The Influence of Donor Body Mass Index on Human Pancreatic Islet Function, Structure and Islet Transplant Outcome

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ABSTRACT

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Background: Pancreatic islet transplantation for type-1 diabetes has resulted in considerable success over the past decade. However, the worldwide shortage of pancreatic donors remains a major challenge. In an attempt to expand the donor pool, pancreases from obese organs donors (>30 kg/m^2) are now routinely offered to islet transplantation in the UK. In addition, it has been suggested that pancreases from donors with early type-2 diabetes mellitus may also be suitable. However, for both these donor groups, although high islet yields (IEQ) may be obtained, it is unclear whether these islets function optimally. An additional approach to the donor shortage is to minimise the number of donors required per islet recipient. One strategy to achieve this is to use different pharmacological agents to enhance post-transplant islet function.

Aims: The aims of this thesis were fourfold; 1) To determine whether islets isolated from high BMI donors function normally *in vitro* and *in vivo*; 2) To establish why islet yields are higher from obese donors; 3) To determine whether islets from donors with type-2 diabetes are suitable for islet transplantation; 4) To investigate whether glucagon-like peptide 1 receptor agonist therapy improves post-transplant islet graft function.

Results: Stimulated insulin and glucagon secretion, and markers of mitochondrial function (intra-islet ATP content and mitochondrial copy number) were compared in islets isolated from obese (BMI>30kg/m^2; n= 27) and non-obese (BMI<28kg/m^2; n=25) donors. No differences in secretory function or intra-islet ATP were observed between the two groups. Pancreatic lipid and intra-islet triglyceride concentrations were higher in the obese group. *In vivo* clinical outcomes of patients transplanted in Oxford and a larger cohort (n=35) in Edmonton, Canada with islets from obese or non-obese donors showed no differences in post-transplant outcomes. Improved islet yields were shown to be a result of improved islet recovery of larger islets, in obese donors. Abnormal insulin and glucagon secretory responses were observed in islets from type-2 diabetic subjects (n= 6) and islet amyloid content was significantly higher in diabetes. The glucagon-like peptide 1 receptor agonist, exenatide, administered for 20 weeks, significantly improved graft function in patients whom islet function was impaired.

Conclusion: The high lipid environment of islets isolated from donors with high BMI appears not to be deleterious to their function either *in vitro* or when transplanted, supporting the use of islets from high BMI donors for clinical islet transplantation. However, islet dysfunction and pathological changes indicate that islets from type-2 diabetic donors are unsuitable. Therapeutic options such as exenatide, improve transplanted islet viability and function, and could have a significant role in the future of beta-cell replacement therapy for type-1 diabetes.

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STATEMENT OF PERSONAL CONTRIBUTION

The work presented in these studies is my own work apart from a selection of experiments, which were conducted in collaboration with other scientists. Their contributions are presented in the appendices and have been clearly acknowledged in the relevant sections. These include the electron micrographs produced by Dr Anne Clark. The electrophysiology studies undertaken by Dr Matthias Braun. The lipid extraction and fatty acid profiling undertaken by Dr Leanne Hodson. Dr Reshma Ramracheya assisted with the initial hormone secretion studies. The islet transplant team in Edmonton undertook the metabolic testing, the data of which are used in this research.

I declare that no part of this thesis has been submitted for any other academic qualification in this University or elsewhere.

PUBLICATIONS AND AWARDS

PUBLICATIONS

Walker JN, Ramracheya R, Zhang Q, Johnson PRV, Braun M, Rorsman P. Regulation of glucagon secretion by glucose: paracrine, intrinsic or both? Accepted for publication in ***Diabetes, Obesity and Metabolism,*** 2011.

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AWARDS ARISING FROM THIS RESEARCH

2011 Diabetes UK: Winner of the Young Diabetologist Award

British Transplantation Society: Medawar Medal Nominee

The Transplantation Society: Young Investigator Travel Award (awarded for highly rated abstract for oral presentation at the 13th World Congress of IPITA)

Poster of distinction 13th World Congress of IPITA

2010 Royal College of Physicians: Samuel Leonard Simpson Fellowship in Endocrinology

University of Oxford: The Trinity College Graduate Scholarship Award for Research

Diabetes UK, Innovators in Diabetes meeting: Oral presentation prize

2009 The Transplantation Society: Young Investigator Travel Award (awarded for highly rated abstract for oral presentation at the 12th World Congress of IPITA)

University of Oxford: The Trinity College Graduate Prize for Progress in Research

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ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine-5'-triphosphate
K_{ATP}-channels	Adenosine-5'-triphosphate-sensitive potassium channels
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CIT	Cold Ischaemia Time
DCD	Donor following cardiac death
DPP-4	Dipeptidyl peptidase 4
Epac	cAMP-Regulated Guanidine Nucleotide Exchange Factor
FFA	Free fatty acids
GLP-1	Glucagon-Like Peptide-1
GLUT-1	Glucose transporter-1
GLUT-2	Glucose transporter-2
GSIS	Glucose-stimulated insulin secretion
H^+	Hydrogen ions
HbA1c	Glycosylated haemoglobin
HBSS	Hank's Balanced Salt Solution
HOMA	Homeostatic model assessment
IAPP	Islet Amyloid Polypeptide
IEQ	Islet Equivalent
IL-6	Interleukin-6
MRS	Magnetic Resonance Spectroscopy

