to assess hyperglycaemia risk (Kovatchev et al., 2002).

The average daily risk range (ADRR) (Kovatchev et al., 2006) takes into account the asymmetric nature of the blood glucose scale and provides a measure of event severity. As a result, it does not just predict outlier blood glucose readings but is also linearly...
associated with progressively higher frequency and severity of events according to blood glucose level. This overcomes the insensitivity to hypoglycaemia and inherent bias towards hyperglycaemia of some blood glucose variability measures.

4.1.4 Aims of CGM assessment in islet recipients
The relationship between endogenous C-peptide secretory capacity and parameters of glycaemic control was assessed by undertaking CGM in islet recipients one week prior to a mixed meal C-peptide response (Palmer et al., 2004) at multiple time intervals post-transplant. As well as defining the duration of time spent within defined blood glucose ranges, a detailed assessment of blood glucose variability, hyperglycaemia risk and hypoglycaemia risk was undertaken to show how this related to endogenous insulin production.

4.2 Methods
See section 2.2 entitled ‘Continuous glucose monitoring in islet recipients to assess relationship between glycaemic control and endogenous insulin secretion’ for a description of study methods.
4.3 Results

4.3.1 Islet recipient characteristics and collected CGM data

Twelve consecutive islet transplant recipients were recruited (9 islet transplant alone, 3 islet after kidney) (Figure 4.1, Table 4.1). Participants received a total of 20 islet transplants (single graft: n=5; two: n=6; three: n=1; median islet equivalents per kilogram (IEQ/kg) per recipient 11232 (IQR 8577-12267) IEQ/kg; per graft 5830 (4943-7118) IEQ/kg). Median follow-up time was 18 (12-29) months post-transplant, and during this time participants underwent 5 (4-9) clinical assessments. Timing of clinical assessments during follow-up is shown in Figure 4.1.

Restoration of C-peptide positivity was associated with recovery of hypoglycaemia awareness, resolution of recurrent severe hypoglycaemia and improvement in HbA1c (Table 4.1). One recipient achieved and maintained insulin independence, with the remainder continuing on reduced dose insulin therapy post-transplant (insulin requirement pre-transplant 0.60 (0.49-0.72) units/kg; post-transplant 0.42 (0.25-0.54) units/kg).

74 CGM records were obtained, providing 7211 hours CGM data for analysis: 70 records were paired with meal tolerance tests, with a further 4 records obtained between assessments. Records were obtained in recipients across a range of graft functions (Table 4.2): 19 records were obtained in 8 recipients at a time when graft function was ‘low’ (<200 pmol/L, total group duration of CGM analysed 1608 hours, median 90 (58-120) hours/recipient/visit) (Figure 4.3A); 15 records were obtained in 7 recipients when graft function was ‘moderate’ (200-500 pmol/L, total group duration of CGM analysed 1487 hours, median CGM duration 108 (90-120) hours/recipient/visit) (Figure 4.3B); 17 records were obtained in 7 recipients when function was ‘good’ (500-1000 pmol/L, total group duration of CGM analysed 1592 hours, median CGM duration of 96 (78-120) hours/recipient/visit) (Figure 4.3C) and 23 records were obtained in 4 recipients when function was ‘excellent’ (>1000 pmol/L, total group duration of CGM analysed 2524 hours, median CGM duration 120 (96-120) hours/recipient/visit) (Figure 4.3D).

4.3.2 Relationship of blood glucose control with endogenous C-peptide production

There was a progressive reduction in mean glucose and standard deviation of blood glucose with higher levels of graft function. Duration of hypoglycaemia,
normoglycaemia, hyperglycaemia and measures of hypoglycaemia and hyperglycaemia risk all improved with increasing graft function (Jonckheere-Terpstra analysis, p<0.001) (Table 4.2, Figures 4.2, 4.3). Improvements in HbA1c and reduced insulin requirements were also associated with increasing levels of graft function (Table 4.2, Figure 4.4).
Figure 4.1 – Bar chart of islet graft function from time of first islet transplant in individual recipients, indicating timing of subsequent transplants. Endogenous C-peptide (median, range) and CGM hours analysed (median, range) from post-transplant assessments are also reported for each recipient.
<table>
<thead>
<tr>
<th></th>
<th>Pre-transplant</th>
<th>Post-transplant</th>
<th><em>p</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Sex (Female / Male)</td>
<td>10 / 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recipient Age (years)</td>
<td>51.5 (47.5-57)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>38.5 (26-42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-treatment insulin regimen – CSII / MDI</td>
<td>5 / 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recipient Weight (kg)</td>
<td>61.7 (55.9-72.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITA / IAK</td>
<td>9 / 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total islet infusions received</td>
<td></td>
<td>20 (1-2)</td>
<td></td>
</tr>
<tr>
<td>Number of islet infusions / recipient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transplant mass (IEQ/kg) / recipient</td>
<td></td>
<td>11232 (8577-12267)</td>
<td></td>
</tr>
<tr>
<td>Donor Age (years)</td>
<td>48 (41-53)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor BMI (kg/m²)</td>
<td>31.0 (29.2-33.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follow-up post-transplant (months)</td>
<td>18 (12-29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assessments post-transplant</td>
<td>5 (4-9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annualised severe hypoglycaemia rate (episodes per patient-year)</td>
<td>12 (4-50)</td>
<td>1 (0-2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Clarke score</td>
<td>6 (5-7)</td>
<td>3 (1-5)</td>
<td>0.03</td>
</tr>
<tr>
<td>Gold score</td>
<td>6 (5-7)</td>
<td>2 (1-4)</td>
<td>0.01</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>9.7 (8.9-10.6)</td>
<td>7.4 (6.5-8.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(mmol/mol)</td>
<td>83 (74-92)</td>
<td>57 (48-66)</td>
<td></td>
</tr>
<tr>
<td>Insulin requirement post-transplant (units/kg)</td>
<td>0.60 (0.49-0.72)</td>
<td>0.42 (0.25-0.54)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>91 (69-119)</td>
<td>99 (89-109)</td>
<td>0.13</td>
</tr>
<tr>
<td>MTT90 C-peptide (pmol/L)</td>
<td>483 (178-853)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1 – Islet recipient pre-transplant characteristics and metabolic outcomes pre-transplant versus post-transplant.

*Comparison of pre-transplant versus outcome post-transplant, Wilcoxon Signed Rank test used for statistical analysis.
Abbreviations: CSII - continuous subcutaneous insulin infusion, ITA - islet transplant alone, IAK - islet after kidney transplant, IEQ/kg - islet equivalents/kilogram recipient weight, MDI - multiple daily insulin injections, MTT90 C-peptide - stimulated C-peptide at 90 minutes in meal tolerance test.
| Graft function at  
<table>
<thead>
<tr>
<th>time of CGM</th>
<th>Glycaemia duration</th>
<th>Glucose variability</th>
<th>Quality of glycaemic control</th>
<th>Clinical Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trace analysis per recipient (Hours)</td>
<td>Hypo (&lt;3 mmol/l) (%)</td>
<td>Normal (3-10 mmol/l) (%)</td>
<td>Hyper (&gt;10 mmol/l) (%)</td>
</tr>
<tr>
<td>Low (&lt;200 pmol/l) (n=19)</td>
<td>90 (58-120)</td>
<td>0.5 (0-2.4)</td>
<td>47.3 (31.8-76.0)</td>
<td>51.7 (24.8-71.1)</td>
</tr>
<tr>
<td>Moderate (200-500 pmol/l) (n=15)</td>
<td>108 (90-120)</td>
<td>0 (0-1.5)</td>
<td>57.7 (43.0-72.7)</td>
<td>41.7 (26.7-57.0)</td>
</tr>
<tr>
<td>Good (500-1000 pmol/l) (n=17)</td>
<td>96 (78-120)</td>
<td>0 (0-0.6)</td>
<td>75.4 (59.9-96.7)</td>
<td>21.3 (3.1-40.1)</td>
</tr>
<tr>
<td>Excellent (&gt;1000 pmol/l) (n=23)</td>
<td>120 (96-120)</td>
<td>0 (0-0.3)</td>
<td>94.7 (87.4-98.8)</td>
<td>5.3 (1.2-12.2)</td>
</tr>
</tbody>
</table>

*Comparison of outcomes across all groups of graft function, using Jonckheere-Terpstra significance test.

**Table 4.2** – Islet recipient pre-transplant characteristics and metabolic outcomes pre-transplant versus post-transplant

|  


All values expressed as median (IQR).
Figure 4.2A & 4.2B Continuous glucose monitoring profiles recorded over 72 hours in islet recipients with low graft function (A), moderate graft function (B)
Figure 4.2C & 4.2D Continuous glucose monitoring profiles recorded over 72 hours in islet recipients with good graft function (C) and excellent graft function (D).
Figure 4.3 (next pages) - Box plots of time spent in normoglycaemic range (A), hyperglycaemic range (B), hypoglycaemic range (C), mean glucose (D), standard deviation of glucose (E), average daily risk ratio (F), high blood glucose index (G) and low blood glucose index (H) in islet recipients according to continuous glucose monitoring outcomes taken when graft function is low (n=19), moderate (n=15), good (n=17) and excellent (n=23). Box indicates median, upper quartile and lower quartile ranges. Whiskers plotted with the lowest datum within 1.5 times the interquartile range of the lower quartile and the highest datum within 1.5 times the interquartile range of the upper quartile. *P < 0.01 vs. low graft function, Mann-Whitney U-test.
**E**

Box plots showing the standard deviation of blood glucose (mmol/L) across different graft functions: Low, Moderate, Good, and Excellent.

**F**

Box plots showing the average daily risk ratio across different graft functions: Low, Moderate, Good, and Excellent.
Figure 4.4 - Box plots of HbA1c (A) and insulin dose (B) in islet recipients when graft function is low, moderate, good and excellent. Box indicates median, upper quartile and lower quartile ranges. Whiskers plotted with lowest datum within 1.5 times interquartile range of the lower quartile and highest datum within 1.5 times interquartile range of the upper quartile. *P < 0.01 vs. pre-transplant, Mann-Whitney U-test.
4.3.3 Relationship of endogenous C-peptide with proportion of time spent within glucose range 3.0 to 10.0 mmol/L, glucose variability, average daily risk ratio, hyperglycaemia and hypoglycaemia risk

There was a strong association between C-peptide and time spent outside the normal glycaemic range, blood glucose standard deviation, average daily risk ratio and high blood glucose index. For each increase of 100 pmol/L in endogenous C-peptide, proportion of time spent outside a glucose range of 3.0 to 10.0 mmol/L reduced by 12.9% (95% CI: 12.6% to 13.2%) (Figure 4.5A), standard deviation of blood glucose reduced by 4.92% (95% CI: 3.41%, 6.40%) (Figure 4.5B), average daily risk ratio reduced by 6.71% (95% CI: 4.33%, 9.02%) (Figure 4.5C), and mean high blood glucose index reduced by 9.52% (95% CI: 6.76%, 12.19%) (Figure 4.5D).

Hypoglycaemia risk was reduced with increasing level of C-peptide (Figure 4.5E), although a random effects normal regression model provided no evidence of a significant linear association between the log transformed low blood glucose and C-peptide.
Figure 4.5 (next pages) – Regression model plots showing relationship of endogenous C-peptide production with proportion of time glucose 3.0 to 10.0 mmol/L (A), blood glucose standard deviation (B), average daily risk ratio (C), high blood glucose index (D) and low blood glucose index (E).

**Figure Notes:**

The relationship between C-peptide and blood glucose control was investigated using a mixed Poisson regression model. The dependent variable in each analysis was the measure of blood glucose control, either fitted directly or after an appropriate transformation. Variation between patients and variation between observations within patients were included as a random effects; C-peptide was included as a fixed effect.

The 95% confidence intervals for each plot are for the estimated population parameter. For example, in Figure 4.5A the mean proportion of time spent with glucose 3.0 to 10.0 mmol/L corresponds to the value of C-peptide production given on the x-axis. At a 90 minute C-peptide production of 200 pmol/L, it is 95% certain that in the population of patients from which this sample is drawn, the mean proportion of time spent with glucose 3.0 to 10.0 mmol/L will be between 41% and 64%. It is not implied that 95% of patients with this level of graft function will have observed levels of blood glucose that fall in this range; it is assumed that across all patients the proportion of time spent in the normal range will vary randomly about the population mean. The observations that correspond to values of C-peptide production provide a slope parameter of -0.0013799 (with 95% CI: -0.0014134 to -0.0013465) on the exponential scale. The relationship suggests that for every increase of 100 pmol/L in C-peptide, the proportion of time spent outside glucose 3.0 to 10.0 mmol/L will be reduced by 12.9% (with 95% CI: 12.6% to 13.2%).
C

![Graph showing the relationship between graft function 90 minute C-peptide (pmol/L) and average daily risk ratio.](image)

D

![Graph showing the relationship between graft function 90 minute C-peptide (pmol/L) and high blood glucose index.](image)

108
E

![Graph showing the relationship between graft function 90 minute C-peptide (pmol/L) and low blood glucose index.](image)
4.4 Discussion

4.4.1 Restoration of endogenous C-peptide production improves CGM outcomes

This study used regular assessment of C-peptide and CGM outcomes in islet transplant recipients to demonstrate a continuous relationship between stimulated C-peptide and multiple clinical and metabolic parameters of blood glucose control. It provides clear evidence that restoration of C-peptide improves CGM outcomes, and quantifies how much improvement in blood glucose control might be expected with increases in endogenous C-peptide secretory capacity.

CGM has been used to assess blood glucose control post-islet transplantation previously to demonstrate that duration of hypoglycaemia and hyperglycaemia is reduced following re-establishment of endogenous C-peptide production post-transplantation (Kessler et al., 2002, Vantyghem et al., 2012, Geiger et al., 2005, Paty et al., 2006, Faradji et al., 2006, Gorn et al., 2008). One group used the beta-score as measure of graft function and suggested that improvements in hypoglycaemia duration might be anticipated at lower levels of graft function before improvements in mean glucose, glucose variability and hyperglycaemia (Vantyghem et al., 2012, Ryan et al., 2005b).

A greater understanding of the impact of endogenous C-peptide capacity on blood glucose parameters is now provided by application of the additional CGM analysis techniques used in this study. Stratification of outcomes according to endogenous C-peptide production allowed initial assessment of the relationship between endogenous C-peptide and metabolic and clinical outcomes. It was noted that when measured level of endogenous C-peptide reduced in a recipient, paired CGM revealed a deterioration in blood glucose control parameters at the time of C-peptide assessment, even if excellent graft function and desirable glucose control outcomes had been achieved previously. Re-transplantation and increase in endogenous C-peptide production resulted in improvement in CGM outcomes once more, suggesting endogenous C-peptide was integral to clinical outcome.

4.4.2 This study supports the original findings of the DCCT

Eleven out of twelve recipients remained on insulin post-transplant to achieve the best possible level of blood glucose control. Increased C-peptide production was still associated with improved clinical outcomes, with less insulin required to achieve a
better HbA1c at higher endogenous C-peptide levels. The benefits of endogenous insulin production on blood glucose control were originally confirmed in the DCCT, in which the intensively treated group could be subdivided according to residual C-peptide secretion at entry into the trial: the subgroup with the highest stimulated C-peptide (>200 pmol/L) had a significantly lower HbA1c over the first four years of the study, 50% less risk of progression of retinopathy and 65% less risk for severe hypoglycaemia with seizure or coma (The Diabetes Control and Complications Trial Research Group (1998)). Beyond outcomes from the DCCT, the evidence base for the advantages of maintained C-peptide production in individuals with type 1 diabetes has been scarce. The outcomes of this study support the original conclusions of the DCCT (Lachin et al., 2013, Steffes et al., 2003): glycaemic control is improved and risk of hypoglycaemia is reduced with increasing levels of endogenous C-peptide production.

4.4.3 Endogenous C-peptide has a continuous relationship with parameters of blood glucose control

In our islet recipient cohort, endogenous C-peptide was subsequently found to have a continuous relationship with duration of blood glucose levels between 3.0 to 10.0 mmol/L, blood glucose variability and hyperglycaemia / hypoglycaemia risk down to the lowest levels of detectable C-peptide. Subanalysis of data in assessments obtained only from individuals with a C-peptide of <200 pmol/L showed that the continuous relationship between these outcomes and measured levels of graft function was maintained. This supports the findings of a recently published study that used glucose clamps to assess functional beta-cell mass in islet recipients, and demonstrated a correlation between HbA1c, insulin dose and glycaemic variability even at very low levels of functional beta-cell mass (Gillard et al., 2013). Even low levels of endogenous C-peptide production may be of clinical benefit, supporting the hypothesis that glycaemic control might be easier in the ‘honeymoon period’ of type 1 diabetes, and providing a possible explanation as to why individuals who retain low levels of endogenous C-peptide may find blood glucose control easier than C-peptide negative individuals in the longer-term.