MtDNA	mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide
ND	Non-Diabetic
NEFA	Non-esterified fatty acids
NucDNA	Nuclear DNA
ODR	Organ Donor Register
PAK	pancreas after a kidney
PCR	Polymerase Chain Reaction
PDX-1	Pancreatic and duodenal homeobox 1
PTA	pancreas transplant alone
qRT-PCR	Quantitative Real-Time PCR
ROS	Reactive oxygen species
SI	Stimulation Index
SPK	simultaneously with a kidney
T1DM	Type-1 Diabetes
T2DM	Type-2 Diabetes
TBS	Tris-Buffered Saline
TCA	Tricarboxylic acid
TNF-alpha	Tumor Necrosis Factor- alpha
TRL	Triglyceride
UKITC	UK Islet Transplantation Consortium
UW	University of Wisconsin
WHO	World Health Organisation
WIT	Warm Ischemia Time

CHAPTER 1 INTRODUCTION

1.1 TYPE-1 DIABETES MELLITUS AND THE NEED FOR BETA-CELL REPLACEMENT

Type-1 diabetes (T1DM) affects several million individuals worldwide [1, 2], with the majority of patients being diagnosed before the age of twenty. It is an autoimmune disease which results in the destruction of the insulin-producing pancreatic beta-cells within the islets of Langerhans [3]. First described by Gepts in 1965 [4], two morphological factors clearly distinguished what we now refer to as T1DM from Type-2 Diabetes Mellitus (T2DM). First, in the majority of islets at the time of diagnosis, there is a significant reduction of beta-cells per islet and second, a chronic inflammatory cell infiltrate affecting the islets, termed 'insulitis', is noted. Within this insulitis, T-cells have been identified as the predominant inflammatory cell, but macrophages and B-lymphocytes are also present [5].

Within the western world the acute stage of this disease (diabetic ketoacidosis) is now rarely fatal [6], but the chronic complications of diabetes (nephropathy, retinopathy, neuropathy and cardiovascular disease) are a major cause of morbidity and mortality [7]. To prevent these chronic complications, the aim of treatment is to attain tight glycaemic control soon after diagnosis. At present, the main strategies for achieving this are though the use of intensive exogenous insulin regimes. These can involve either multiple subcutaneous injections of long-acting and rapid-acting insulin, or alternatively a subcutaneous insulin pump. The Diabetes Control and Complications Trial (DCCT) [8] was a major clinical study conducted from 1983 to 1993. It involved 1,441 volunteers, aged 13 to 39, with T1DM and compared the effects of standard control of blood glucose versus intensive control on the complications of diabetes. The

patients were followed-up for a mean of 6.5 years and the intensive therapy group was found to have a reduced risk for the development of retinopathy (76%) as compared with conventional therapy. Intensive therapy was also found to slow the progression of diabetic retinopathy, nephropathy, and neuropathy in T1DM patients. Longer-term follow-up of these patients showed that the reduction in the risk of progressive retinopathy and nephropathy was found to be maintained [9]. More recently, a further review of the patients undergoing intensive control showed long-term beneficial effects on the risk of cardiovascular disease [10].

These studies show that when tight glycaemic control is achieved, microvascular diabetic complications can be improved. However, the main concern with these therapies is the observed significant increase in hypoglycaemic episodes [8]. Recurrent hypoglycaemia leads to a diminished ability to perceive the onset of hypoglycaemia [11]. Patients with impaired awareness of hypoglycaemia (IAH) and erratic daily glycaemic excursions are therefore highly susceptible to severe hypoglycaemic events, which are at times life threatening. Studies of patients with IAH with normal renal function, who were waiting for a pancreas transplant, showed a mortality rate of approx 8% after being on the waiting list for 4 years [12]. To reduce the incidence of hypoglycaemia, it is now possible for patients to have continuous glucose sensors, which alarm when the blood glucose goes low. The concept of an artificial pancreas has also been developed. This has a sensing arm that measures the blood glucose repeatedly or continuously, an insulin delivery arm that injects insulin upon command and a computer that makes the decisions of when and how much insulin to deliver [13]. These closed-loop systems offer great potential however; several fundamental challenges need to be overcome. These include the asymmetric risk of clinical complications associated

with low and high glucose levels due to mechanical failure and the irreversibility of insulin action when using only insulin [14].

Although a number of regimes for administering exogenous insulin are able to achieve glycaemic stability and reduce the risk of long-term microvascular complications, these do not replicate the complexity or efficacy of endogenous insulin. Beta-cells are able to make delicate adjustments on a regular basis (approx 15 granules released per minute per beta-cell during first phase response [15]). Replacement of insulin-secreting beta-cells can potentially restore metabolic control without the risk of hypoglycaemia, but at present this comes at the expense of immunosuppression and its associated side-effects.

1.2 PANCREATIC ISLET REPLACEMENT

Transplantation of pancreatic islets can occur either via transplantation of the whole pancreas, as a vascularised graft or via transplantation of isolated islets.

1.2.1 Whole organ transplantation

The first attempt to cure T1DM using whole pancreas transplantation was at the University of Minnesota, in Minneapolis, on December 17, 1966; however, due to the lack of potent immunosuppressive drugs and high incidence of post operative infections, progress was restricted [16]. With advances in immunosuppression and surgical techniques, whole pancreas transplantation became a more viable option. It is now

established as a successful long-term therapeutic option for patients suffering from T1DM, with approx 28,000 transplants undertaken worldwide [17]. The pancreas is most commonly transplanted simultaneously with a kidney (SPK), but can also be transplanted after a kidney (PAK) or alone (PTA). Although transplantation outcomes have significantly improved [18] the procedure still has a relatively high peri-operative morbidity (~30% re-laparotomy) and mortality rate (~4%) [19] and is unlikely to be applicable in the early stage of the disease before microangiopathic complications become irreversible. It is therefore also unlikely that this mode of transplantation will be suitable for children.

1.2.2 Pancreatic Islet transplantation

Islet transplantation on the other hand can be regarded as a safe and well tolerated therapy with significantly improved co-morbidity and mortality rates compared with whole pancreas transplantation.

The first recorded transplant in humans using pancreatic fragments, was performed in the UK on December 20, 1893 [20]. A freshly slaughtered sheep's pancreas was implanted subcutaneous into a 15-year-old boy. Glycosuria was lowered, however the patient died after a few days. This paved the way for further work on the concept of transplanting non-vascularised pancreatic tissue. Significant advances have been made since that time and purified islets can now be isolated and transplanted with significant success [21]. Islet transplantation involves two stages namely islet isolation and the transplant itself.

1.2.2.1 Islet isolation

The pancreatic islets account for ~1.3% of the total pancreas volume and a pancreas is estimated to contain at least 1million islets >23μm in diameter [22]. As mentioned above, the beta-cells of the pancreatic islets in T1DM patients become damaged; however the exocrine pancreas remains functional. The aim of the islet isolation procedure is to liberate islets from the exocrine tissue of a donated cadaveric pancreas and then transplant these into the liver, via the hepatic portal vein of a T1DM recipient.

Initial techniques to isolate islets from the exocrine pancreas involved micro-dissection under a dissecting microscope [23]. This enabled small numbers of islets to be isolated but was clearly impractical for large scale isolation. Other mechanical methods for disruption of the pancreas where employed, but with limited success [24]. In 1964, Moskalewski [25] reported that using collagenase to enzymatically digest the pancreas resulted in the isolation of intact islets, free from the surrounding connective tissue. The technique involved cutting the rodent pancreas into blocks followed by the addition of crude collagenase blends to disrupt the collagen matrix which surrounds the islets (**Figure 1-1**). It was not however, until intra-ductal administration of collagenase was established that significantly higher yields could be obtained on a reproducible basis in both dogs [26] and humans [27]. The use of collagenase digestion assisted by mechanical dissociation at present remains the most effective method of isolating the islets of Langerhans.

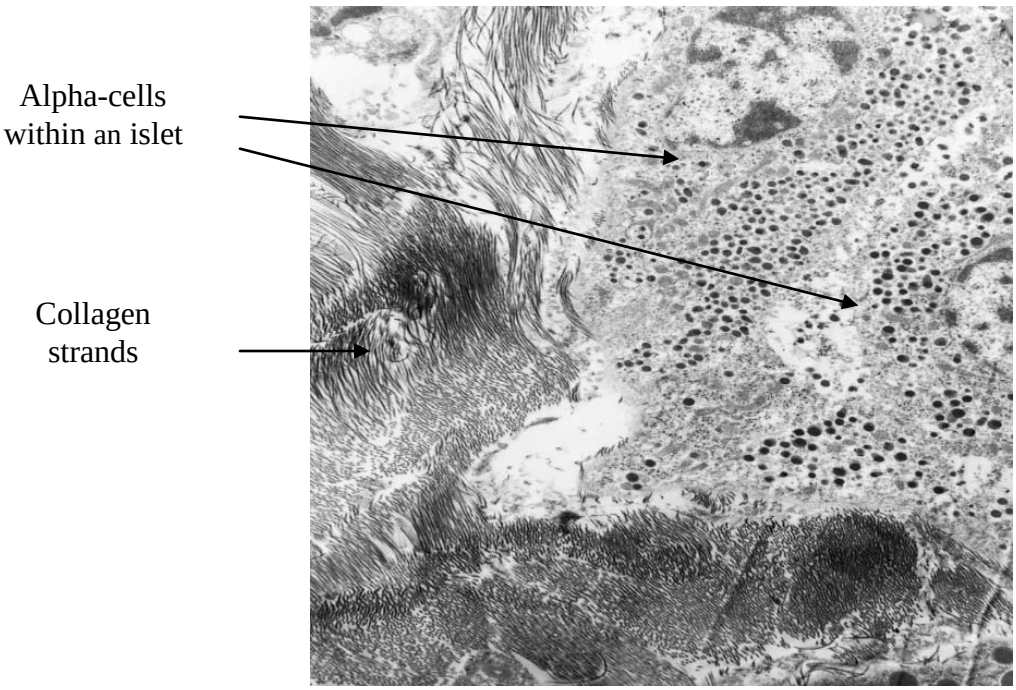


Figure 1-1: Electron micrograph demonstrating the collagen matrix surrounding the pancreatic islet.
Arrows indicate collagen strands and an alpha-cell within an islet (courtesy of Prof PRV Johnson)

Pancreases are typically procured from brain-dead, heart beating donors. The pancreas is perfused and preserved in cold storage solution and transported to the islet isolation laboratory. On arrival the duodenum and spleen are removed and the excess peripancreatic fat trimmed (**Figure 1-2a**). The pancreatic duct is cannulated and blends of highly purified collagenase and neutral protease are infused (**Figure 1-2b**). Following this distension phase, the pancreas is dissected into blocks and placed in a digestion chamber [28]. A combination of enzymatic and mechanical digestion helps separate islets from exocrine and ductal tissues and results in the liberation of islets detached from the surrounding tissue (**Figure 1-2c-d**). After digestion the islets are then purified from the digested exocrine tissue by continuous density gradient purification [28] (**Figure 1-2e**). (As explained in detail in *Chapter 2.2.2*)