Improvement in endogenous C-peptide may have immediate implications for predisposition to hypo or hyperglycaemia. LBGI and HBGI split overall glucose variation into two independent sections related to excursions into hypoglycaemia and hyperglycaemia, equalising the amplitude of the excursions with respect to the risk they
carry (Kovatchev et al., 2002). ADRR is designed to be equally sensitive to hypoglycaemia and hyperglycaemia risk (Kovatchev et al., 2006), taking into account the asymmetric nature of the blood glucose scale and providing a measure of event severity. At levels of excellent function (>1000 pmol/L), HBGI, LBGI and ADRR were reduced to low risk scores. Regression analysis in this study demonstrated that ADRR, HBGI and LBGI have a continuous relationship with endogenous C-peptide capacity, although higher levels of endogenous C-peptide production were required to influence LBGI significantly post-transplant and the risk reduction in LBGI achieved with an increase in endogenous C-peptide capacity was less predictable than that for HBGI or ADRR.

4.4.4 Relationship between endogenous C-peptide secretion and incidence of severe hypoglycaemia is complex

Prevention of severe hypoglycaemia may be more complex than simply re-establishing ‘metabolic memory’ following a period of freedom from hypoglycaemia in islet transplant recipients. Improvement in HbA1c and insulin dose occurred with increasing graft function but although islet transplantation dramatically reduced the frequency of severe hypoglycaemia in the recipient cohort, a relationship between endogenous C-peptide secretion and incidence of severe hypoglycaemia was more difficult to establish. Severe hypoglycaemia is an infrequent ‘all-or-nothing’ event that is often reported as an annualised rate; it is therefore difficult to establish a relationship with graft function, which might vary throughout the year. There remains the possibility that only a very low level of endogenous C-peptide is required to substantially reduce risk of severe hypoglycaemia.

4.4.5 Precautions regarding CGM interpretation

Caution must be applied to interpretation of this study’s results, given the small number of participants in the study and the limitations of current CGM sensors. The presence of a time lag between interstitial glucose values and actual plasma blood glucose must be accepted, and this has been reported to be 7.94+/−6.48 minutes for the iPro Enlite sensor worn in the abdominal area (Keenan et al., 2012). The MAD between the iPro Enlite sensor glucose and blood sample plasma glucose has been reported to be 13.86% in adults (Keenan et al., 2012). These inconsistencies have been shown to be increased outside of the normoglycaemic range, with clinical accuracy between actual interstitial
glucose and that recorded by the monitoring device as low as 62%, suggesting the error to be due to engineering rather than physiological difficulties (Clarke and Kovatchev, 2007). Although CGM systems might generate statistically acceptable results, there is particular concern regarding their accuracy during the hypoglycaemic blood glucose range (Clarke et al., 1987, Zung and Zadik, 2002).

CGM records that exceeded an MAD of 28% over a 24-hour period were excluded from statistical analysis but caution must remain with regards to the interpretation of our results, especially outside of the normoglycaemic range. However, this study has analysed over 7,000 hours of CGM data before drawing its conclusions, and continuous measurement of interstitial glucose levels by CGM has allowed assessment of blood glucose variability, hyperglycaemia risk and hypoglycaemia risk in individuals with varying levels of endogenous C-peptide secretory capacity (Hill et al., 2011, Clarke and Kovatchev, 2009).
CHAPTER FIVE

Quantitative assessment of hepatic steatosis in islet recipients
Chapter 5 - Quantitative assessment of hepatic steatosis by magnetic resonance imaging in islet recipients

5.1 Introduction

5.1.1 Hepatic steatosis may be a surrogate marker of graft function

Islet graft function suffers attrition over time (Ryan et al., 2005a), and there is a need to develop techniques for monitoring graft function using surrogate markers of rejection. Accurate measurement of liver fat content would permit evaluation of steatosis post-transplantation, and allow long-term assessment of whether its development does indeed impact on graft function. Steatosis seen in islet recipients is heterogeneous in distribution, and therefore the selected mode of investigation must assess the liver in its entirety. A non-invasive imaging modality that can be used to quantitatively assess the whole liver in post-transplant recipients is required, without unnecessarily exposing the assessed individual to ionizing radiation.

5.1.2 Magnetic resonance imaging (MRI) for liver fat assessment

MRI can be used to measure the difference in resonance frequencies between water and fat to obtain in-phase (water + fat signal) and opposed phase (water – fat signal) images: radiologists routinely use loss-of-signal on the opposed phase to interpret diffuse or focal steatosis. However, such methods cannot quantify liver fat owing to spatially varying imperfections in the magnetic and radiofrequency fields (Hu and Kan, 2013).

Additional imaging using chemical shift based water-fat separation methods are required to overcome these problems (Reeder et al., 2011). This method separates MR signals from water and fat that can be used to produce water-only and fat-only images, corrected for inhomogeneities in the magnetic and radiofrequency fields; these images can be recombined to create a proton density fat signal fraction map. Unlike in and out-of-phase imaging, full separation of water and fat signals permits a 0-100% assessment of fat signal fraction.

5.1.3 Study aims for hepatic steatosis in islet recipients

Previous assessments of liver fat content in islet recipients have utilised imaging modalities to make qualitative measures of hepatic fat content. This study aimed to use MRI to make the first quantitative assessment of liver fat in islet recipients. A complex-
based MR imaging technique was utilised, with magnitude and phase information from images acquired at echo times selected to achieve accurate separation of water and fat signals, so that a fat fraction estimate with a range of 0-100% (Yu et al., 2008) was provided.

The specific aims of this study were:

1) To develop a technique to accurately quantify hepatic fat content in islet transplant recipients, and to assess inter-user variability of this technique.

2) To identify whether intra-hepatic islet transplantation results in morphological fatty change within the liver.

3) To compare hepatic fat content in islet recipients with two control groups: individuals with a known diagnosis of NAFLD and adults with type 1 diabetes on continuous subcutaneous insulin infusions (CSII).

4) To assess the impact of any hepatic fat changes in islet recipients on graft function.

5.2 Methods

See section 2.3 entitled ‘Hepatic steatosis assessment in islet recipients’ for a description of study methods (See also Appendix Three).
5.3 Results

5.3.1 Study recruitment and baseline characteristics

Fifteen islet recipients, 15 patients with type 1 diabetes on CSII and 15 control patients with a previous clinical diagnosis of NAFLD were recruited to the study. Islet recipients were transplanted at NHS centres in Newcastle (11 recipients / 19 transplants), Manchester (2 recipients / 3 transplants), the Royal Free Hospital, London (1 recipient / 2 transplants) and King’s College Hospital, London (1 recipient / 2 transplants) between September 2008 and November 2013. Islet graft mass per recipient was 10919 (5953-12846) islet equivalents/kg (IEQ/kg). No steatosis was identified in any islet recipients on pre-transplant ultrasounds. Timing of MRI scans in the islet recipients is shown in Figure 5.1.

Baseline characteristics (median, interquartile range) of islet recipients pre-transplant and post-transplant and the two control groups are given in Table 5.1. There was no significant difference in age, weight, BMI, waist circumference, insulin dose, alanine transaminase (ALT), total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) or triglycerides between islet recipients and CSII controls. NAFLD controls had a significantly greater weight, BMI, waist circumference and ALT and lower HDL than islet recipients (p<0.001, Mann-Whitney U-test). C-peptide was negative (<50 pmol/l) in all islet recipients prior to transplantation and all but one of the insulin pump controls (one patient random C-peptide = 357 pmol/l).
Figure 5.1 – Timing of MRI scans (black arrows) and additional islet transplants (unfilled arrows) up to 24 months post-transplant.
<table>
<thead>
<tr>
<th></th>
<th><strong>M:F</strong></th>
<th><strong>Age (years)</strong></th>
<th><strong>Weight (kg)</strong></th>
<th><strong>BMI (kg/m²)</strong></th>
<th><strong>Waist (cm)</strong></th>
<th><strong>Insulin (units/kg)</strong></th>
<th><strong>HbA₁c (%)</strong></th>
<th><strong>ALT (IU)</strong></th>
<th><strong>Lipid profile</strong></th>
<th><strong>Hepatic Fat Quantification (%)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Islet recipients pre-transplant (n=5)</strong></td>
<td>Median</td>
<td>2:3</td>
<td>52</td>
<td>72.4</td>
<td>NA</td>
<td>0.48</td>
<td>9.6</td>
<td>23</td>
<td>5.3</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>IQR</td>
<td>(47-55)</td>
<td>(60.0-75.9)</td>
<td>(23.4-26.8)</td>
<td>NA</td>
<td>(0.40-0.86)</td>
<td>(8.0-11.4)</td>
<td>(19-36)</td>
<td>(4.0-6.1)</td>
<td>(1.4-2.0)</td>
</tr>
<tr>
<td><strong>Iset recipients 1st scan post-transplant (n=15, median follow-up time 3 months)</strong></td>
<td>Median</td>
<td>4:11</td>
<td>50</td>
<td>61.0</td>
<td>24.0</td>
<td>85</td>
<td>0.34</td>
<td>7.2</td>
<td>25</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>IQR</td>
<td>(48-57)</td>
<td>(56.4-72.5)</td>
<td>(22.2-25.3)</td>
<td>(79-91)</td>
<td>(0.21-0.56)</td>
<td>(6.6-8.5)</td>
<td>(16-30)</td>
<td>(4.0-5.75)</td>
<td>(1.5-1.9)</td>
</tr>
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<td></td>
<td>¥P</td>
<td>0.41</td>
<td>0.71</td>
<td>0.12</td>
<td>0.56</td>
<td>0.27</td>
<td>0.08</td>
<td>0.95</td>
<td>0.36</td>
<td>0.06</td>
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<td></td>
</tr>
<tr>
<td><strong>Continuous subcutaneous insulin infusion (n=15)</strong></td>
<td>Median</td>
<td>7:8</td>
<td>49</td>
<td>69.1</td>
<td>22.1</td>
<td>83</td>
<td>0.50</td>
<td>8.4</td>
<td>24</td>
<td>2.3</td>
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<tr>
<td></td>
<td>IQR</td>
<td>(26-61)</td>
<td>(55.6-75.1)</td>
<td>(20.6-75.1)</td>
<td>(79-86)</td>
<td>(0.43-0.78)</td>
<td>(7.8-9.0)</td>
<td>(12-33)</td>
<td>(4.5-5.8)</td>
<td>(1.8-2.9)</td>
</tr>
<tr>
<td></td>
<td>¥P</td>
<td>0.41</td>
<td>0.71</td>
<td>0.12</td>
<td>0.56</td>
<td>0.27</td>
<td>0.08</td>
<td>0.95</td>
<td>0.36</td>
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</tr>
<tr>
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<td></td>
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</tr>
<tr>
<td><strong>Clinical Diagnosis of NAFLD (n=15)</strong></td>
<td>Median</td>
<td>8.7</td>
<td>54</td>
<td>101.1</td>
<td>34.7</td>
<td>106</td>
<td>NA</td>
<td>7.3</td>
<td>36</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>IQR</td>
<td>(42-57)</td>
<td>(86.0-110.1)</td>
<td>(31.2-37.0)</td>
<td>(97-113)</td>
<td>NA</td>
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<td>(29-56)</td>
<td>(3.6-4.6)</td>
<td>(1.0-1.4)</td>
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<tr>
<td></td>
<td>¥P</td>
<td>0.74</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>0.01</td>
<td>0.10</td>
<td>&lt;0.001</td>
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</tbody>
</table>

**Abbreviations:**
NAFLD – non-alcoholic fatty liver disease, IQR – Interquartile range, M – male, F – female, BMI – Body Mass Index, HbA₁c – glycated haemoglobin, ALT – alanine transaminase, C-cholesterol, HDL – high density lipoprotein, LDL – low density lipoprotein, Trigs – triglycerides, NA – result not available.

¥P is Mann-Whitney test of significance versus islet recipient group at time of first scan post-transplant

**Table 5.1** - Baseline characteristics & hepatic fat quantification in islet recipients (pre and post-transplant) and CSII and NAFLD control groups
5.3.2 Hepatic fat assessment and precision of quantification

Hepatic fat fraction was quantified in the islet recipients and both control groups (see Figures 5.2A and 5.2B, Table 5.1).

Sixty-five paired MRI hepatic fat measurements were available for Bland-Altman analysis of hepatic fat quantification comparison between assessors AB and AA (see Figure 5.3). The correlation coefficient R value for AA’s values plotted against AB’s values was 0.997 (p<0.001). Mean difference between both sets of results was calculated at 0.081 %, with standard deviation of the differences 0.825 %. The coefficient of repeatability (twice the standard deviation) was 1.65 %, giving limits of agreement for the hepatic fat measurements by the two assessors to be -1.536 % to 1.698 %.

5.3.3 Comparison of hepatic fat in islet recipients versus control groups

No difference in hepatic fat content was noted between islet recipients’ first post-transplant scan (median time to scan post-transplant = 4 (1-12) months) and insulin pump controls (p=0.13, Mann-Whitney U-test) (Figure 5.4). Liver fat fraction was significantly greater in NAFLD patients compared to either of these groups (p<0.001 versus islet recipients and CSII controls, Mann-Whitney U-test).
Figure 5.2 – ImageJ software screenshots showing ‘water-only’ (A) and ‘fat-only’ (B) signals from the liver. The region of interest for analysis (indicated by the dotted yellow line) was selected manually on water-only images initially, as these images highlight areas of vascularity, hepatic ducts, liver capsule and artefact which must be avoided for hepatic fat quantification (these areas alter signal intensity irrespective of fat content). Regions of interest were subsequently overlaid onto the corresponding fat-only image for hepatic fat quantification.
Figure 5.3 – Scatter plot showing correlation coefficient (A) and Bland-Altman plot (B) for comparison of inter-user variability for hepatic fat quantification by assessors AA and AB.
Figure 5.4 – Box plot showing comparison of hepatic fat content in insulin pump users (n=15), islet recipients post-transplant (n=15) and individuals with NAFLD (n=15). *P < 0.01 vs. islet recipients, Mann-Whitney U-test.
5.3.4 Variation in hepatic fat with time post-transplant in islet recipients

Pre-transplant, mean liver fat fraction was 2.42 (2.05-4.41) % (n=5). No significant difference in liver fat fraction was noted pre-transplant compared to 1 month (2.16 (1.85-3.99) %, n=6), 3 months (2.43 (1.77-3.04) %, n=6), 6 months (2.86 (1.41-3.62) %, n=4), 12 months (2.27 (1.41-3.62) %, n=6) and 24 months post-transplant (2.53 (1.95-5.38) %, n=4) (p=0.94, Kruskal-Wallis test) (Figure 5.5).

Of the 15 islet recipients, 10 had > 1 scan (Figure 1). 5 recipients had a pre-transplant and a post-transplant scan, whilst 5 recipients had sequential scans post-transplant. The median time between scans was 8.5 (3-15) months for all 10 recipients in the post-transplant period. The median change in hepatic fat in islet recipients undergoing repeat scanning post-transplantation was 0.32 (-1.13-1.60) % between scans (Figure 5.6).

5.3.5 Development of focal steatosis distribution

Two islet recipients were noted to develop a focal steatosis distribution post-transplant (Figure 5.7). In the first recipient (NE014), hepatic fat fraction increased from 2.4 % pre-transplant to 5.7 % on a 1 month post-transplant scan showing the focal steatosis changes. This patient subsequently declined further MRI scanning. In the second recipient (NE010), hepatic fat fraction was quantified at 3.9 % on a 3 month post-transplant scan showing the focal steatosis changes. This recipient went on to have a second islet transplant 6 months after the first, and resolution of the focal steatosis distribution was noted on a repeat MRI scan at 18 months from the first transplant: at no stage did this recipient have loss of graft function and liver fat fraction fell to 1.4 % on the follow-up scan.
Figure 5.5 – Box plot showing change in hepatic fat content in islet recipients pre-transplant (n=5) and 1 (n=6), 3 (n=6), 6 (n=4), 12 (n=8) and 24 (n=5) months post-transplant.
Figure 5.6 – Bar chart showing median change in hepatic fat in 10 islet recipients undergoing repeat scanning post-transplantation.
Figure 5.7 - ImageJ software screenshots showing ‘fat-only’ signals from the liver in islet recipient NE014, with fat signals highlighted in purple. Figure A displays fat signals pre-transplant, Figure B displays fat signals one month post-transplant.
A heterogeneity index was calculated for both subjects with focal steatosis changes, and in two further control subjects with type 2 diabetes and a homogeneous hepatic fat distribution on their scans (study codes T2D01 and T2D02). Calculated heterogeneity indices were 2.34 for NE014 and 4.39 for NE010. Total hepatic fat content for T2D01 and T2D02 was 4.85 % and 3.92 % respectively, with calculated heterogeneity indices of 1.29 and 1.52 respectively.

5.3.6 Impact of hepatic fat content on graft outcome

Median 90 minute C-peptide achieved during meal tolerance testing was 485 (68-882) pmol/L in islet recipients at 12 months post-transplant. Liver fat fraction on first scan undertaken post-transplant (median time of scan = 4 (1-12) months post-transplant) did not have a direct correlation with 90 minute C-peptide assessed by meal tolerance test at 12 months post-transplant (R = +0.30, p=0.76 on regression analysis using Pearson’s Correlation Coefficient) (Figure 5.8).