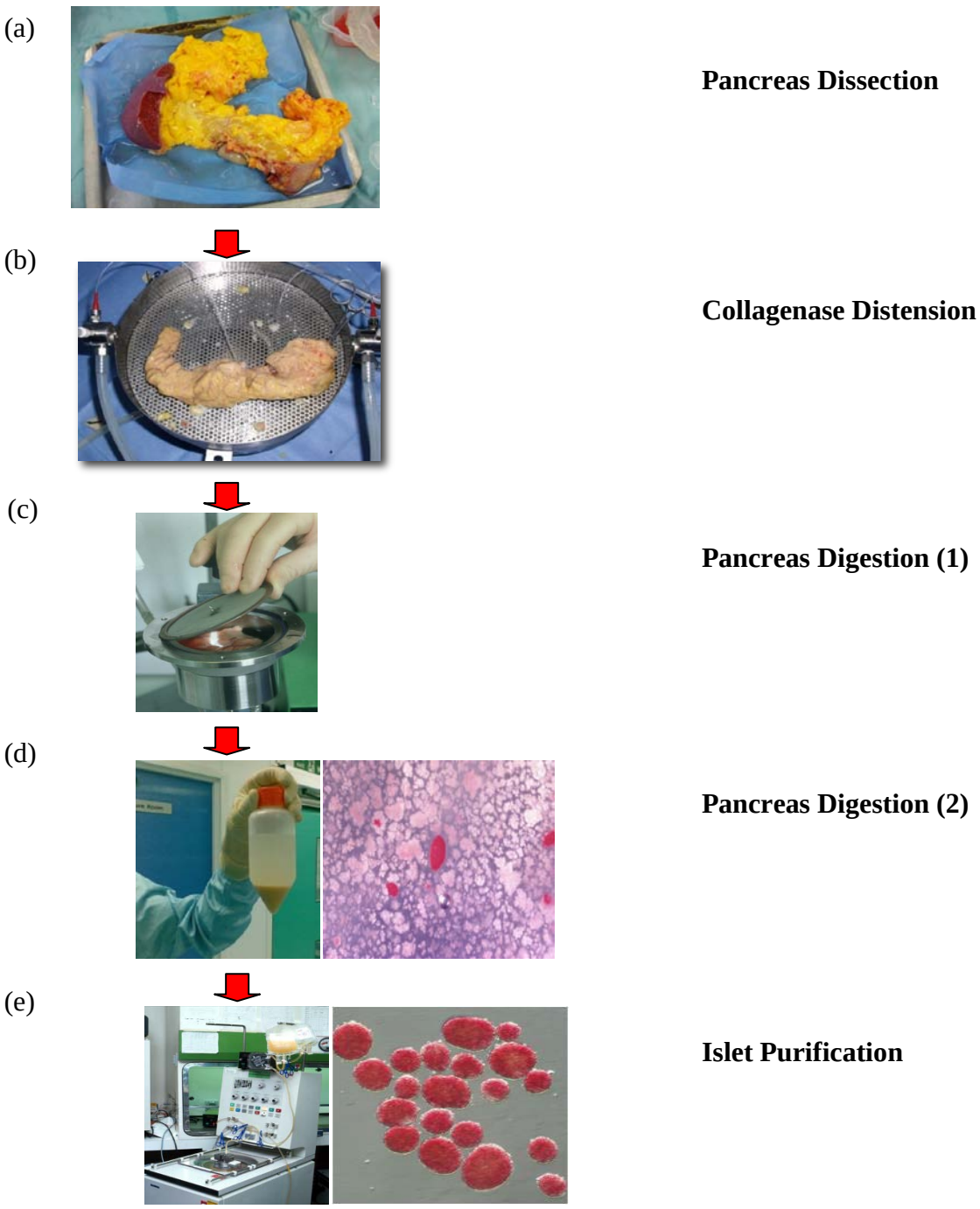


Figure 1-2: The islet isolation process.

The pancreas is retrieved "en block" with the duodenum, spleen and adherent fat (a). The pancreas is dissected so that only the trimmed pancreas remains. Collagenase is infused through the pancreatic duct (b) and the organ becomes distended. Following enzymatic and mechanical digestion the digest (c-d) containing the islets is then purified (e) on a continuous gradient with centrifugation. Islets are stained red with dithizone to facilitate identification and counting

1.2.2.2 Islet transplantation

The transplantation can be done using freshly isolated islets or as is the case with most centres, after a period of time in culture. The purified islets (<5ml packed volume) are transferred to an infusion bag (**Figure 1-3b**) and suspended with transplant medium which is often supplemented with unfractionated heparin (**Figure 1-3c**).

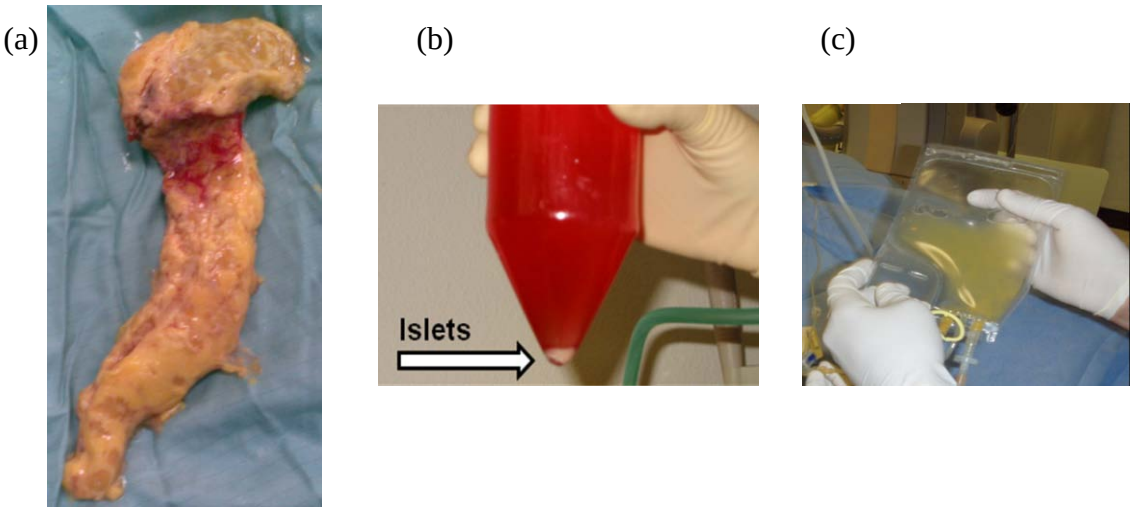
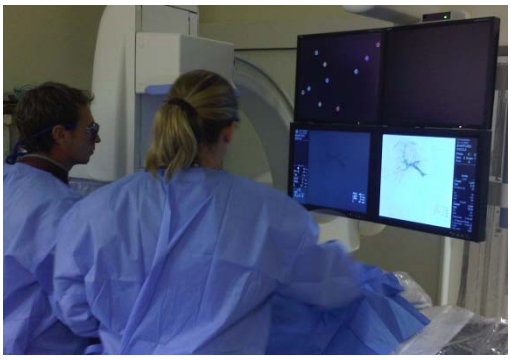


Figure 1-3: (a) Human pancreas before digestion (weight 100grams) (b) Final islet volume of ~2mls following islet isolation (c) suspension of the islets prior to transplantation

The patient receives light sedation and local anesthesia over the transplantation site and under radiological guidance the portal vein is cannulated via the trans-hepatic route (**Figure 1-4**). Islets are then infused under gravity pressure. To minimize the risk of bleeding, the catheter tract is embolised. The patient is then closely observed for the next 48-72 hrs as an inpatient. The transplant recipients all require immunosuppressive therapy. This consists of an induction agent (e.g. Daclizumab or Alemtuzumab) and lifelong maintenance therapy (e.g. Tacrolimus, Mycophenolate mofetil, Sirolimus).

(a) 

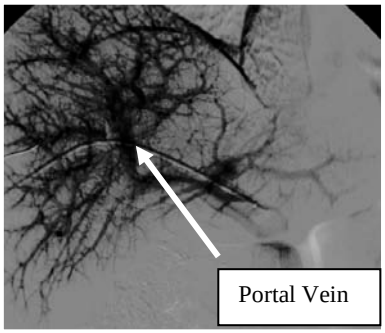
(b) 

Figure 1-4: (a) and (b) Radiological insertion of catheter into the portal vein

1.2.2.3 Graft survival rates

The ability to isolate human islets lead to the first clinical islet transplant which took place in Minneapolis in 1974 [17]. Although the procedure was repeated in centres worldwide, success was limited. It was not until 1990 that insulin independence after islet transplantation was first reported [29].

The International Islet Transplant Registry (ITR), established in 1989, collected data on 200 patients between 1990-97, who had undergone islet transplantation using a combination of single or multiple transplants [30]. The 1-year patient survival rate was 96% and the 1-year islet graft survival rate was 35%. However, insulin independence was achieved in only 10% (for >7 days).

In 2000, a landmark paper from Edmonton, Canada was the first to show prolonged insulin independence in 7 consecutive patients (median follow-up, 11.9 months; range= 4.4- 14.9) [31]. The Edmonton protocol contained a number of changes compared with conventional protocols. A glucocorticoid-free immunosuppressive

regimen consisting of sirolimus, tacrolimus, and daclizumab was administered to the recipients and the use of xenoprotein products (such as fetal-calf serum) was avoided during islet isolation and purification. Islets were transplanted immediately after harvesting and all recipients required islets from at least two donor pancreases.

By 2005, 65 patients had been transplanted by the Edmonton team and longer-term data was available [32]. Patients were deemed to have completed the islet transplant procedure once they gained insulin independence for 4 weeks or if they had received >15,000 islet equivalents (IEQ)/kg, even if they were not insulin independent. The majority of patients received at least two transplants to achieve this goal. For those who attained insulin independence this persisted for a median of 15 months (IQ range= 6.2–25.5) before insulin was once again required. Five year post–islet transplantation graft survival (as measured by C-peptide positivity) was 80%, while insulin independence was ~10%.

The Immune Tolerance Network (ITN) consisting of nine centres in Canada, the U.S., and Europe was established to enable a multicentre trial of allograft islet transplantation using the Edmonton protocol [33]. This consortium reported in 2006 that 44% of T1DM recipients achieved the primary end point of insulin independence 1 year after islet transplantation. It however highlighted, that centres with previous experience in islet isolation and transplantation achieved higher success rates. Currently islet transplantation in experienced centres using similar protocols to Edmonton, can achieve high one-year rates of insulin independence (80-85%), and increasing 5-year rates of partial islet function (C-peptide secretion and protection from hypoglycaemia) are now routine [34]. More recently, reports have shown prolonged graft function (13 years)

[35] and prolonged insulin-independence from donors received multiple transplants [36, 37] and insulin independence using single donors [21, 38].

1.2.2.4 Impact on the secondary complications of diabetes

A reduction in the number of severe hypoglycaemic events experienced in T1DM recipients following transplantation is well documented [32, 39]. Improved quality of life and well being has also been extensively reported [40-42]. Whether other secondary complications of diabetes can be improved however remains unclear. In 2008, one of the first studies which directly compared the diabetic complication rates of islet transplant recipients against intensive medical therapy took place [43]. This study showed that the group receiving islet transplants had reduced progression of diabetic retinopathy compared to the medically managed patients. A recent study [44] confirmed this finding and also found that the rate of decline in glomerular filtration rate is slower after islet transplantation, than on intensive medical therapy. These studies highlight, that islet transplantation may have additional therapeutic benefits in the management of diabetic complications, above what can be achieved with intensive medical management.