In the two recipients noted to have focal steatosis changes on MR scans, 90 minute C-peptide was 794 pmol/l one month post-transplant in the first recipient (NE014) and 492 pmol/l at the time of the changes three months post-transplant in the second recipient (NE010). 90 minute C-peptide achieved at a 12 month post-transplant meal tolerance test was 357 pmol/l in NE014 and 1003 pmol/l in NE010. The number of recipients with focal steatosis changes was too small to assess whether this had any impact on graft function at 12 months.
Figure 5.8 – Scatter plot showing relationship between hepatic fat fraction on first scan post-transplant (median 4 (1-12) months post-transplant) and 12 month graft function determined by 90 minute C-peptide during a meal tolerance test in islet recipients.
5.4 Discussion

5.4.1 Hepatic fat assessment in islet recipients

This study is the first to describe a method of hepatic fat quantification utilising chemical shift based water-fat separation MRI in islet recipients. The technique has previously been used to quantify hepatic fat in individuals with NAFLD and type 2 diabetes (Lim et al., 2011, Yokoo et al., 2011), where the steatosis distribution is diffuse and homogeneous. However, its use in islet recipients presents unique challenges due to the focal and patchy nature of hepatic fat distribution post-transplantation. Previous studies have shown that islets infused into the main portal vein at the time of transplantation embolize within branches of the portal venous system, which is predominantly located in the liver periphery (Sever et al., 1992). The paracrine action of insulin results in steatosis predominantly in perivenular regions and located peripherally in the liver.

Earlier studies have attempted to assess hepatic fat in islet recipients but only a qualitative assessment has been previously reported. Ultrasound is the most commonly used imaging modality for the diagnosis of hepatic steatosis (Mishra and Younossi, 2007) and has been used for assessment of steatosis after islet transplantation (Venturini et al., 2010). Although non-invasive and without radiation risk, the technique is operator and machine-dependent; and the outcomes are not reproducible on repeat testing (Saadeh et al., 2002). The sensitivity of ultrasound for diagnosing hepatic steatosis in islet recipients has been questioned when compared to MRI (Hu and Kan, 2013). Computed tomography (CT) can provide objective assessment of hepatic fat content (Kodama et al., 2007) but inaccuracies can be caused by oedema, fibrosis, glycogen, copper or iron, and CT has a low sensitivity for mild to moderate steatosis (Limanond et al., 2004).

Non-targeted percutaneous liver biopsy with histological evaluation is considered to be the gold standard investigation for diagnosis of hepatic steatosis (Kleiner, 2005). However, biopsy is limited by sampling variability (Ratziu et al., 2005): a biopsy assesses a tissue sample 1/50,000th the overall size of the liver, and therefore is not representative of the whole organ. Biopsy assumes homogeneous distribution of steatosis within the liver – a major shortfall in the assessment of islet recipients as steatosis development is most likely to be heterogeneous in distribution. A biopsy study
in 16 islet recipients found localised steatosis in 8 patients (Toso et al., 2009), but emphasised the difficulties of using a liver biopsy as a diagnostic tool to assess transplanted islets, as islet tissue was found in only 31% of biopsies. In addition, liver biopsy has an associated risk of complications that can lead to hospitalisation (1-3%) and rarely death (1 in 10,000) (Bravo et al., 2001).

This study’s chosen method of MRI utilises a non-invasive modality of investigation that does not expose the subject to ionizing radiation. To capture as much of the heterogeneous hepatic fat distribution as possible, images providing the largest possible area of liver for analysis on three different levels were assessed with ROIs that marked the largest possible area of liver. Our analysis technique did require that areas of vascularity were avoided when selecting the region of interest, so that calculation of hepatic fat content was not inappropriately influenced by fat content of vasculature. However, there remains the possibility that if steatosis distribution following islet transplantation is known to be predominantly perivenular, the described technique could have provided an underestimate of hepatic fat: this was avoided as much as possible by marking regions of interest as close as possible to blood vessels. In addition, the selected technique for this study volume-weights the fat measurements, and therefore extremely localised fat might be hard to detect in the 10 mm slices used for the fat map generation.

5.4.2 Quantification of hepatic fat content in islet recipients

It was necessary to check whether two individuals taught the described technique of hepatic fat quantification obtained comparable results, to verify that the technique described produced reliable and reproducible results and might be used by other scientific teams investigating hepatic fat changes in islet recipients. The two individuals (AA and AB) undertaking hepatic fat quantification were blinded to each other’s work and marked their own regions of interest for hepatic fat analysis on image sections selected independently from one another.

Bland-Altman comparison of the two assessors’ measurements was undertaken. A plot of the difference in hepatic fat measurements against the mean hepatic value for both assessors’ measurements allowed investigation of the relationship between the assessors’ results. The true hepatic fat value for each obtained scan was unknown, and therefore the best available estimate for a true value was the mean of both assessors’
measurements. The mean difference between both sets of results was calculated to be 0.081 % fat, with a standard deviation of the differences of 0.825 %. This gave calculated limits of agreement for the assessors’ results of -1.536 % to 1.698 % (mean difference +/- two standard deviations). No previous quantitative data for hepatic fat in islet recipients has previously been reported to suggest what might be a clinically suitable limit of agreement. We believe that this is a sufficient limit of agreement given the range of fat fractions measured by the technique: mean values for hepatic fat from both assessors’ measurements ranged from 1.25 % to 51.08 %. However, many of the hepatic fat values were low fat fractions: it could be conceivably argued that the limit of agreement reported may not be sufficiently narrow to compare scans with low hepatic fat content values. Even so, the limits of agreement for the described technique suggest that two independent users should be capable of readily detecting shifts in hepatic fat content of >3 %, allowing investigation of whether islet transplantation produces sizeable shifts in hepatic fat content post-transplant.

5.4.3 Hepatic fat outcomes after islet transplantation

Hepatic fat in islet recipients did not differ from CSII controls. Both groups were well matched for BMI, so that detectable differences in hepatic fat content might be attributable to islet transplantation rather than body habitus (Gaba et al., 2012). NAFLD subjects had a substantially greater BMI and hepatic fat content than either of the other two groups.

Hepatic fat content at time-points post-transplant did not change significantly from pre-transplant values within the islet recipient group. These observations suggest that islet transplantation does not result in a marked increase in quantified hepatic fat content.

Hepatic fat content has previously been correlated with body mass index (Gaba et al., 2012). The islet recipients were not noted to have any significant change in weight compared to pre-transplant: a number of patients gained weight post-transplant, whilst a proportion lost weight; no correlation between recipient body mass index and hepatic fat assessment was identified.

Our quantified hepatic fat content results for the CSII group are in keeping with previously calculated values in subjects with type 1 diabetes but perhaps closer to values previously calculated for a population without diabetes (Perseghin et al., 2005).
As hepatic insulin concentration is anticipated to be reduced in individuals with type 1 diabetes, it has been suggested that the low glucagon to insulin ratio may result in abnormally low intrahepatic lipid oxidation. This combined with enhanced whole-body fat oxidation during the fasting state may contribute to a lower intrahepatic fat content in individuals with type 1 diabetes.

Insulin secretion in islet transplant recipients is pulsatile, with glucose-induced stimulation of insulin secretion accomplished by amplification of insulin pulse size (Meier et al., 2006). This may optimise insulin signalling and insulin extraction by the liver, with pulsatile insulin release indirectly governing systemic insulin delivery because of preferential extraction of insulin bursts (Meier et al., 2005). Direct hepatic catheterisation studies have revealed that hepatic first-pass insulin extraction is similar in healthy control subjects and in patients who have undergone islet transplantation, implying that insulin secreted from islet grafts is delivered into hepatic sinusoids rather than into the hepatic central vein (Meier et al., 2006). If venous drainage was via the hepatic central vein, insulin secretion by intrahepatic transplanted islets would be systemic rather than portal and would have implications for hepatic insulin clearance, with a substantial risk of hyperinsulinaemia.

Re-establishing a physiological route of intraportal insulin delivery might putatively expose hepatocytes to secreted insulin via fenestrated sinusoids, inducing lipid deposition by promoting esterification of free fatty acids within hepatocytes. However, it could equally be argued that the afore-mentioned findings suggest that insulin release from transplanted islets avoids hyperinsulinaemia, and insulin might not be present for sufficient time and in concentrations high enough in the liver parenchyma to induce localised fatty change.

5.4.4 Hepatic fat fraction influence on graft function

Hepatic fat fraction in islet recipients calculated from scans taken 4 (1-12) months post-transplant did not correlate with C-peptide achieved during a meal tolerance test at 12 months post-transplant, suggesting that hepatic fat fraction measured soon after transplantation is unlikely to be a surrogate marker for future graft function. This is in keeping with the findings of another recent study that found no relationship between qualitative assessment of hepatic fat content and graft survival (Jackson et al., 2013).
5.4.5 Development of focal steatosis and significance in islet recipients

Although no significant increase in hepatic fat content was found post-transplantation in the islet population as a whole, two islet recipients did develop focal steatosis changes post-transplant. This focal steatosis pattern in islet recipients has been identified by other groups (Markmann et al., 2003, Bhargava et al., 2004, Venturini et al., 2010, Hu and Kan, 2013), although the onset of steatosis post-transplant in this study (1 month and 3 months post-transplant) was considerably quicker than that reported previously.

One of the two subjects with focal changes (NE014) had a pre-transplant MRI and hepatic fat was quantified at 2.42% pre-transplant. This increased to 5.71% on an MRI showing the focal steatosis pattern 1 month post-transplant. Unfortunately, this subject declined further imaging. In the second subject (NE010), focal steatosis changes were noted on a scan taken 3 months post-transplant. This subject received a second islet transplant 6 months after the first transplant to improve endogenous insulin production, and islet graft function improved (90 minute MTT C-peptide was 492 pmol/l at 3 months and 1003 pmol/l at 12 months post-first transplant). At no stage did this recipient have loss of graft function but on a repeat scan undertaken at 18 months post-transplant, the focal steatosis changes had resolved, and liver fat fraction fell from 3.95% to 1.39%.

Previously, it has been noted that localised focal steatosis patterns resolve with loss of graft function (Bhargava et al., 2004, Venturini et al., 2010), and steatosis development has been associated with a higher proportion of islet recipients requiring exogenous insulin compared to those without steatosis. Stimulated C-peptide production has been reported to be significantly lower in patients with positive ultrasound findings for steatosis compared to those with no fatty changes (Venturini et al., 2010). Transplanted islets with a chronic exposure to a uniquely high lipid environment and high glucose levels may become victim to combined glucolipotoxicity, and it can be hypothesised that development of localised hepatic steatosis may reflect islet graft stress and dying islets. β cell apoptosis in the presence of high fatty acid concentrations could result in residual functioning islets having to produce excessive insulin to maintain blood sugar levels within the normal range. Hypersecretion of insulin might therefore promote an environment of glucolipotoxicity that results in further islet death, and localised hepatic steatosis may therefore be a harbinger of graft failure.
This study showed resolution of steatosis with an improvement in graft function following repeat transplantation, suggesting acute lipotoxicity may be reversible with improved blood glucose control using insulin or repeat transplantation. Identification of localised steatosis by MRI scanning may theoretically be used to time medical intervention, such as recommencement of insulin therapy or re-transplantation. However, a recent study in islet recipients with a median follow-up time of 8 years has found no clear relationship between additional islet infusions and the development or resolution of steatosis (Jackson et al., 2013). It is equally possible that only a subgroup of islet recipients have a genetic predisposition to developing localised steatosis post-transplantation, and only a proportion of these patients may experience complications from the finding. Studies utilising MR analysis in a larger number of post-transplant recipients are required before further conclusions and recommendations can be made.

5.4.6 The heterogeneity index as a method of identifying recipients with focal steatosis changes

A measure that might be used to assess whether recipients have developed focal steatosis changes is the heterogeneity index. This measure utilises the heterogeneity of the steatosis distribution identified in islet recipients developing the focal changes. Values of 4.39 and 2.34 were reported in islet recipients, versus 1.29 and 1.52 in control subjects subjectively identified as having homogeneous fatty changes. The results suggest that an index of >2 might be initially used as a measure of whether islet recipients have developed focal steatosis. The index calculation relies on subjectivity in the selection of areas of high and low fat concentration but it does attempt to quantify the heterogeneity seen in recipients developing focal changes. The measure needs to be applied to a larger number of scans from islet recipients before it can be validated as a true measure of whether an individual has developed focal steatosis post-transplant.
CHAPTER SIX

*De novo* donor specific antibody development in islet recipients
Chapter 6 - *De novo* donor specific antibody development in islet recipients and impact on graft function

6.1 Introduction

6.1.1 The role of the humoral immune system in graft rejection

Islet graft function has been noted to suffer attrition with time, with 14\% of recipients maintaining insulin independence and 30\% of recipients experiencing graft failure two years post-transplant in an international trial (Shapiro et al., 2006). Assessments of metabolic function have been used to define when graft failure has occurred but surrogate markers might allow prediction of graft failure, allowing interventions that maintain endogenous insulin production (Lacotte et al., 2011).

6.1.2 HLA matching pre-islet transplantation

Pre-transplant sensitisation to donor HLA has previously been associated with rapid loss of islet graft function (Mohanakumar et al., 2006). Ideally, HLA matching would be performed to prevent sensitisation and problems associated with the immune response to transplanted islets. However, a limited pool of donors and suitable recipients makes allocation of HLA matched donor organs to recipients unworkable. Islets are allocated primarily on ABO compatibility and a sufficient number of islet equivalents based on recipient weight. Multiple infusions of islets from different donor organs are generally required to reach adequate transplanted islet mass to attain insulin independence. Recipients usually have multiple mismatches with donor epitopes, and each HLA mismatch may result in the development of *de novo* antibodies to donor tissue and previously mismatched HLA antigens. It has been shown that *de novo* HLA antibodies act differently to preformed antibodies and - as they are not present at the time of infusion - do not result in hyperacute rejection. However, their presence may be associated with gradual injury and repair steps that might lead to graft failure (Terasaki and Mizutani, 2006).

6.1.3 HLA antibody and DSA detection

Historically, detection of HLA antibodies was performed using complement-dependent cytotoxicity (CDC). However, this relatively insensitive method underestimates the presence of circulating antibodies (Christiaans et al., 2000, Muro et al., 2005a). The molecular structure of HLA was identified in 1987 (Bjorkman et al., 1987), and it
became possible to make recombinant lines containing only a single HLA specificity. Current methods for the detection of HLA antibodies utilise newer methods such as the use of the Luminex® assay. This assay is the most sensitive test currently available and detects both complement fixing and non-complement fixing antibodies using either soluble or recombined HLA molecules bound to polystyrene beads. Beads are mixed with patient serum in order to allow for antibody binding, then a second anti-human antibody linked to a fluorescent reporter molecule is added. Binding of alloantibodies to the beads is then detected by a dual laser flow cytometer, increasing both sensitivity and accuracy of HLA antibody detection (Tambur et al., 2000, El-Awar et al., 2005). This allows a semi-quantitative interpretation of the level of antibody binding present using Median Fluorescence Intensity (MFI). Single antigen Luminex tests can be used to determine reactions against specific HLA antigens.

6.1.4 The role of the autoimmune response in graft rejection

The autoimmune response may also have a significant role in islet graft rejection. Type 1 diabetes is an autoimmune disease characterised by T cell mediated destruction of β cells, in which T helper cells seem to play a pivotal role (Roep, 2003). Transplantation in recipients with type 1 diabetes is performed in the presence of an active or memory autoimmune response to islet autoantigens that could theoretically contribute to rejection. In SPK recipients, a rise in autoantibody titre and T cell response post-transplant has been correlated with subsequent pancreas graft failure (Braghi et al., 2000). The presence of autoreactive T cells in the failed allografts of pancreas transplant alone (PTA) recipients lends further support to the hypothesis that autoimmunity may play a significant role in graft failure (Vendrame et al., 2010).

In this study, islet graft function was assessed in parallel with DSA screening to compare graft function in recipients developing DSA against those without DSA development. At each clinical follow-up, blood was taken to assess alloimmune and autoimmune response in order to explore the temporal relationship between antibody onset and graft failure in islet recipients followed up for 12 months post-transplant.

Available donor information (age, body mass index, HLA match, pancreas condition, harvesting and cold ischaemia time) was retrospectively reviewed to see if there was any influence on the characteristics of the isolated islets (islet yield and total number
transplanted, islet purity and viability), and whether this in turn had a correlation with DSA formation and duration of graft function.

This study assessed the impact of alloantibody and autoantibody development on graft outcome. The aim of the work was to identify potential surrogate markers that might predict islet graft failure, with particular emphasis on whether DSA formation in islet recipients might be used as a prognostic tool for predicting future islet transplant function.