1.2.2.5 Mortality and morbidity following islet transplantation

As mentioned previously the early reports from the ITR showed a 4% mortality 1 year after transplantation [30]. In 2008 however, the Collaborative Islet Transplant Registry [39] reported the mortality rate at 1 year to be < 0.5%.

Procedure-related morbidity following intraportal islet transplantation is low [45]. The most common serious adverse events within the first year after an islet allograft infusion are (1) elevated liver function tests (9% of all allograft recipients) (2) neutropaenia (9%) (3) procedural haemorrhage (6%) [39]. The occurrence of malignancy as a result of immunosuppression therapy has been reported to be elevated compared to background prevalence although this increase is relatively small [39].

Islet transplantation can therefore be generally regarded as a safe and well tolerated therapy with significantly improved co-morbidity and mortality rates compared with whole pancreas transplantation. This also makes it a very attractive option for children with T1DM.

1.3 THE SHORTAGE OF PANCREATIC DONORS

A number of challenges need to be addressed before islet transplantation becomes a widespread clinical reality; however, one of the immediate challenges is the worldwide shortage of pancreas donors. It is estimated that in the UK there are ~25,000 individuals under the age of 25 with T1DM [46] and the number of new cases reported each year is increasing [2]. It is apparent from these figures that the number of suitable deceased human pancreas donors available (386 pancreases offers between 2009-10 in the UK, NHS blood and transplant service, personal communication) will never match the demand. Islet transplantation in the UK is therefore only indicated for highly selected patients with unstable T1DM, experiencing recurrent, potentially life-threatening hypoglycaemia.

Alternative sources of islets are being explored which include stem cell therapy and cross-species transplantation (xenotransplantation). Several advances have been made in stem cell biology and tissue engineering. A number of human embryonic stem (hES) cell lines have been established [47] and inducible pluripotent stem (iPS) cell lines are rapidly evolving [48]. Recently beta-like cells have been derived from iPS cells [49]. These were found to secrete insulin in response to glucose and following an iPS cell transplant, successfully corrected hyperglycaemia in two mouse models of type 1 and 2 diabetes. This work provides an initial proof of principle for potential clinical applications of reprogrammed somatic cells in the treatment of T1DM. One of the major concerns however, is the risk of tumorigenicity resulting from uncontrolled hESC/iPSC proliferation [50]. Significant efforts are still required before the use of these cell lines can be used as an established treatment for patients with T1DM.

The use of islets from healthy, designated pathogen-free, and potentially genetically modified, pig donors presents unique opportunities for improving the availability and outcomes of islet replacement therapies in diabetes [51]. Pig islets are available either as islets isolated from adult pigs [52] or as fetal [53] or neonatal [54] islets which require ~4 weeks to mature into glucose-sensing and insulin-producing cells. Prolonged restoration of insulin independence (for > 3 months) after pig islet cell xenotransplantation into non-human primates has been demonstrated [55, 56]. This work has paved the way for a purpose built facility to raise designated pathogen-free pigs with genetics for high islet cell yield to be used for clinical islet xenotransplantation trials (The Springpoint project http://www.springpointproject.org). Islet cell xenotransplantation holds great promise, however the significant immune

response generated to inter-species tissue needs to be overcome before this treatment can be used in the clinical setting.

Until alternative sources of islets do become available, several strategies can be implemented to increase the number of pancreases within the donor pool for islet transplantation. These include: 1) increasing pancreas donation rates, 2) identifying organs not suitable for whole pancreas transplantation, but potentially suitable for islet transplantation, 3) extending the donor acceptance criteria to include more donors and 4) achieving long-term diabetes stability from a single donor pancreas, therefore reducing the need for sequential transplants.

1.3.1 Increasing organ donation rates

Nearly 15 million people are now registered on the NHS Organ Donor Register (ODR) [57]. However, the UK continues to have one of the worst records for organ donation in Western Europe. Two main strategies are open to the government to improve the number of donated organs; first, increasing publicity and awareness for organ donation and second, to consider a change in the system of donation, to an opt-out system.

1.3.1.1 Increase awareness and publicity

In a significant number of cases, patients die without clearly expressing their wishes as to whether they would like to donate their organs. Therefore, families are relied upon in the difficult time around the death to decide whether organ donation should take place. In a high proportion of cases (~45%) [58] the families may refuse if the patient is not

registered. Increased awareness and publicity by the government aims to increase peoples understanding of the need for organ donation and encourages registration on the Organ Donor Register to aid families in making the decisions at the time of death. Other strategies include, improving training and education for all clinical staff likely to be involved in the treatment of potential organ donors. In addition, honouring the gift of donation personally and publicly thereby recognizing individual organ donors has also been proposed to help increase organ donation rates [57].

1.3.1.2 Opt-out policies

An opt-out system is one where adults are considered to be willing to donate unless they opt out by joining a national register, shifting the default position to presumed willingness to donate. The most recent government opinion poll in 2007 showed 64% of the public support a change to an opt-out system [59]. An Organ Donation Taskforce was therefore established to determine if policy should be changed [60]. The Taskforce concluded that an opt-out system should not be introduced at the current time for several reasons namely; that it has the potential to undermine the concept of donation as a gift and that it may erode trust in NHS professionals as there would be a conflict of interest between care of the dying and those waiting for organs.

Despite evidence from other European countries showing that an opt-out policy has resulted in an increase in organ donation [61] and support of the UK opinion polls, the government is currently focusing efforts to urgently increase public awareness and understanding.

They predict that through this a 50% increase in organ donation is possible and achievable in the UK within five years [57]. Currently however, only 28% of the population is registered on the ODR and the rise in registration seen in the last few years has not translated into increases in organ donation; indeed, the number of heart-beating donors has fallen since 2004 [58].

1.3.2 Identifying organs not suitable for whole pancreas transplantation, but potentially suitable for islet transplantation

The worldwide shortage of donated organs is a problem for all types of transplantation [62]. For islet transplantation, this is compounded further by the fact that whole organ pancreas transplant programmes and islet transplant programmes compete for the limited resources available.

1.3.2.1 Obese donors

Donor obesity has been shown to have a significant detrimental effect on surgical complications after whole pancreas transplantation [63]. A large series published by Humar et al [63] stratified donors into normal (BMI <25 kg/m^2), overweight (BMI 25-30kg/m^2) and obese (BMI >30 kg/m^2) groups. Surgical complications, most notably infections and thrombosis were significantly more frequent in the obese donor group (incidence of infection 21.0% vs 11.7% in the >30kg/m^2 vs <25 kg/m^2 groups). Technical failure rates and short-term graft survival were also noted to be inferior in the obese group. Several reasons have been suggested for these complications. Obese donors are more likely to have a higher incidence of underlying vascular disease and this may affect the graft vasculature, contributing to a higher risk of thrombosis after transplantation [63]. Obese donors are also known to have a greater fat content both

surrounding and within the pancreas [64]. This fat, which is poorly vascularised, is likely to be more susceptible to ischemia-reperfusion injury after transplantation, resulting in pancreatitis in the early post-transplant period. Clumps of external fat left on the pancreas graft may subsequently undergo necrosis and serve as a focus for infection [65].

In islet transplantation, the factors which preclude obese donors from use in whole pancreas transplantation can be overcome, as the pancreas is fully digested during the islet isolation process. Ischaemia-reperfusion injury as a result of excess fat is therefore not a concern and for a similar reason macro-vasculature damage to the graft vessels does not affect the utilisation of the organ. It is for these reasons that a new deceased donor pancreas allocation scheme within many centres worldwide has been developed where donors with a BMI>30kg/m^2 are now preferentially offered to islet transplantation programmes, thus providing an opportunity to utilise more organs and increase the donor pool for islet transplantation. The effect of increased BMI on islet function is less clear and there is only limited previous research which has addressed this issue.

The concern in using islets from obese donors for transplantation however, is whether these islets are potentially suboptimal in function. The potential reasons for this are: 1) in addition to obesity, the majority of pancreatic donors are of middle age (between 40-60years old) (NHS Blood and Transplant services, personal communication) and together these provide a classic pre-T2DM phenotype; and 2) higher levels of non-esterified free fatty acids and triglyceride concentrations in the circulation of obese individuals, may be potentially damaging to islets.

1.3.2.2 Normal glucose control and potential reasons why an obese environment may have a detrimental effect of islet function

Normal glucose control and stimulus-secretion coupling of insulin

In the normal physiological state plasma glucose concentration is tightly regulated. This is achieved by multiple regulatory factors, the most notable of which is the secretion of insulin from the pancreatic beta-cells. Insulin is stored in a crystalline form bound to Zn^{2+} [66, 67] and ultra-structural studies have shown that a single beta-cell contains more than 10,000 insulin granules [68] (**Figure 1-5**).

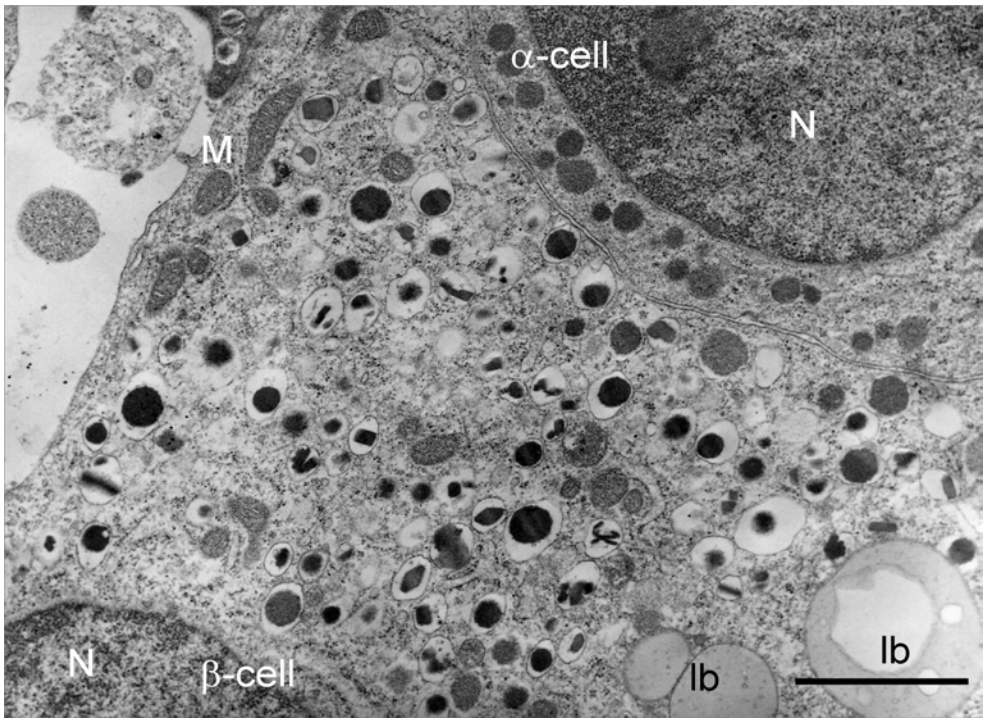


Figure 1-5: Densely granulated beta and alpha-cells within an islet.
M, mitochondria; N, nucleus; lb, lipofuscin body Scale bar 1.0 micron

Insulin granules are secreted in response to increases in blood glucose and this is modulated by both neuronal [69] and hormonal inputs [70]. Insulin release takes a biphasic pattern with a first-phase in insulin release lasting 5-10 minutes after exposure to an elevated glucose level [15]; this is then followed by a more prolonged second phase. Insulin acts primarily at liver, muscle, and fat to promote energy storage [71]. A number of molecular mechanisms are involved in insulin secretion [72] (**Figure 1-6**).