6.2 Methods

See section 2.4 entitled ‘De novo donor specific antibody assessment in islet recipients and assessment of impact on graft function’ for a description of study methods.
6.3 Results

6.3.1 Islet recipient population

Fourteen individuals (12 females and 2 males; 9 islet transplant alone, 3 islet after kidney) followed up for 12 months post-first transplant received a total of 23 islet transplants (single graft: n=6; two: n=7; three: n=1) between October 2008 and March 2013 at Newcastle-upon-Tyne Hospitals NHS Foundation Trust. Recipient age (median [IQR]) was 51.5 (48.3-54.0) years with diabetes duration 38.5 (33.5-42) years and weight 59.8 (54.3-70.1) kg at time of first transplant. Total transplant mass per recipient was 11232 (6222-12802) IEQ/kg, per graft was 6032 (4872-7186) IEQ/kg (Table 6.1).

6.3.2 DSA response to individual grafts

DSA was detected following transplantation in 5 / 23 (21.7%) grafts, with median time to DSA onset of 1 (1-2) month post-transplant of the provoking graft (Figure 6.1). In three cases, DSA class I antibodies alone were identified; in two cases both class I and class II DSA were detected simultaneously in recipient serum.

6.3.3 Impact of DSA presence on graft outcome

All five grafts associated with a DSA positive response failed within eight months post-transplantation, with three grafts failing within two months of DSA detection (Figures 6.1, 6.2). Stimulated C-peptide at 3 months post-transplant was significantly less in recipients developing DSA to a graft compared to recipients who remained DSA negative (Table 6.1, Figures 6.2, 6.3, 6.4).
<table>
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Table values given as median (interquartile range).

*Comparison of 12 month outcomes in DSA positive grafts vs DSA negative grafts, Mann-Whitney test used for statistical analysis.

Abbreviations: ALT – alanine transaminase, BMI - Body mass index, DBD – donors after brain death, DCD – donors after circulatory death, DSA – Donor specific antibody, HLA - Human Leukocyte Antigen, ICH – intracranial haemorrhage, IEQ/kg - islet equivalents/kilogram recipient weight, MTT90 C-peptide - stimulated C-peptide at 90 minutes in meal tolerance test.

Table 6.1 – Comparison of clinical characteristics of grafts provoking DSA negative and DSA positive responses.
Figure 6.1 Bar graph of graft function post-transplant, indicating DSA formation in islet recipients and temporal relationship with graft failure. All DSA positive recipients developed graft failure within 12 months follow-up, one of nine DSA negative recipients developed graft failure.
Figure 6.2 Line graph showing time course of graft function (as determined by 90 minute C-peptide during a meal tolerance test) in recipients developing DSA up to 12 months following first transplant.
Figure 6.3 Line graph showing time course of graft function (as determined by 90 minute C-peptide during a meal tolerance test) in recipients without DSA up to 12 months following first transplant.
Figure 6.4 Box plot of stimulated C-peptide (pmol/L) at 90 minutes during a meal tolerance test three months post-transplant for islet grafts provoking a DSA negative (n=18) versus a DSA positive response (n=5). Boxes indicate median, upper quartile and lower quartile ranges. Whiskers plotted with the lowest datum within 1.5 times the interquartile range of the lower quartile and the highest datum within 1.5 times the interquartile range of the upper quartile. *P < 0.01 vs. DSA negative grafts, Mann-Whitney U-test.
6.3.4 Factors associated with DSA development

The clinical characteristics of donors and recipients in grafts developing DSA were compared to grafts with a DSA negative response (Table 6.1).

There were 22 donors after brain death (DBD) and one donor after circulatory death (DCD), with the DCD donor associated with later DSA formation. Intracranial haemorrhage was the cause of death in 20 of the 23 donors.

No difference was noted in donor age, BMI, renal function, liver function tests, amylase, duration of ventilation, HLA A / B / DR / total mismatches, HLA C / DP / DQ mismatches, IEQ received, pre-transplant islet viability or purity between grafts that subsequently developed DSA and those that did not (Table 6.1). Cold ischaemia time was shorter in grafts that subsequently developed DSA (p<0.05).

6.3.5 Induction immunosuppression

In grafts developing a DSA response, induction was with alemtuzumab (n=2), basiliximab (n=2) or daclizumab (n=1). In grafts with no DSA formation, induction was with alemtuzumab (n=11) or basiliximab (n=7).

6.3.6 Autoantibody assessment

Pre-transplant, five recipients were GAD positive only, one recipient was IA-2A antibody positive only, one recipient was GAD positive and ICA positive, and the remaining recipients were autoantibody negative. There was no difference in three month post-transplant graft function according to pre-transplant autoantibody status (p=0.61).

A weakly positive ICA response was detected following two of the graft transplantations at three months post-transplant but this was not sustained and ICA response was negative at 12 months. Follow-up of each graft with an ICA response indicated that one developed DSA and subsequent graft failure (MTT90 12 months = 7 pmol/L), whilst the second remained DSA negative and achieved insulin independence (MTT90 12 months = 2254 pmol/L). IA-2A positivity developed in a third graft at one month post-transplant; this recipient did not develop DSA and good graft function was maintained post-transplant (MTT90 6 months = 692 pmol/L). GAD positivity developed
following four transplants but was sustained to 12 months post-transplant in only two cases. Of these cases, one patient with a sustained rise in GAD antibodies and one patient with a temporary rise in GAD antibodies developed graft failure; in both cases DSA formation preceded GAD positivity. In the remaining two recipients with GAD positivity, DSA remained negative and graft function was maintained at 12 months post-transplant (MTT90 12 months = 1003 pmol/L and 357 pmol/L).

There was no difference in three month graft function when comparing post-transplant autoantibody positive versus post-transplant autoantibody negative grafts (p=0.61), and no difference in graft function outcomes when comparing transplants that resulted in a change in autoantibody status (n=7) versus those that did not (n=16) (p=0.53).
6.4 Discussion

In this study, *de novo* DSA formation occurred following 21.7% grafts. Formation occurred soon after transplantation and was associated with rapid graft failure, occurring within two months in three out of five cases. Little change in autoantibody levels occurred post-transplant, and no association with graft function was found.

6.4.1 Measured DSA rate is comparable to other forms of organ transplant

The *de novo* DSA rate of 21.7% to individual islet grafts is comparable to other reports in solid organ transplantation: DSA rates are usually below 25% after several years in renal transplant populations (Everly et al., 2013). However, our study differs from these previous studies in that eight of our recipient population received more than one transplant, making the DSA rate in the recipient population 35.7% (5 / 14). All five recipients who developed DSA remained on maintenance immunosuppression at the time of antibody detection, with regular monitoring of tacrolimus levels and full blood counts to ensure that adequate immunosuppression with tacrolimus and mycophenolate was achieved. A previous study has reported a comparable DSA incidence of 30% in 98 islet recipients (Campbell et al., 2007b), with the majority of recipients remaining on immunosuppression at the time of DSA detection. It has been suggested that solid organ transplants may adsorb antibodies, preventing their detection on peripheral blood sampling (Adeyi et al., 2005). Islet tissue mass is likely to be insufficient to adsorb antibody and therefore DSA will be detectable if present in the blood. Biopsy of islet transplant tissue to confirm this hypothesis is difficult due to the heterogeneous nature of liver architecture post-islet transplantation (Toso et al., 2009).

6.4.2 Association of DSA with graft failure

In this study, DSA onset was rapid in grafts stimulating DSA formation, with detection within two months of transplantation in all five cases. Their presence was associated with rapid graft failure and significantly reduced graft function at three months post-transplant, with subsequent graft failure within eight months of DSA detection.

The impact of DSA on islet graft outcome is debatable: not all studies demonstrate an association between DSA detection and graft failure. Pre-transplant DSA has been detected in 49% of islet recipients in a recent study, and this was associated with improved graft outcomes (Piemonti et al., 2013). However, the same study also reported
that graft function was reduced in recipients developing *de novo* DSA post-transplant. Resynthesis of DSA has been reported to have no impact on graft function (Cardani et al., 2007), although it has also been shown that outcome for islet recipients is poorer when HLA antibody formation is detected both pre and post-transplant (Campbell et al., 2007a, Campbell et al., 2007b). In keeping with our findings, the largest available database for islet transplantation reported that *de novo* HLA antibody (class I) formation was associated with subsequent graft failure (Naziruddin et al., 2012).

### 6.4.3 Factors influencing DSA development

On assessment of donor characteristics, cold ischaemia time was found to be significantly shorter in grafts that subsequently demonstrated DSA. This is unexpected and contradicts previously published literature, which suggests that an increased incidence of DSA formation is associated with increased cold ischaemia times (Lebranchu et al., Abu-Elmagd et al., Gilligan et al., 2004). An extended cold ischaemia time might have been expected to impact on islet purity or viability, and reduced islet purity has been suggested to be a possible risk factor for DSA formation as it may lead to increased presentation of donor material to host antigen presenting cells (Hilbrands et al., 2013). However, in our patient population no difference in pre-transplant purity or viability was identified between DSA positive and DSA negative recipients. Although cold ischaemia time was prolonged in DSA negative recipients, the increased duration may not have been sufficient to impact on islet isolate quality (median 320 minutes versus 254 minutes), and the role of cold ischaemia time in influencing graft outcomes in this study remains questionable. Whether reduced cold ischaemia time might conceivably be responsible for DSA formation remains uncertain, and caution must be taken in interpreting results from this small study reporting on outcomes on 23 grafts in 14 recipients.

There was no difference in recipient characteristics in the DSA positive group when compared to the DSA negative group. When comparing HLA mismatches, the median number of total mismatches was five per recipient and four per graft, again with no difference found for recipients developing DSA. Other studies have reported a higher median number of mismatches per recipient (Campbell et al., 2007b, Piemonti et al., 2013) but these studies failed to show a significant relationship with the development of DSA.
Reduced islet purity has been suggested as a possible risk factor for DSA formation, as it may lead to increased presentation of donor material to host antigen presenting cells (Hilbrands et al., 2013). However, no difference in pre-transplant purity or viability was identified between DSA positive and DSA negative recipients in this study.

6.4.4 No relationship between graft outcome and autoimmune antibody levels identified

Two recipients temporarily became ICA positive at three months post-transplant, four recipients became GAD positive (one of which was also one of the recipients developing ICA positivity) and a further recipient developed IA-2A positivity. However, no temporal relationship with subsequent graft function was identified, and only in individuals developing DSA was graft function later compromised, regardless of autoantibody status.

Previous studies have suggested that autoantibody status might influence islet graft function. Autoantibody and DSA response was recently assessed in 59 islet recipients and an increase in autoantibody or DSA was associated with graft loss, with a hazard ratio five times that of recipients developing no antibodies (Piemonti et al., 2013). Although our autoantibody tests were negative, the findings do not completely rule out that an autoimmune response occurred post-transplantation: we did not measure ZnT8 antibody response, and this was the only autoantibody to increase in five recipients in the afore-mentioned study. In addition, our autoantibody findings do not rule out that an autoimmune response occurred post-transplantation. A cytotoxic T lymphocyte precursor assay has allowed the development of T cell studies to assess the autoimmune response to transplantation (Bouma et al., 1992). Poor outcome has been associated with the presence of pre-transplant autoreactive T cells, with no influence observed regarding pre-transplant or post-transplant autoantibody status (Roep et al., 1999, Huurman et al., 2008). Whilst our study agrees with the finding that autoimmune antibody levels might not be a surrogate marker of graft failure, the practical aspects of monitoring T cell mediated autoimmunity remain challenging; evidence suggests that cellular reactivity to autoantigens and alloantigens following transplantation might not correlate with future graft function (Huurman et al., 2008).
6.4.5 DSA detection might guide therapeutic intervention

DSA development might reflect inadequate induction immunotherapy. Improved islet transplant outcomes have been reported with newer lymphocyte depleting agents (Cai and Terasaki, 2010, Bellin et al., 2012), with an insulin independence rate of 50% at five years approaching that of pancreas transplant alone recipients (Bellin et al., 2012). In this study, DSA was detected following grafts where campath (n=2), basiliximab (n=2) and daclizumab (n=1) were used at induction. DSA formation therefore occurred in recipients receiving both lymphocyte depleting agents and the older anti-CD25 agents. Tacrolimus and mycophenolate mofetil were used as maintenance immunosuppression in all recipients, and therefore no difference in regimen was implicated in DSA susceptibility. Mycophenolate targets de novo purine biosynthesis, and therefore can inhibit human T and B lymphocytes more efficiently to reduce allograft rejection episodes (Cai and Terasaki, 2005).

All our recipients developing DSA experienced graft failure within 8 months. If DSA is a genuine surrogate marker for future graft rejection, therapeutic interventions that might improve outcome upon its detection should be considered. Steroids are often used as the first line drug upon detection of organ rejection in transplant medicine and they can inhibit production of alloantibodies (Planey and Litwack, 2000); however, they cannot be used in islet transplant recipients as they are detrimental to islet graft function (Kaufman et al., 1991). Rituximab – a chimeric mouse / human monoclonal antibody - specifically targets B cell surface marker CD20 and induces B cell lysis. Anecdotal reports suggest that its use might prevent humoral rejection (Garrett et al., 2002). In an islet transplant recipient who developed DSA and lost insulin independence, insulin independence was restored following treatment with rituximab and intravenous immunoglobulin (Kessler et al., 2009). Bortezomib - a proteasome inhibitor that affects plasma cells – has been shown to reduce antibody levels and improve graft function in renal transplant recipients developing DSA (Walsh et al., 2010). High dose intravenous immunoglobulin acts by blocking HLA antibody activity and inhibiting complement, as well as inducing significant B cell and T cell apoptosis to prevent the mechanisms underlying transplant rejection (Toyoda et al., 2003). Intravenous immunoglobulin used in conjunction with plasmapharesis has been used to reverse established antibody mediated rejection in renal recipients (Rocha et al., 2003), and could potentially reverse established antibody mediated rejection in islet recipients. However, intensive, prolonged immunosuppression interventions do not always successfully prevent graft
rejection in the presence of DSA (Hodges et al., 2012), and there are risks associated with their use. In addition, outcomes from this study suggest that DSA detection might be subsequently followed by loss of graft function that is too rapid to allow these interventions to be clinically effective. If this is the case, alternative strategies need to be contemplated.
CHAPTER SEVEN

Concluding Remarks
Chapter 7 - Concluding Remarks

7.1 The UK islet transplant program has achieved its primary outcome goals

The primary goals of the UK islet transplantation program were to prevent recurrent life-threatening hypoglycaemia and restore optimal glycaemic control in islet recipients, and this study has shown that these goals have been attained up to 36 months post-transplant.

Islet transplantation has often been perceived as an experimental procedure which is prohibitively costly - both financially and in terms of deceased donor pancreas usage - and which cannot deliver sustainable insulin independence (Khan and Harlan, 2009). The UK program shows how integration of a limited number of isolation facilities with satellite centres providing all aspects of assessment, transplantation and post-transplant follow-up can be achieved without the major costs of establishing and maintaining their own isolation teams and facilities.

Clinical success has been confirmed in the UK islet transplant program in tandem with avoidance of multiple transplants. This has underpinned sharing of donor organs between isolated islet and solid organ recipients listed according to congruent criteria, providing a platform for further innovation and definitive comparison of outcomes. The therapeutic goal of preventing recurrent severe hypoglycaemia in those at highest risk in combination with restoration of glycaemic control to a level proven to prevent long-term hyperglycaemic complications (The Diabetes Control and Complications Trial Research Group (1993)) has been realised.

7.2 Assessment of long-term outcomes is now required for islet transplantation

This study has shown that islet transplantation achieves short-term goals for diabetes care by improving glycaemic control and reducing the incidence of severe hypoglycaemia. Further studies assessing islet transplantation must now determine the intervention’s impact on microvascular and macrovascular complications that are attributed to the long-standing hyperglycaemia associated with diabetes. Islet transplantation has only achieved widespread recognition as an acceptable intervention for the management of recurrent severe hypoglycaemia in patients with type 1 diabetes since the landmark Edmonton study of 2000 (Shapiro et al., 2000); time is therefore required before conclusions can be drawn about the procedure’s long-term outcomes.
The Collaborative Islet Transplant Registry (CITR) has been compiling a comprehensive international database for islet transplantation from 27 North American, European and Australian centres since 1999, including outcomes for retinopathy, nephropathy, neuropathy and macrovascular disease. CITR have recently published data on outcomes for islet transplantation in 677 ITA and IAK recipients receiving 1375 islet infusions between 1999 and 2010 (Barton et al., 2012). The study focused on five primary efficacy outcomes that are not dissimilar to the clinical outcomes reported in this study: assessment of graft function, (with C-peptide ≥0.3 ng/ml indicative of sustained graft function), reduction in HbA1c, resolution of severe hypoglycaemia, fasting blood glucose stabilisation and insulin independence rate. Graft function was retained for longer in the most recent era of islet transplantation from 2007-2010: graft survival was 92% at 1 year and 83% at 3 years, with duration of insulin independence 66% at 1 year and 44% at 3 years. However, to date CITR have not formally published long-term outcome data for microvascular and macrovascular outcomes following islet transplantation.

Islet transplantation has been shown to improve microvascular disease outcomes in small-scale short-term studies by the Edmonton islet transplant team (Thompson et al., 2011, Warnock et al., 2008). These studies demonstrated that rate of decline of glomerular filtration rate and progression of retinopathy were slower following islet transplantation in comparison to individuals receiving intensive medical therapy for management of type 1 diabetes; these studies also found a non-significant trend for improvement in nerve conduction velocity after islet transplantation.

The comorbidities associated with long-term immunosuppression therapy must also be taken into consideration when assessing long-term outcomes from transplantation therapies. Transplant recipients receiving immunotherapy to reduce the potential for graft rejection have a substantially increased lifetime incidence of lymphoproliferative disorders (Singavi et al., 2015) and skin cancer (Stoff et al., 2010). Squamous cell cancer relative risk is increased 100-fold compared to the non-transplanted population, whilst basal cell carcinoma relative risk is increased 10-16 times in renal transplant recipients. Following a diagnosis of skin cancer, early preventative and aggressive therapeutic interventions are required in transplant recipients to reduce mortality risk (Martinez et al., 2003).
Reduced renal function is common following solid organ transplantation as a consequence of immunosuppression nephrotoxicity, predominantly from the use of calcineurin inhibitors. The steroid free immunosuppression regimen used by the Edmonton team to achieve insulin independence in islet recipients included the use of the calcineurin inhibitor tacrolimus: this medication remains a recommended maintenance immunosuppressant following islet transplantation and was regularly used in the UK cohort of islet recipients reported in this study. No decline in renal function was seen in our recipients but long-term follow-up of the UK cohort is required to assess the impact of immunosuppression therapy on renal function following islet transplantation.

A second immunosuppressant originally recommended by the Edmonton team was sirolimus, which suppresses the immune system’s IL-2 response by its action as an mTOR (mechanistic Target Of Rapamycin) inhibitor. Sirolimus also has adverse effects on renal function and has been implicated in the development of proteinuria in a cohort of 41 islet recipients (Senior et al., 2007, Senior et al., 2005). Median glomerular filtration rate decline was 0.39 ml/min/1.73m²/month at a median of 29.8 months follow-up. Substantial inter-individual variation was noted, although in the majority of individuals, glomerular filtration rate was lower at 24 months post-transplantation. Albuminuria progressed in 10 individuals, with regression of microalbuminuria in only one recipient despite improved glycaemic control post-transplant. Rate of decline of glomerular filtration rate and progression of albuminuria was very difficult to predict, and proteinuria was reversed on withdrawal of sirolimus (Senior et al., 2005). Symptomatic small bowel ulceration was also noted to resolve on stopping sirolimus therapy (Molinari et al., 2005).

Sirolimus has now been dropped as a maintenance immunosuppressant of choice for islet transplant recipients following the Edmonton team’s findings and replaced by mycophenolate mofetil, which was used in the majority of this study’s UK islet recipients as the second maintenance immunosuppressant alongside tacrolimus. ITA is now only considered in individuals with an isotopic glomerular filtration rate of >60 ml/min/1.73m² with no evidence of macroalbuminuria. The Edmonton team’s reported experiences with sirolimus highlight the need for caution when contemplating the potential consequences of immunosuppression therapy, and underline the need for
Islet transplantation is an intervention where the short-term risks of the procedure are low, and short-term outcomes for the management of patients with type 1 diabetes have now been demonstrated by this study. However, given the potential associated risks of immunotherapy, islet transplantation should only be used as an intervention for those most at risk of harm from recurrent severe hypoglycaemia, where the intervention can bring about an immediate improvement in quality of life and reduce mortality risk such that this outweighs any potential long-term risks from immunosuppression therapy.

Similarly, it should be acknowledged that type 1 diabetes is a disease that can result in long-term complications that have a substantial impact on quality of life, and assessment of any intervention that might improve long-term clinical outcomes should be supported so that the potential risks and benefits of the intervention can be established prior to a decision on its place in routine clinical practice. Following this study’s reported findings for the first three years of the UK islet transplant programme, commissioning of multicentre, randomised trials should be considered to assess the impact of islet transplantation on long-term clinical outcomes in individuals with type 1 diabetes.

7.3 Endogenous C-peptide secretory capacity has a continuous relationship with clinical outcome measures of blood glucose control and might determine long-term outcomes in individuals with type 1 diabetes

CGM analysis techniques used in this study have provided a greater understanding of the impact of endogenous C-peptide secretory capacity on blood glucose control, demonstrating a continuous relationship between stimulated C-peptide and multiple clinical and metabolic parameters including blood glucose variability, hyperglycaemia risk and hypoglycaemia risk. Work undertaken during this research has quantified just how much each of these parameters might improve with increases in endogenous C-peptide secretory capacity.

This study’s results go beyond the original conclusions of the DCCT by showing that these relationships are maintained at levels of endogenous C-peptide well below the 200
pmol/L cut-off originally quoted in the DCCT. Even low levels of endogenous C-peptide production might provide clinical benefits.

Ultra-sensitive assays capable of detecting C-peptide under 5 pmol/l now allow very low levels of C-peptide to be detected using an electrochemiluminescence assay, and a recent study in 74 volunteers with diabetes (median duration 30 (19-41) years) has suggested that individuals with long-standing diabetes may be insulin microsecretors with functioning beta cells (Oram et al., 2014). Absolute C-peptide levels were reported to fall with increasing disease duration, although prevalence of detectable C-peptide remained high (68% in 25/37 volunteers with >30 years disease duration). A mixed meal tolerance test was administered to study participants, and C-peptide increased in 80% of those with detectable C-peptide, suggesting the presence of functional beta cells. This finding of residual endogenous C-peptide secretion in individuals with long-standing diabetes has subsequently been repeated in a larger cohort study of 924 individuals using urine C-peptide to creatinine ratio assessment, where 80% of study participants were defined as insulin microsecretors (Oram et al., 2015).

The techniques described for CGM interpretation in this study could be applied to the microsecretor population to assess what benefits might be attained from retaining low levels of endogenous insulin production. CGM could be utilised in this population to establish whether the relationship between outcome measures of glycaemic control and endogenous C-peptide secretory capacity is maintained in subjects with extremely low levels of endogenous C-peptide production. It might then be possible to elucidate whether factors other than those assessing overall glycaemic control - such as the degree of glucose variability or hyperglycaemia / hypoglycaemia risk - have any determination on the subsequent risk of developing long-term microvascular and macrovascular complications associated with diabetes. Findings from such a proposed study and this study’s reported findings of a continuous relationship between C-peptide secretory capacity and outcome measures of glycaemic control will establish the benefits of residual endogenous insulin secretion, and may have substantial implications for present and future therapeutic interventions aimed at maintaining or restoring endogenous insulin production in individuals with type 1 diabetes.
7.4 Hepatic fat content quantified for the first time in islet recipients and future studies for the assessment of localised hepatic steatosis in islet recipients

This study is the first to demonstrate how a quantitative assessment of hepatic fat content might be made in islet recipients by use of chemical shift based water-fat separation MRI. For centres with access to this technique, MRI settings can be readily recreated at different sites to standardise the hepatic images produced. If it is agreed that the method of hepatic fat quantification from obtained images in islet recipients is reproducible, centres at different geographical locations might be able to adopt the same analysis techniques to quantify hepatic fat in their own islet recipient cohorts.

Scan analysis has shown that islet recipients do not have a significantly increased level of hepatic fat compared to individuals with type 1 diabetes on CSII therapy, and levels of hepatic fat are substantially less than that seen in individuals with NAFLD.

A focal steatosis pattern that develops in a subgroup of islet recipients post-transplantation has been identified. In the presence of this change, hepatic fat quantification has shown that these recipients appear to have increased levels of hepatic fat. Resolution of steatosis occurred with improvement in graft function following repeat transplantation in one of our recipients, suggesting acute lipotoxicity may be reversible with improved blood glucose control. Identification of focal steatosis on MRI may therefore have a role in determining time of re-transplantation. A heterogeneity index might be used as a measure of focal steatosis, although its use for the assessment of heterogeneous hepatic fat changes requires validation in larger studies.

Ultimately, one of the requirements of islet monitoring tests should be that they allow a clinical intervention to be made if there is concern that the graft might be failing. Novel markers of islet graft stress at an early stage may allow early rescue therapies or repeat transplantation to prevent total graft failure. The initial work of this study has not shown a relationship between hepatic fat content and graft function measured at 12 months post-transplant, suggesting that quantification of hepatic fat may not be a novel marker for islet graft failure. However, the causation and significance of focal steatosis warrants further investigation in larger scale studies before further conclusions and recommendations can be made regarding the use of MRI in islet recipients to assess hepatic fat.
7.5 Strategies to prevent DSA formation could be key in improving islet graft outcomes, with focus on early inflammatory responses following transplantation

All islet recipients developing DSA in this study experienced graft failure within 8 months of its detection, suggesting that DSA may be a genuine prognostic marker for future graft rejection, and that strategies should be implemented to prevent its formation post-transplantation.

Investigation of factors that promote DSA formation in the peri-transplant period could be key in improving islet graft outcomes. During islet isolation, tissue digestion of the donor pancreas leads to disruption of the extracellular matrix and loss of cell adhesion molecules, reducing islet function and survival (Hammar et al., 2004). Isolation requiring tissue digestion at ‘high’ temperatures adds another level of injury to the islets (Noguchi et al., 2010). Notably, injured islets undergo shedding of donor antigens, which may contribute to the first steps of initiating an adaptive immune response.

Islets constitutively express HLA class I antigens but it has been demonstrated that they can be induced to express HLA class II molecules following stimulation with inflammatory markers tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) (Jackson et al., 2009); this might precede HLA class II DSA formation and play a critical role in islet allograft rejection. Studies assessing allograft specific cytokine profiles by IFNγ:IL-10 ratio have shown that when the profile is skewed towards a T\textsubscript{H}2 T helper cell profile with raised concentrations of IL-10, post-transplant insulin independence rates are higher than recipients with a T\textsubscript{H}1 T helper cell profile (Huurman et al., 2009). IL-10 stimulates regulatory T cells and is inversely correlated with the proliferation of alloreactive cytotoxic T cell precursors; it also restricts the antigen presenting functions of dendritic cells, and IL-10 modified dendritic cells might induce transplant tolerance (Lai et al., 2009). Therefore, interventions encouraging a T\textsubscript{H}2 T helper cell response and increased IL-10 concentrations might reduce the risk of DSA formation and subsequent graft failure.

T cell studies measure T cell response to transplant alloimmune and diabetes-related autoimmune antigens, and these responses may provide an earlier view of the pathogenic processes underlying transplant rejection prior to DSA and autoantibody formation. Lymphopenia that follows induction immunosuppression has been associated with increased IL-7 and IL-15 release: IL-7 expands the CD4+ helper T cell population;
IL-15 expands the number of CD8+ cytotoxic T cells, including highly enriched GAD-65 specific autoreactive T cell clone populations. The presence of autoreactive T cells proliferating after incubation with islet-specific autoantigens has been shown to be predictive of post-transplant insulin independence (Huurman et al., 2008), and it has been suggested that T cell studies might be used to measure autoreactive effector T cell IFNγ production pre-transplant as a prognostic tool for determining future islet transplant outcomes (Van Belle and von Herrath, 2008). An increase in alloreactive cytotoxic T cell lymphocyte precursors has also been associated with poorer islet transplant outcomes (Roep et al., 1999), and it may be this response that precedes DSA formation and might be used as an earlier prognostic marker of future graft dysfunction. However, the challenge of using T cell studies to predict future clinical outcomes are that they are time consuming and notoriously difficult to undertake, being reliant on the provision of substantial blood samples (typically 50 mls) for processing by expertly trained staff prior to interpretation.

Initial inflammatory challenges might result in antigen exposure that leaves islet cells susceptible to secondary immunological responses, provoking allogeneic rejection. Future studies should therefore focus on the mechanisms that underlie early islet damage following transplantation, as it may be that these are the initial steps that later provoke DSA formation and subsequent islet graft loss. Animal studies with radiolabelled islets have utilised positron emission tomography and computed tomography techniques to demonstrate that half the transplant mass is lost within the first minutes to hours post-transplant, with <50 % of transplanted islets engrafting two hours after intraportal infusion (Eich et al., 2007, Eriksson et al., 2009). Early losses are primarily non-specifically immunological within the liver, secondary to ischaemia-reperfusion injury and non-specific inflammatory phenomena, such as the instant blood mediated inflammatory reaction (IBMIR) (Barshes et al., 2005, Tjernberg et al., 2008, Lai et al., 2009, Grotting et al., 1978).

The IBMIR is triggered by naturally occurring IgG and IgM, with islets subjected to a double-peak attack: rapid complement activation induced by naturally occurring antibodies is followed by a slower coagulation reaction triggered by Tissue Factor (TF) (Tjernberg et al., 2008). TF is a transmembrane glycoprotein expressed by islets that initiates the extrinsic coagulation system (Dahlback, 2000) and binds factor VIIa to cause inflammation (Versteeg et al., 2001). Innate defence cells trigger the IBMIR in
the presence of TF, with release of IL-1B, TNFα, and IFNγ (Gysemans et al., 2008). Activated platelets bind islet surfaces for assembly of coagulation factors (Ozmen et al., 2002), with subsequent macrophage and neutrophil stimulation mediated by TF and monocyte chemoattractant protein-1 (MCP-1). Heparin is now administered in the immediate post-transplant period in islet recipients in an attempt to reduce the severity of this early inflammatory response.

TF might be a potential target for improving islet transplant outcomes given its role in initiating the IBMIR, and use of humanised anti-TF monoclonal antibodies (CNTO 859) has been shown to enhance engrafted islet mass and function (Berman et al., 2007). Other potential inflammatory marker targets for improving islet transplant outcomes include macrophage migration inhibitory factor (MIF). MIF inhibits macrophage migration and mediates cellular immunity, inducing macrophages to produce TNFα and IL-1B; high levels of MIF have been associated with subsequent graft loss in SIK patients (Pfleger et al., 2011). Recently, efforts have focused on the encapsulation of islets in protective barriers to reduce harm from early inflammatory responses but to date these strategies have largely failed due to the size of the protective capsules and the materials used to encase islet isolates (Scharp and Marchetti, 2014).

Inflammatory changes identified in islet isolate preparations pre-transplant might also predict future DSA formation and islet graft function. MCP-1, TNFα, IL-1 and IFNγ have been reported to be released by islets following isolation from a donor pancreas (Lai et al., 2009). MCP-1 is produced by multiple cell types in response to pro-inflammatory stimuli, and it is produced constitutively in human islet preparations. High levels of isolate MCP-1 pre-transplant (measured by mRNA expression using polymerase chain reaction) have been associated with poor clinical outcomes following transplantation, with increased coagulation and inflammation in the immediate post-transplant period (Piemonti et al., 2002, Melzi et al., 2010). MCP-1 assessment might therefore be a useful tool for preselecting islet preparations for engraftment success, and it has been suggested that pre-transplant exposure of isolated islets to nicotinamide might reduce TF and MCP-1 expression and improve islet recipient outcomes (Moberg et al., 2003).

Rejection of islet transplants is multifactorial but identification of markers predictive of graft success or failure will allow interventions to be made that improve outcome. This
study has shown that routine screening for DSA in recipients might predict future graft failure, suggesting that elimination of DSA might improve clinical outcome. However, the rapidity with which graft function was lost on detection of DSA and the inability of current treatments to selectively eliminate alloantibody indicates that alternative therapeutic strategies might need to be considered in the peri-transplant period to improve islet graft outcomes in the longer term.

Assessment of inflammatory markers in the peri-transplant period is likely to be the next crucial step in the search for prognostic markers of graft function, with the goal being identification of a marker that will also be amenable to medical interventions that improve clinical outcomes for future islet recipients. Identification and management of the correct inflammatory marker may start even before the point of transplantation, with assessment and treatment of islet isolates pre-transplant.

TNFα is known to be cytotoxic to human islets in culture (Rabinovitch et al., 1990), and the use of etanercept – a TNFα inhibitor – in the peri-transplant period has been associated with improved long-term insulin independence (Bellin et al., 2012); it remains to be seen whether etanercept use might reduce DSA formation post-transplant. Future studies should identify which inflammatory markers in the immediate post-transplant period are associated with graft dysfunction, and investigate the means by which these responses might be suppressed to improve graft outcomes.
Appendix One

Islet transplantation: Service specifications
(Non-Research Requirements)

The National Commissioning Group (NCG) requirements for islet transplantation list the **investigations and follow-up that must be arranged for all patients** undergoing islet cell transplantation, regardless of whether they are participating in additional research studies.

**Indications for referral for islet transplantation**

The overall aim of the islet transplantation service is to provide patients with freedom from episodes of recurrent severe hypoglycaemia. Referral is anticipated from secondary care but can come directly from primary care. Post-transplant follow up necessitating regular outpatient attendance will be undertaken by the multidisciplinary team at each transplant site. Patients to be considered for referral:

1. Recurrent severe hypoglycaemia in patients with type 1 diabetes who have persistent hypoglycaemia despite full education and multidisciplinary team intervention.

2. Patients with type 1 diabetes who are already immunosuppressed following renal transplantation and continue to have problematic blood glucose control.
Attendance at first outpatient appointment

Patient selection criteria

Acceptable indications for listing for islet transplantation:

1. Patients experiencing ≥1 episode of severe hypoglycaemia requiring third party intervention per year AND ≥2 episodes of severe hypoglycaemia within the last 2 years or 1 episode in the last year.

Usually the rate of severe hypoglycaemia will be higher but this criterion is used to allow inclusion of patients who begin to experience recurrence of severe hypoglycaemia having previously obtained benefit from optimization of therapy and in those who have relaxed control in an attempt to avoid hypoglycaemia.

2. Evidence of impaired awareness of hypoglycaemia.

3. Marked glycaemic lability as defined by the Ryan Lability Index or continuous subcutaneous glucose monitoring profiles.

Requirements for patients being listed for islet transplantation

1. Established C-peptide negative diabetes (Type 1 diabetes; monogenic diabetes; post-pancreatectomy)
   a. Male and female patients aged 18 years or older
   b. Insulin dependence for at least 5 years
   c. Negative C-peptide (below the limit of detection on local assays in the absence of hypoglycaemia – blood glucose ≥ 4 mmol/l)

Note: Children will not ordinarily be considered for transplant given the potential long-term complications of immunosuppression.
2. **Intensive diabetes management**
   a. Evidence of compliance with expert medical advice
   b. Glucose testing 3 or more times daily
   c. Evidence of diabetes self-management and re-education
   d. Optimized insulin regimens – this would usually include a trial of continuous subcutaneous insulin infusion (CSII) via an insulin pump

3. **Absence of insulin resistance**
   a. Insulin requirement of <0.7 units/kg body weight per day to achieve an HbA1c <9%.
   b. BMI <28 kg/m²

   (Maximum insulin dose usually < 55 units/day and weight usually <80 kg)

4. **Absence of contraindications to the use of the immunosuppressants**
   a. Impaired renal function
      i. Isotopic GFR <60 mls/min/1.73m² (unless previous renal transplant, GFR 60-90 mls/min/1.73m² to be discussed with nephrologist)
         
      *or*
      
      ii. Macroalbuminuria (AER >300 mg/24hr) or overt proteinuria (unless previous renal transplant))
   
   b. Uncontrolled hypertension
   c. Uncontrolled dyslipidaemia
   d. Active infection including HBV, HCV, HIV, TB or aspergillus within previous year
   e. Any history of malignancy except completely resected squamous or basal cell carcinoma of skin
   f. High index of suspicion of non-compliance with conventional therapy
   g. Pregnancy or plans for pregnancy (including fatherhood)

5. **Absence of contraindications to surgery**
   a. Untreated proliferative retinopathy
   b. Recent myocardial infarction or uncorrected myocardial ischaemia
   c. Portal hypertension, gall stones or liver haemangioma on baseline ultrasound
   d. Anaemia / leucopenia / thrombocytopenia; coagulopathy
e. On anticoagulants (excluding aspirin)
f. Active gastric or duodenal ulcer; pancreatitis
g. Abnormal liver function tests (persistently >1.5 x upper limit of normal)

6. Other contraindications
   a. Addison’s disease (untreated)
   b. Malabsorptive disease (untreated)
   c. Evidence of alcohol excess or other drug abuse
   d. Concomitant conditions requiring steroid therapy (>15 mg prednisolone daily)

**NOTE: Indications for pancreas transplant alone versus islet transplant alone**

The indications for pancreas transplant alone (PTA) are the same as those for islet transplant alone (ITA). PTA may be considered in patients with a low cardiovascular risk and in patients who are fit for major surgery, especially if insulin independence is the desired primary outcome of intervention over and above that of the achievement of freedom from severe hypoglycaemia. The morbidity and mortality risk from PTA is much higher than that from ITA, and these risks must be weighed up against the likelihood and benefits of achieving / maintaining insulin independence post-intervention.

**Requirements in individuals with sub-optimal control despite a functional renal graft (Islet After Kidney [IAK] transplantation)**

Patients should have a functional renal transplant and be clinically stable on immunosuppression (with prednisolone dose ≤5mg daily).

There are no absolute renal contraindications to listing for islet after kidney transplant, but caution if GFR <40 ml/min/1.73m² / serum creatinine >175µmol/L.

The following renal transplant recipients would be eligible for islet transplantation:

1. Severe hypoglycaemia or altered hypoglycaemia awareness
2. HbA1c >7% (>53 mmol/mol)

3. Evidence of marked glycaemic lability

This group of patients may also be considered for pancreas after kidney transplantation provided they do not have a high cardiovascular risk precluding the more major whole organ transplant procedure.
Patient assessment for islet transplantation

Patients fulfilling selection criteria are put through a series of investigations designed to assess suitability for the procedure.

1. **Hypoglycaemia assessments**: standardised recording of number and impact of severe hypoglycaemic events
   a. Nature and number of severe hypoglycaemia events requiring third party assistance over last 12 months should be recorded
   b. Visits should be preceded by one month’s glucose diary, minimum of 4 point day profile and a single 8 point profile with a complete log of all glucose levels <4 and/or hypo episodes per month
   c. Modified Clarke & Gold impaired hypoglycaemia awareness assessments
   d. Completion of Ryan Hypo Score / Lability Index *(optional)*
   e. Performance of 3 to 7 day continuous sub-cutaneous glucose monitoring profile

2. **Blood tests**: to exclude concurrent pathology and characterise diabetes control/complication
   a. Full biochemistry profile to include
      i. Renal profile – inc eGFR
      ii. Bone profile
      iii. Liver profile
      iv. Bicarbonate
      v. CRP
      vi. Immunoglobulins
      vii. Glucose
      viii. HbA1C
      ix. Lipid profile
      x. Thyroid function
      xi. Addison’s assessment of serum cortisol ± short synacthen test
      xii. Coeliac screen
      xiii. Ferritin, B12 and folate
      xiv. PSA (if male)
      xv. β-HCG (if female of reproductive age)
b. Haematology
   i. Full blood count
   ii. Clotting screen

c. Assessment of residual β-cell function
   i. Undetectable C-peptide (below the limit of detection on local assays in the absence of hypoglycaemia – blood glucose ≥ 4 mmol/l)

d. Microbiology and serology
   i. Toxoplasmosis
   ii. VDRL

e. Virology
   i. Hepatitis B
   ii. Hepatitis C
   iii. HIV
   iv. VZV
   v. CMV
   vi. EBV
   vii. HSV

f. Islet auto-immunity
   i. GAD antibodies
   ii. IA-2 antibodies
   iii. Islet cell antibodies

g. Studies of activated T cells (Tim Tree / Mark Peakman, KCH)

h. Blood bank
   i. Group and save serum
   ii. Tissue typing
      i. Tissue type
      ii. PRA and HLA specific antibodies

3. Renal workup
   a. Serum creatinine
   b. 24 hour proteinuria collection
   c. Isotopic GFR

4. Dental health (assessed by qualified dental practitioner)
5. **Ensure no history of previous malignancy**
   a. Breast examination; in women >40 yrs mammography required
   b. Cervical smear will be required

6. **Psychological evaluation**
   Includes semi-structured interview with a clinical psychiatrist or psychologist to assess understanding of risks / benefits of and suitability for transplantation.

   Patient reported psychosocial outcomes are critical in the evaluation of islet transplantation. If the patient consents to the Biomedical and Quality of life study, a Psychosocial questionnaire pack should be provided to the patient for completion *(see islet transplant research protocol)*. The answers to these questionnaires will be received and collated by the Applied Health Psychology team for a psychological evaluation that meets NCG requirements.

7. **Cardiac evaluation**: history of cardiovascular; peripheral vascular; cerebrovascular disease
   a. ECG
   b. Stress echocardiography or stress isotope perfusion scan
   c. Where indicated, coronary angiogram. This is not costed in the programme: if indicated by earlier investigations, needs to be undertaken independent of any transplantation plans

8. **Lung Function assessment**
   a. Chest x-ray

9. **Liver ultrasound**
   a. Exclude hepatic pathology
   b. Confirm patency of and direction of flow in portal vein
   c. Exclude vascular malformations

10. **Eye evaluation**: 
    a. Arrange dilated 4 field retinal photography
    b. Consider obtaining opinion on retinopathy from ophthalmologist

Recommend quantitative assessment through:

a. Biothesiometry

and

b. Autonomic function tests

*Note:* Nerve conduction studies are costed into NCG budget but have not been routinely undertaken to date

12. Clinical assessment of diabetic foot disease

Acute ulceration / infection / ischaemia / Charcot foot will require suspension from the transplant waiting list until resolution.

*Multi-disciplinary team meeting for listing for islet transplantation*

After physical and psychological assessment a clinical consensus is reached by the multi-disciplinary transplant team of a patient’s suitability for transplantation.
Listing Visit for islet transplantation

The patient will be required to attend for a listing visit to discuss the outcome of investigations and an MDT meeting will be required before a final decision on whether to proceed to islet transplantation is made.

All assessment investigations will be reviewed with the patient at the listing visit with confirmation of understanding of risks / benefits and desire to proceed.

Fulfilment of listing criteria will be reconfirmed and it is recommended that a 4 week blood glucose diary is specifically completed before this visit.

Standardised recording of the number and impact of severe hypoglycaemic events should be made. The nature and number of severe hypoglycaemia events requiring third party assistance over last 12 months must be documented, and a pre-transplant Clarke and Gold hypoglycaemia score recorded.

If the decision is to pursue transplantation, the patient is listed locally for islet transplantation:

1. Complete NHS blood and transplant kidney/pancreas/islet recipient registration (including the supplementary form) at time of listing

2. On-line registration for transplantation at ODT
If no transplant is undertaken within 6 months, then **6 monthly review** is required to assess ongoing suitability for islet transplantation. At each visit the following must be provided by the patient:

1. **One month blood glucose diary readings.** A minimum of 4 day profiles, a single 8 point profile and a complete log of all glucose levels <4 and/or hypo episodes per month should be recorded.

2. **3 to 7 day CGM download**

3. **Modified Clarke & Gold impaired hypoglycaemia awareness assessments**

At each review, an assessment must be made as to whether any investigations must be repeated to assess a patient’s fitness for islet transplantation.

**At each 6 monthly visit, MDT to review that patient remains suitable to remain on islet transplant waiting list.**
Admission for transplant

On being made aware of a donor for islet transplantation, the patient should attend the islet transplant unit for bloods whilst islet isolation is being undertaken.

Bloods include:
H&I Crossmatch, FBC, U&E, LFTs, TFTs, clotting, HbA1C, glucose

Discuss cross-match with Histopathology & Immunocytogenetics prior to transplantation.

Pre and post-transplant, follow local islet transplant protocols for peri-operative management.

NCG requirements at the time of transplantation:
Ensure the following documentation is completed during the admission:

1. Standard hospital consent for transplantation (NICE requirement for audit purposes)

2. Obtain and record donor UKT / ODT Number (found on islet infusion bag).
   
   *NHSBT donor pancreas form is completed by the isolation labs and returned to ODT.*

3. Return Product Release Form to the isolation centre after filing a copy in the local patient records.

   
   Return form to NHSBT at ODT Data services, NHS Blood and Transplant, Fox Den Road, Stoke Gifford, Bristol. BS34 8RR

5. Ensure HTA B form completed & signed and sent to ODT.
   
   Return form to ODT Data services, NHS Blood and Transplant, Fox Den Road, Stoke Gifford, Bristol. BS34 8RR

At the time of transplantation, ensure a donor islet sample is sent for Gram stain and culture both from the islet isolation centre and the transplantation centre. Regular liaison with the microbiology teams at the site of isolation and the site of transplantation.
must occur in the peri-transplant period to identify whether any additional treatments are required in the event of positive culture results from any of the samples at each centre.

It is anticipated that patients will remain in hospital for 3 days following each transplant, with close monitoring of blood glucose levels, adjustment of insulin doses and immunosuppressant medications, measurement of C-peptide and monitoring for bleeding following portal vein cannulation (including a liver ultrasound on day +1).

It is imperative that a standard discharge summary is sent to the patient’s GP to inform them of the patient’s treatment and medications on discharge (especially immunosuppressant therapy). On discharge, arrange appointment to see patient for next blood test and to review immunosuppressant levels and dose.
Immediate post-discharge management

Regular structured review should be organized, including monitoring of immunosuppression levels and assessment for complications over the first 3 months. Following discharge, a UKITC glucose diary should be given to the patient to complete.

Suggested blood test schedule post discharge:

**Day 7**
FBC, U& E, LFT, TFT, CRP, Tacrolimus level
Random glucose & C-peptide

**Day 10**
FBC, U& E, LFT, Tacrolimus level
CRP if previous result not within normal range

**Day 21**
FBC, LFT, U& E, Tacrolimus level
CRP if previous result not within normal range

**Day 28**
FBC, LFT, U& E, Tacrolimus level
CRP if previous result not within normal range
Fasting glucose and C-peptide, HbA\textsubscript{1C}
1 month post-transplant:

Patient to receive clinical review by managing team

This visit will allow a review of the patient’s progress post-transplantation, an assessment of their insulin dosing and adjustment of their immunosuppression if required.

1 month – 3 months
Review every 2 weeks up to 3 month post-transplant
HbA1c, FBC, U&E, Tacrolimus level to be checked at each review visit
CRP and LFT should be checked at review visits if the previous results have not been within normal range

In addition, at 3 month visit:
Fasting glucose & C-peptide, HbA1c, antibody testing for ICA, anti-GAD and IA-2,
fasting lipid profile & TFTs

Month 3 – Month 6
Review every 4 weeks
FBC, U&E, Tacrolimus level to be checked at each review visit
CRP and LFT should be checked at review visits if the previous results have not been within normal range

In addition, at 6 month visit:
Fasting glucose & C-peptide, HbA1c, antibody testing for ICA, anti-GAD and IA-2,
fasting lipid profile & TFTs

Month 6 onwards
Review every 6 weeks
FBC, U&E, Tacrolimus level to be checked at each review visit
CRP and LFT should be checked at review visits if the previous results have not been within normal range
Fasting glucose & C-peptide, HbA1c, fasting lipid profile & TFT at 6 monthly visit
Scheduled follow-up assessment at 3, 6 and 12 months

NCG requirements specify that follow-up and review should at least be organized for 3, 6 and 12 months post-transplant. **If a patient has a further transplant, an extra follow-up one month after discharge must be organized for an additional patient review.**

At each scheduled follow-up:

1. Complete NHSBT forms at the each timepoint. 
   Return form to NHSBT at ODT Data services, NHS Blood and Transplant, Fox Den Road, Stoke Gifford, Bristol. BS34 8RR

2. Hypoglycaemia assessments at 3, 6 and 12 months
   a. Standardised recording of number and impact of severe hypoglycaemic events. Visits should be preceded by one month’s glucose diary, minimum of 4 point day profile and a single 8 point profile with a complete log of all glucose levels<4 and/or hypo episodes per month
   b. Continuous glucose monitoring assessment (CGMS) at 3, 6, 12 months. CGMS device should be attached to patient 7 days before each review. 1 single 8 point profile to be completed while wearing the CGMS sensor

3. Urine albumin-creatinine ratio assessment

4. Retinal screening assessment – dilated 4-field retinal photography

5. Clinical assessment of peripheral neuropathy and foot risk - quantitative assessment with nerve conduction studies and autonomic nerve function tests should be considered at each review

6. T cell studies – autoreactive T cell profiles may be a predictive marker for graft function and loss and they are thus a clinical requirement in all islet transplant patients. This assessment is dependent on the provision of a service that can undertake T cell study analysis, and this is currently provided by Tim Tree at King’s Hospital.
After 12 months, follow-up should be arranged on a 6 monthly basis for ongoing review of the patient post-transplantation.

*Secondary endpoints: to be determined at 3 months post-transplant and then annually.*

1. Insulin independence / insulin requirement

2. \( \text{HbA}_1c \)

3. Clarke survey / Hypo Score / Lability Index

4. Measurement of glucose, insulin and C-peptide. Consider patient for standardised meal tolerance test with fasting and 90 minute values.

5. Serum creatinine / urinary protein estimation

6. Dilated 4 field Retinal photography

7. Detailed clinical assessment of diabetes / immunosuppressant complications status

8. Quality of life and health status questionnaires – alternative arrangements for psychological assessment must be made if the patient is not participating in the Biomedical and Quality of Life research study and completing the Psychosocial questionnaire packs (*see research protocol*).
Appendix Two

Islet transplant research manual: Research summary for patients undergoing islet transplantation

Pre transplant

First clinic visit

Please see NCG requirements section for work-up and required investigations pre-transplant; these assessments will take place in all patients being considered for islet transplantation, whether they consent to research or not.

After the first clinic visit and when all investigations are available, a multi-disciplinary team (MDT) meeting will take place and a decision on whether to offer to list the patient for islet transplantation will then be made. The patient will then return to outpatients for their listing visit to be informed of the outcome of the MDT meeting and make their final decision.

Listing Visit

If the patient is listed for an islet transplant, the following should be arranged:

1. The opportunity to participate in research should be offered to the patient and they should be provided with the participant information sheet for biomedical and quality of life research in islet transplantation.

2. Arrange research visit.

• The research visit can be at the same time as the listing visit, depending on the preferences of the patient and the local islet transplant team.
• The aim of the Research Visit is an opportunity for the transplant team to explain and consent patients for research projects associated with islet transplantation.

• The patient is at liberty not to consent for research projects but must understand that some investigations are still required as part of the service specific requirements for islet transplantation (National Commissioning Group [NCG] requirement. A screening log entry of the patient’s decision not to participate in research should be made locally.

**Research Visit**

The research visit is to allow the patient to discuss the aims of the research and ask any questions regarding research, prior to signing consent forms. Patients do not have to undertake all aspects of the available research.
**Study Consent Forms**

1. **Biomedical and quality of life consent form** - Consenting to completion of adult healthcare psychology questionnaire pack for centralized analysis and analysis of their collated biomedical data for research presentations / publication.

2. **Islet function assessment consent form** - Includes mixed meal tolerance testing [MMTT] post transplant. Covers blood samples sent for metabolic and inflammatory marker analysis at Cambridge and King’s, and urine C-peptide analysis at Exeter.

3. **Liver Magnetic Resonance Imaging (MRI) scan consent form.** If consent is given for MRI scanning, send a letter to the patient’s GP informing them of participation in the study.

At the research visit, if patients have consented to participate in studies, please ensure the following:

1. **Assign patient a Study Number**

2. **Pre-transplant Case Report Forms (CRF)** – History, examination, investigation forms. Return copies to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

3. **Psychosocial questionnaire pack.** Add the study number to the packs before giving to the participant. Participants return these in the reply paid envelope direct to Applied Health Psychology (AHP) Research. Ensure that study participant returns it to: UKITC Project, AHP Research, 16 Walden Way, Hornchurch, Essex RM11 2LB

4. **Modified Clarke & Edinburgh (includes Gold score) Hypoglycaemia questionnaire.** These are an NCG requirement but please ensure that a copy of the completed questionnaire is returned to Ruth Wood at Newcastle Clinical Trials Unit. Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH
5. **Arrange for patient to have pre-transplant MRI of liver if they consent to the liver MRI study**

6. Patient to have a further 50 ml blood sample taken on two separate occasions for **T-cell studies – sample must be taken on a Monday** and sent to Shereen Sabbah at Guy’s Hospital via DX Drop box delivery system. Using DX delivery system, ensure samples are sent to Dr Shereen Sabbah, Central Specimen Reception, 4th Floor, Guy's Hospital Southwark Wing, London SE1 9RT (DX number 6571004, DX Exchange 6571000)

7. **Collection of a sample for urine C-peptide.** This is a research urine sample to assess endogenous insulin production by a patient prior to islet transplantation. A urine ‘pack’ can be supplied by Richard Oram at the Royal Devon & Exeter Hospital. All samples to be returned to address Clinical Biochemistry, Area A2, Royal Devon & Exeter Hospital NHS Foundation Trust, Barrack Road, Exeter, Devon EX2 5DW
If no transplant after 6 months –

6 monthly assessments required from date of listing visit

A 6 monthly research assessment visit is required whilst awaiting transplantation. This could take place alongside the 6 monthly assessment that is an NCG requirement in patients on the waiting list for transplantation.

At each appointment:

1. **Complete CRF forms** – Subsequent history, examination and investigations. Return copies to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

2. Give out **Psychosocial questionnaire pack**. Participants return these in the reply paid envelope direct to AHP Research. Ensure that study participant returns the form to: UKITC Project, AHP Research, 16 Walden Way, Hornchurch, Essex RM11 2LB

3. Further 50 ml blood sample taken for **T-cell studies – sample must be taken on a Monday** and sent to Shereen Sabbah at King’s College London via DX Drop box delivery system. Using DX delivery system, ensure samples are sent to Dr Shereen Sabbah, Central Specimen Reception, 4th Floor, Guy’s Hospital Southwark Wing, London SE1 9RT (DX number 6571004, DX Exchange 6571000)

4. **Modified Clarke & Edinburgh (includes Gold score) Hypoglycaemia questionnaire.** A copy of the completed questionnaire is returned to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH
Admission for transplant

Please follow NCG requirements for all patients admitted for transplantation. See NCG section.

For all patients participating in research, please ensure the following:

1. **Complete CRF forms** – Subsequent History, examination, investigations. Return copies to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

2. **On discharge:**
   a. **Complete the CRF Discharge Summary.** Donor ODT number must be recorded on the CRF sheet. Return a copy to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH
   b. Enter the **UKT recipient number** on the CRF discharge summary form
   c. On discharge, give the patient an appointment time and date for their one month post-transplant **Meal Tolerance Test**
   d. **Arrange Liver MRI** for one month post-transplant if participating in MRI study

**Early post-transplant**

1. For research patients, please ensure that **Research samples for C-peptide are taken peri-transplant and most importantly at 7 days post-transplant.** These are stored and then sent to Cambridge as a batch on dry ice for C-peptide analysis. (Consent for transporting these to Cambridge is taken as part of the islet function assessment. Samples can be stored, but not shipped until consent has been given).

**Ensure that blood form has relevant Cambridge labelling prior to being sent to local laboratory to ensure Cambridge receive sample.** Cambridge Address for
samples: Clinical Biochemistry, Box 232, Cambridge University Hospitals NHS Foundation Trust, Cambridge Biomedical Campus, Hills Road, Cambridge CB2 0QQ

2. **Collection of samples for urine C-peptide** following discharge should be started one week after transplant. Once a week, patients should provide a urine specimen on waking (first void of the day) followed by a second sample two hours after breakfast. Ensure patient has sufficient supply of urine ‘packs’ until one month meal tolerance test and samples are returned to Richard Oram at Royal Devon & Exeter Hospital. All samples to be returned to address Clinical Biochemistry, Area A2, Royal Devon & Exeter Hospital NHS Foundation Trust, Barrack Road, Exeter, Devon EX2 5DW
1 month post first transplant

NOTE:

- The 1 month follow-up is a research requirement

- Patients should have a one month research follow-up after any second / subsequent transplant and then revert back to 3 / 6 / 12 monthly follow-up from first transplant

- The NCG specifies that scheduled follow-up of islet transplant patients should be at 3, 6 and 12 months. Please ensure that all NCG requirements are met at each follow-up appointment (see NCG section)

At the one month research follow-up, please ensure the following are undertaken:

1. **Completion of 1 month post-transplant CRF forms** – Subsequent History, examination, investigation. Return copies to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

2. The visit should be preceded by **one month’s glucose diary**. A minimum of 4 day profiles, a single 8 point profile and a complete log of all glucose levels <4 and/or hypo episodes per month should be recorded.

3. The visit should be preceded by a **1 week CGMS download**

4. **Modified Clarke/Edinburgh Hypoglycaemia questionnaire**. A copy of the completed questionnaire is returned to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH
5. **Give out the Psychosocial questionnaire pack.** Participants return these in the reply paid envelope direct to AHP Research. Ensure that study participant returns the form to: UKITC Project, AHP Research, 16 Walden Way, Hornchurch, Essex RM11 2LB

6. **Attend for 1 month post-transplant Meal Tolerance Test.** Samples are separated, stored, batched and sent to Cambridge for analysis in line with the Cambridge SOP (Cambridge finally send aliquots to King's for cytokine analysis). Cambridge address for samples: Clinical Biochemistry, Box 232, Cambridge University Hospitals NHS Foundation Trust, Cambridge Biomedical Campus, Hills Road, Cambridge CB2 0QQ

7. **Collection of samples for urine C-peptide.** Ensure patient has sufficient supply of urine ‘packs’ until next meal tolerance test and samples returned to Richard Oram at Royal Devon & Exeter Hospital. All samples to be returned to address Clinical Biochemistry, Area A2, Royal Devon & Exeter Hospital NHS Foundation Trust, Barrack Road, Exeter, Devon EX2 5DW

8. **Liver MRI** if participating in MRI study. **Arrange MRI visit** for three months, six months and twelve months post-transplant, if consented to this part of study.
3 months post transplant

NOTE:
• Please ensure that all NCG requirements are met at each follow-up appointment (see NCG section)

The NCG specify that the following are required at 3 months:

1. The visit should be preceded by **one month’s glucose diary**. A minimum of 4 day profiles, a single 8 point profile and a complete log of all glucose levels <4 and/or hypo episodes per month should be recorded.

2. The visit should be preceded by **1 week CGMS download** recorded.

3. **Modified Clarke/Edinburgh Hypoglycaemia questionnaire.** A copy of the completed questionnaire is returned to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

Research patients will also require:

1. **Complete CRF forms** – Subsequent History, examination, investigation. Return copies to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

2. Attend for **3 month post-transplant Meal Tolerance Test.** Samples are then batched and sent to Cambridge for analysis in line with the Cambridge SOP (Cambridge finally send aliquots to King's for cytokine analysis). Cambridge address for samples: Clinical Biochemistry, Box 232, Cambridge University Hospitals NHS Foundation Trust, Cambridge Biomedical Campus, Hills Road, Cambridge CB2 0QQ
3. Patient to have a further 50 ml blood sample for **T-cell studies** – **sample must be taken on a Monday** and sent to Shereen Sabbah at Guy’s Hospital via DX Drop box delivery system. Using DX delivery system, ensure samples are sent to Dr Shereen Sabbah, Central Specimen Reception, 4th Floor, Guy's Hospital Southwark Wing, London SE1 9RT (DX number 6571004, DX Exchange 6571000)

4. Review **weekly urine C-peptide** completion. Ensure patient has sufficient supply of urine ‘packs’ until next meal tolerance test and samples returned to Richard Oram at Royal Devon & Exeter Hospital. All samples to be returned to address Clinical Biochemistry, Area A2, Royal Devon & Exeter Hospital NHS Foundation Trust, Barrack Road, Exeter, Devon EX2 5DW

5. **Liver MRI** if participating in MRI study

   No psychosocial questionnaire pack at 3 months
6 months post transplant

NOTE:
- Please ensure that all NCG requirements are met at each follow-up appointment (see NCG section)

The NCG specify that the following are required at 6 months:

1. The visit should be preceded by **one month’s glucose diary**. A minimum of 4 day profiles, a single 8 point profile and a complete log of all glucose levels <4 and/or hypo episodes per month should be recorded.

2. The visit should be preceded by **1 week CGMS download** recorded.

3. **Modified Clarke/Edinburgh Hypoglycaemia questionnaire.** A copy of the completed questionnaire is returned to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

Research patients will also require:

1. **Complete CRF forms** – Subsequent History, examination, investigation. Return copies to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

2. Attend for **6 month post-transplant Meal Tolerance Test**. Samples are then batched and sent to Cambridge for analysis in line with the Cambridge SOP (Cambridge finally send aliquots to King's for cytokine analysis). Cambridge address for samples: Clinical Biochemistry, Box 232, Cambridge University Hospitals NHS Foundation Trust, Cambridge Biomedical Campus, Hills Road, Cambridge CB2 0QQ
3. Patient to have a further 50 ml blood sample for T-cell studies – sample must be taken on a Monday and sent to Shereen Sabbah at Guy’s Hospital via DX Drop box delivery system. Using DX delivery system, ensure samples are sent to Dr Shereen Sabbah, Central Specimen Reception, 4th Floor, Guy's Hospital Southwark Wing, London SE1 9RT (DX number 6571004, DX Exchange 6571000)

4. Review weekly urine C-peptide completion. Ensure patient has sufficient supply of urine ‘packs’ until next meal tolerance test and samples returned to Richard Oram at Royal Devon & Exeter Hospital. All samples to be returned to address Clinical Biochemistry, Area A2, Royal Devon & Exeter Hospital NHS Foundation Trust, Barrack Road, Exeter, Devon EX2 5DW

5. Give out Psychosocial questionnaire pack. Participants return these in the reply paid envelope direct to AHP Research. Ensure that study participant returns it to: UKITC Project, AHP Research, 16 Walden Way, Hornchurch, Essex RM11 2LB

6. Liver MRI if participating in MRI study
12 months post transplant

NOTE:
- Please ensure that all NCG requirements are met at each follow-up appointment (see NCG section)

The NCG specify that the following are required at 12 months:

1. The visit should be preceded by one month’s glucose diary. A minimum of 4 day profiles, a single 8 point profile and a complete log of all glucose levels <4 and/or hypo episodes per month should be recorded.

2. The visit should be preceded by 1 week CGMS download recorded.

3. Modified Clarke/Edinburgh Hypoglycaemia questionnaire. A copy of the completed questionnaire is returned to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

Research patients will also require:

1. Complete CRF forms – Subsequent History, examination, investigation. Return copies to Ruth Wood at Newcastle Clinical Trials Unit, Human Nutrition Research Centre, Room M1 151, 1st Floor William Leech Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

2. Attend for 12 month post-transplant Meal Tolerance Test. Samples are then batched and sent to Cambridge for analysis in line with the Cambridge SOP (Cambridge finally send aliquots to King's for cytokine analysis). Cambridge address for samples: Clinical Biochemistry, Box 232, Cambridge University Hospitals NHS Foundation Trust, Cambridge Biomedical Campus, Hills Road, Cambridge CB2 0QQ

3. Patient to have a further 50 ml blood sample for T-cell studies – sample must be taken on a Monday and sent to Shereen Sabbah at Guy’s Hospital via DX Drop
box delivery system. Using DX delivery system, ensure samples are sent to Dr Shereen Sabbah, Central Specimen Reception, 4th Floor, Guy's Hospital Southwark Wing, London SE1 9RT (DX number 6571004, DX Exchange 6571000)

4. Review weekly urine C-peptide completion. Ensure patient has sufficient supply of urine ‘packs’ until next meal tolerance test and samples returned to Richard Oram at Royal Devon & Exeter Hospital. All samples to be returned to address Clinical Biochemistry, Area A2, Royal Devon & Exeter Hospital NHS Foundation Trust, Barrack Road, Exeter, Devon EX2 5DW

5. Give out Psychosocial questionnaire pack. Participants return these in the reply paid envelope direct to AHP Research. Ensure that study participant returns it to: UKITC Project, AHP Research, 16 Walden Way, Hornchurch, Essex RM11 2LB

6. MRI at 12 months to be done at local centre if participating in MRI study
More than 12 months post transplant

Patients should continue to be followed up 3 monthly if the local team have the capacity to do so. Otherwise, 6 monthly full research follow-up visits are acceptable to remain a participating centre for the Diabetes UK research study.

Psychosocial questionnaire packs should be completed every 6 months.

Ensure all NCG requirements are completed at each 6 month appointment.

Research patients should continue to have 6 monthly testing as listed in the previous sections whilst they are considered to have ongoing graft function by the lead clinician (detectable serum C-peptide >50 pmol/l).
Appendix Three

Protocol for MRI analysis in islet recipients

A) Conversion of Original Scans into “fat” and “water’ files

From University Login:

1) Open ‘MATLAB 2011a’ in Scientific Software File
2) In ‘Current Directory’, select “X:\” or “Z:\” (as appropriate according to “T1Tx“ location)
3) Select ‘Analysis Program’ – Right click – select ‘Add to Path’ – select ‘Folders and subfolders’
4) In command window, type ‘fatcalc’
5) Select ‘3’ PAR files for analysis
6) Select MRI file for analysis, then pick 3 files in order of ‘inferior, middle, superior’ or ‘3, 2, 1’
7) Analyse
8) Look for abdominal fat in fat window to ensure correct analysis (x3)
9) File conversion complete

B) Fat analysis of converted files

From University Login

1) Open ‘Image J’
2) Select subfile for analysis ending in’fatPC.hdr’
   NB: For water images (& to be blinded to fat content but to see vessels) use ‘water.hdr’ file
3) Check image to ensure has been converted from original scan appropriately (check no inversion)
4) Select 3 of 18 files with largest liver surface are available for analysis
5) Mark Region Of Interest (ROI) for analysis: using water file, choose rhomboid symbol and draw area for analysis, avoiding:
   a. Liver Capsule
b. Vessels (where possible)

6) Avoid kidney / other organs

7) Press “T” – Save file with name denoting ‘Subject/Time post-Tx/Slice No’

8) Check ROI transfers okay to fat image to make sure not over artefacts (ENSURE SLICE NUMBERS MATCH UP FOR WATER AND FAT FILES)

9) Save ROI outline:
   a. Press “Ctrl”
   b. Select all
   c. Right click
   d. Save in relevant T1Tx file labelled “Gus ROI” or “Afsara ROI” as appropriate

10) Analyse fat slice:
   a. If desired, change view to ‘Fire’ to see liver more clearly
      i. On Image J title menu, select ‘Image’ then ‘Lookup Tables’
      ii. Select ‘Fire’ view
   b. Image J – select ‘analyze’, then ‘measure’
   c. Gives mean/min/max values

11) Repeat entire process for total of 3 slices

12) In results box, select ‘edit’ then ‘summarise’ – this will give mean / min / max / SD for all 3 slices analysed

   a. This file contains tabs with ‘Sheet 1 / Slice 1 / Slice 2 / Slice 3 / Mean 20 bin / Mean 100 bin’
   b. Paste ‘1, 2, 3, mean, SD’ into sheet 1
   c. Rename Slice Numbers

14) For Histograms:
   a. Press ‘H’ whilst analysing fat file
   b. On menu, type in ‘100 bins’ , unclick ‘min/max’, X min ‘0’, X max ‘100’, Y max ‘Auto’
   c. Copy down SD into Xcel file ‘Sheet 1’
   d. Copy Histogram to Xcel file for relevant slice
      i. Paste data on ‘0’ for 100 bin histogram – this gives you 100 bin and 20 bin data automatically
      ii. Rename Slice tab
iii. Repeat for next 2 slices
e. On ‘Mean 20 bin tab’, paste 20 bin values (include all values >0 only) for each slice, pasting ‘123 paste option’
   i. Rename all columns appropriately
   ii. Empty boxes need to be zero (affects mean column values)
f. Repeat step ‘e.’ for ‘Mean 100 bin tab’ and repeat for all 3 slices
   i. 3 x columns
   ii. Empty boxes need to be zero (affects mean column values)

15) On ‘islet’ and ‘Pump patient’ Xcel tabs, entered values will produce graphs

16) Xcel file for scan analysis now complete for selected T1Tx file

17) Before finishing, copy data to reference table file for all data
   a. On “Summary Sheet” “Sheet 1” of file, copy ‘Slice file values (eg 11, 12, 13), mean & SD’, ‘Copy’
   b. Open ‘T1Tx’
   c. Select ‘Afsara_outputs_summer_2012_legacy’
   d. Select ‘Tables’
   e. Copy Means & SD in 3 slices fat PC into appropriate ‘Gus’ or ‘Afsara’ file

C) Converting T1Tx files to ‘In and out phase’ files

Looks at difference between fat and water phases to highlight areas of fat
Allows identification of fat pattern quickly

From University Login:
1) Need to reimage in Matlab
2) Choose appropriate ‘X’ or ‘Z’ drive
3) Select ‘Analysis NMRC 20’, Right click and select ‘Folders and subfolders’
4) In command window, type ‘out of phase’
5) Choose ‘3’ files for analysis
6) Select files in order of inferior, middle and superior
7) This will give you 2 ‘out of phase’ images and 1 ‘in phase’ image in “Image J”:
   a. Select appropriate patient file:
      i. Choose ‘inphase.hdr’ / ‘outofphase.hdr’ / ‘outofphase2.hdr’
      ii. Black lines are more clear on ‘out of phase’ images
8) Looking for exclusive fat pattern on ‘out of phase’ images that are not present on ‘in phase’ images
   a. Note decision of ‘yes’ or ‘no’ for whether exclusive fat pattern is present
   b. Record yes/no decision in appropriate Xcel file tab of ‘Gus’ or ‘Afsara’ file

**D) Altering Colour % on files to enhance fatty contrast on fat files**

From University Login:

1) Open Matlab, select appropriate drive and then file
2) Select ‘Analysis Program NMRC 20’
3) Select ‘Afsara_read’
4) Look for numbers in brackets (eg ‘[1,25]’), and set values as desired, then save
5) Type ‘Afsara_read’ in command menu, then select ‘fatPC.hdr’ file for analysis
6) This program gives 1-18 slices with altered % contrasts in image J
   a. Select the same 3 slices that were used for analysis
   b. Save images as ‘jpeg’ file in ‘image file’ appropriate T1Tx file
      i. In name of file, include Slice No / Min % / Max % (Lower percentage and upper percentage borders for colour range)
      ii. Select to save as ‘jpeg’ file
REFERENCES
REFERENCES


BAIDAL, D. A., FARADJI, R. N., MESSINGER, S., FROUD, T., MONROY, K.,
RICORDI, C. & ALEJANDRO, R. 2009. Early metabolic markers of islet allograft

BALLINGER, W. F. & LACY, P. E. 1972. Transplantation of intact pancreatic islets in

12, 141-6.

insulin autoantibody levels in the Diabetes Prevention Trial Type 1 oral insulin study.
*Diabetologia*, 50, 1603-6.

BARSHES, N. R., LEE, T., GOODPASTURE, S., BRUNICARDI, F. C.,
ALEJANDRO, R., RICORDI, C., SOLTES, G., BARTH, M., HAMILTON, D. &
GOSS, J. A. 2004. Achievement of insulin independence via pancreatic islet
transplantation using a remote isolation center: a first-year review. *Transplant Proc*, 36,
1127-9.

dysfunction and apoptosis in pancreatic islet transplantation: implications for

BARTON, F. B., RICKELS, M. R., ALEJANDRO, R., HERING, B. J., WEASE, S.,
NAZIRUDDIN, B., OBERHOLZER, J., ODORICO, J. S., GARFINKEL, M. R.,


CHRISTIAANS, M. H., NIEMAN, F., VAN HOOFF, J. P. & VAN DEN BERG-LOONEN, E. M. 2000. Detection of HLA class I and II antibodies by ELISA and


region contributes to susceptibility to IDDM in the Finnish population. Childhood Diabetes in Finland (DiMe) Study Group. *Diabetologia*, 37, 937-44.


GREENBAUM, C. J., MANDRUP-POULSEN, T., MCGEE, P. F., BATTELINO, T.,
HAASTERT, B., LUDVIGSSON, J., POZZILLI, P., LACHIN, J. M. & KOLB, H.
2008a. Mixed-meal tolerance test versus glucagon stimulation test for the assessment of

GREENBAUM, C. J., MANDRUP-POULSEN, T., MCGEE, P. F., BATTELINO, T.,
HAASTERT, B., LUDVIGSSON, J., POZZILLI, P., LACHIN, J. M., KOLB, H., TYPE
Mixed-meal tolerance test versus glucagon stimulation test for the assessment of beta-

the fog: recent clinical trials to preserve beta-cell function in type 1 diabetes. *Diabetes*,
61, 1323-30.

GROTTING, J. C., ROSAI, J., MATAS, A. J., FRENZEL, E. M., PAYNE, W. D.,
islets in diabetic rats. A morphologic and immunohistochemical study. *Am J Pathol*, 92,
653-70.

GRUESSNER, A. C. 2011. 2011 update on pancreas transplantation: comprehensive
trend analysis of 25,000 cases followed up over the course of twenty-four years at the

Cytokine signalling in the beta-cell: a dual role for IFNγamma. *Biochem Soc Trans*, 36,
328-33.


HODGES, A. M., LYSTHER, H., MCDERMOTT, A., RICE, A. J., SMITH, J. D.,
ROSE, M. L. & BANNER, N. R. 2012. Late antibody-mediated rejection after heart
transplantation following the development of de novo donor-specific human leukocyte
antigen antibody. Transplantation, 93, 650-6.

HOI-HANSEN, T., PEDERSEN-BJERGAARD, U. & THORSTEINSSON, B.
Classification of hypoglycemia awareness in people with type 1 diabetes in clinical

HOPKINS, D., LAWRENCE, I., MANSELL, P., THOMPSON, G., AMIEL, S.,
CAMPBELL, M. & HELLER, S. Improved biomedical and psychological outcomes 1
year after structured education in flexible insulin therapy for people with type 1

Pancreatic beta-cell responsiveness during meal tolerance test: model assessment in
normal subjects and subjects with newly diagnosed noninsulin-dependent diabetes

HU, C. Y., RODRIGUEZ-PINTO, D., DU, W., AHUJA, A., HENEGARIU, O.,
antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest, 117, 3857-
67.

NMR Biomed, 26, 1609-29.


INTERNATIONAL ASSOCIATION OF, D., PREGNANCY STUDY GROUPS
CONSENSUS, P., METZGER, B. E., GABBE, S. G., PERSSON, B., BUCHANAN, T.
A., CATALANO, P. A., DAMM, P., DYER, A. R., LEIVA, A., HOD, M.,
KITZMILER, J. L., LOWE, L. P., MCINTYRE, H. D., OATS, J. J., OMORI, Y. &
SCHMIDT, M. I. 2010. International association of diabetes and pregnancy study
groups recommendations on the diagnosis and classification of hyperglycemia in
pregnancy. *Diabetes Care*, 33, 676-82.

IRVING, C., CARTER, V., PARRY, G., HASAN, A. & KIRK, R. 2011. Donor-
specific HLA antibodies in paediatric cardiac transplant recipients are associated with

receptor with HLA DRB1 0301 in type 1 diabetes patients from North India. *PLoS One*,
4, e8023.

JACKSON, A. M., CONNOLLY, J. E., MATSUMOTO, S., NOGUCHI, H., ONACA,
N., LEVY, M. F. & NAZIRUDDIN, B. 2009. Evidence for Induced Expression of HLA
Class II on Human Islets: Possible Mechanism for HLA Sensitization in Transplant

JACKSON, S., MAGER, D. R., BHARGAVA, R., ACKERMAN, T., IMES, S.,
HUBERT, G., KOH, A., SHAPIRO, A. M. & SENIOR, P. A. 2013. Long-term follow-
up of hepatic ultrasound findings in subjects with magnetic resonance imaging defined
hepatic steatosis following clinical islet transplantation: a case-control study. *Islets*, 5,
16-21.


KAUFMAN, D. L., ERLANDER, M. G., CLARE-SALZLER, M., ATKINSON, M. A.,

KEENAN, D. B., MASTROTOTARO, J. J., ZISSER, H., COOPER, K. A.,
RAGHAVENDHAR, G., LEE, S. W., YUSI, J., BAILEY, T. S., BRAZG, R. L. &
SHAH, R. V. 2012. Accuracy of the Enlite 6-day glucose sensor with guardian and Veo

KELLY, W. D., LILLEHEI, R. C., MERKEL, F. K., IDEZUKI, Y. & GOETZ, F. C.
1967. Allotransplantation of the pancreas and duodenum along with the kidney in

KEMP, C. B., KNIGHT, M. J., SCHARP, D. W., LACY, P. E. & BALLINGER, W. F.
*Nature*, 244, 447.

KESSLER, L., BUCHER, P., MILLIAT-GUITTARD, L., BENHAMOU, P. Y.,
BERNEY, T., PENFORNIS, A., BADET, L., THIVOLET, C., BAYLE, F.,
OBERHOLZER, J., RENOULT, E., BRUN, M. J., RIFLE, G., ATLAN, C., COLIN, C.,
allotransplantation in type 1 diabetic patients within the Swiss-French GRAGIL

KESSLER, L., PARISSIADIS, A., BAYLE, F., MOREAU, F., PINGET, M.,
FROELICH, N., CAZENAVE, J. P., BERNEY, T., BENHAMOU, P. Y., HANAU, D.


hypoglycemia among adults with IDDM: validation of the low blood glucose index. 

*Diabetes Care*, 21, 1870-5.


*Diabetes Care*, 29, 2433-8.


living related liver donors: correlation between CT and histologic findings. *Radiology*, 230, 276-80.


LOWE, C. E., COOPER, J. D., BRUSKO, T., WALKER, N. M., SMYTH, D. J., BAILEY, R., BOURGET, K., PLAGNOL, V., FIELD, S., ATKINSON, M.,


secretes insulin in a coordinate pulsatile manner directly into the liver. *Diabetes*, 55, 2324-32.


2015. Most people with long-duration type 1 diabetes in a large population-based study are insulin microsecretors. *Diabetes Care*, 38, 323-8.


PANERO, F., NOVELLI, G., ZUCCO, C., FORNENGO, P., PEROTTO, M., SEGRE, O., GRASSI, G., CAVALLO-PERIN, P. & BRUNO, G. 2009. Fasting plasma C-


with increased whole-body lipid oxidation in patients with type 1 diabetes.


PIEMONTI, L., LEONE, B. E., NANO, R., SACCANI, A., MONTI, P., MAFFI, P.,
BIANCHI, G., SICA, A., PERI, G., MELZI, R., ALDRIGHETTI, L., SECCI, A., DI
CARLO, V., ALLAVERA, P. & BERTUZZI, F. 2002. Human pancreatic islets produce
and secrete MCP-1/CCL2: relevance in human islet transplantation. Diabetes, 51, 55-
65.

PLANEY, S. L. & LITWACK, G. 2000. Glucocorticoid-induced apoptosis in

type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. Endocrinology, 143,
339-42.

response to a standard mixed meal in a group of Brazilian type 1 diabetic patients. Braz

POZZILLI, P. 2002. The DPT-1 trial: a negative result with lessons for future type 1

PUGLIESE, A., ZELLER, M., FERNANDEZ, A., JR., ZALCBERG, L. J.,
BARTLETT, R. J., RICORDI, C., PIETROPAOLO, M., EISENBARTh, G. S.,
thymus and transcription levels correlated with allelic variation at the INS VNTR-

RATZIU, V., CHARLOTTE, F., HEURTIER, A., GOMBERT, S., GIRAL, P.,
BRUCKERT, E., GRIMALDI, A., CAPRON, F., POYNARD, T. & GROUP, L. S.
2005. Sampling variability of liver biopsy in nonalcoholic fatty liver disease.
*Gastroenterology*, 128, 1898-906.


& TEFF, K. L. 2005. {beta}-Cell function following human islet transplantation for

RICKELS, M. R., SCHUTTA, M. H., MUELLER, R., KAPOOR, S., MARKMANN, J.
F., NAJI, A. & TEFF, K. L. 2007. Glycemic thresholds for activation of


ROCHA, P. N., BUTTERLY, D. W., GREENBERG, A., REDDAN, D. N., TUTTLE-
NEWHALL, J., COLLINS, B. H., KUO, P. C., REINSMOEN, N., FIELDS, T.,


utility of radiological imaging in nonalcoholic fatty liver disease. *Gastroenterology*, 123, 745-50.


SMITH, R. N., KAWAI, T., BOSKOVIC, S., NADAZDIN, O., SACHS, D. H.,
in chronic alloantibody-mediated renal allograft rejection in Cynomolgus monkeys. *Am
J Transplant*, 8, 1662-72.

lessons learned from prevention and recent-onset type 1 diabetes immunotherapy trials.
*Diabetes*, 62, 9-17.

function and the development of diabetes-related complications in the diabetes control


Dermatopathology of skin cancer in solid organ transplant recipients. *Transplant Rev
(Orlando)*, 24, 172-89.

TAMBUR, A. R., BRAY, R. A., TAKEMOTO, S. K., MANCINI, M., COSTANZO,
M. R., KOBASHIGAWA, J. A., D'AMICO, C. L., KANTER, K. R., BERG, A., VEGA,
J. D., SMITH, A. L., ROGGERO, A. L., ORTEGEL, J. W., WILMOTH-HOSEY, L.,


VANTYGHEM, M. C., RAVERDY, V., BALAVOINE, A. S., DEFRANCE, F., CAIAZZO, R., ARNALSTEEN, L., GMYR, V., HAZZAN, M., NOEL, C., KERR-CONTE, J. & PATTOU, F. 2012. Continuous glucose monitoring after islet transplantation in type 1 diabetes: an excellent graft function (beta-score greater than 7) is required to abrogate hyperglycemia, whereas a minimal function is necessary to suppress severe hypoglycemia (beta-score greater than 3). J Clin Endocrinol Metab, 97, E2078-83.


VERGE, C. F., STENGHER, D., BONIFACIO, E., COLMAN, P. G., PILCHER, C.,
autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell
antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. *Diabetes*,
47, 1857-66.

effects of tissue factor: a possible role for factor VIIa-induced intracellular signalling?

VILLASENOR, J., BENOIST, C. & MATHIS, D. 2005. AIRE and APECED:
molecular insights into an autoimmune disease. *Immunol Rev*, 204, 156-64.

WAKI, K., TERASAKI, P. I. & KADOWAKI, T. Long-term pancreas allograft survival
in simultaneous pancreas-kidney transplantation by era: UNOS registry analysis.
*Diabetes Care*, 33, 1789-91.

WALSH, R. C., EVERLY, J. J., BRAILEY, P., RIKE, A. H., AREND, L. J.,
MOGILISHETTY, G., GOVIL, A., ROY-CHAUDHURY, P., ALLOWAY, R. R. &
WOODLE, E. S. 2010. Proteasome inhibitor-based primary therapy for antibody-

WARNOCK, G. L., THOMPSON, D. M., MELOCHE, R. M., SHAPORE, R. J., AO, Z.,
KEOWN, P., JOHNSON, J. D., VERCHERE, C. B., PARTOVI, N., BEGG, I. S.,
A multi-year analysis of islet transplantation compared with intensive medical therapy