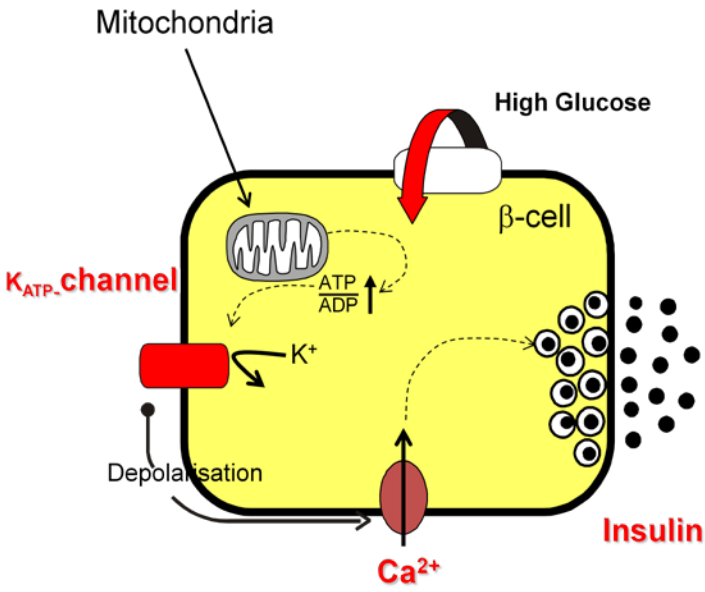


Figure 1-6: The molecular mechanisms involved in insulin secretion.
Elevations in plasma glucose results in a rise in the ATP/ADP ratio through increased mitochondrial metabolism. This in turn leads to closure of the K_{ATP} channels, subsequent cell depolarisation and an influx of intracellular calcium which triggers insulin granule exocytosis. (Courtesy of Dr Carina Ammala, unpublished)

This begins with the glucose being rapidly transported into beta-cells through glucose transporters (GLUT-1 in man but GLUT-2 in mice). After glucose has entered the beta-cell, it is phosphorylated to glucose-6-phospate by glucokinase. Phosphorylated glucose is then metabolized by glycolysis to produce pyruvate, and to a lesser degree

NADH and ATP. Pyruvate serves as a substrate for the tricarboxylic acid (TCA) cycle in the mitochondria resulting in the production of NADH which leads to stimulation of the electron transport chain and the production of hydrogen ions (H^+) which are pumped out of the mitochondrial matrix. The flux of H^+ down an electrochemical gradient results in the generation of ATP by ATP synthase. ATP thus generated, is then transported into the cytosol, increasing cytosolic ATP concentration and hence the ATP/ADP ratio increases. Elevation in the ATP/ADP ratio induces closure of cell-surface ATP-sensitive K^+ (K_{ATP}) channels, leading to cell membrane depolarization. Cell-surface voltage-dependent Ca^{2+} channels are opened, facilitating extracellular Ca^{2+} influx into the beta-cell. A rise in free cytosolic Ca^{2+} then triggers the exocytosis of insulin granules (**Figure 1-7**).

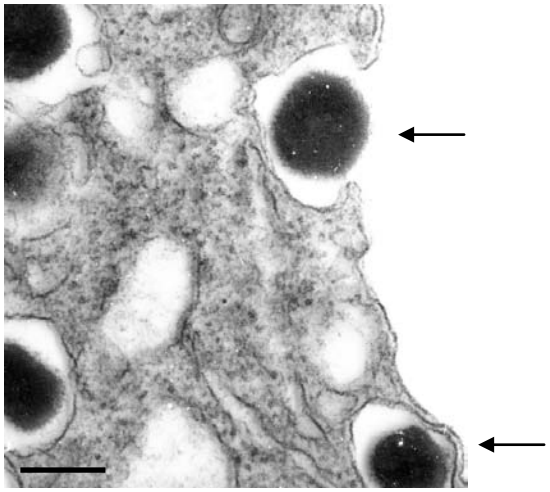


Figure 1-7: Electron micrograph showing insulin granule exocytosis from the beta-cell. Arrows indicate insulin granule. Scale bar represents 300 nm. Adapted from [73].

One of the other major cell types in the human islet is the alpha-cell; this produces the counter-regulation hormone glucagon. These cells comprise 15–20% of

the total islet cells and secrete glucagon in response to a fall in plasma glucose levels (hypoglycaemia). However, release is also stimulated by beta adrenergic stimulation [74], lipids [75] and amino acids [76]. Control of glucagon secretion from alpha-cells involves paracrine (intra-islet), neuronal and intrinsic mechanisms [77]. Under normal conditions, a complex interplay of insulin and glucagon acts to maintain plasma glucose within a narrow range despite large variations in the availability of glucose, particularly during transition from the fasting to fed state [78].

Pre-Diabetes.

Although the beta-cells are remarkably plastic in their ability to regulate insulin release [79], studies have shown that a forty-five year old with a BMI >30kg/m^2, compared with normal-weight subjects, has a predicted increased lifetime risk of developing diabetes from 17.7 to 50.9% [80]. This occurs as a result of the beta-cells inability to compensate with adequate insulin secretion, in the face of increasing insulin resistance. A progressive deterioration in beta-cell function is thought to occur well before the onset of diabetes [81]. Although the cause of this decline is likely to be multi-factorial, one of main hypotheses is that the obese environment may cause lipid-induced pancreatic beta-cell dysfunction; this phenomenon has been termed “lipotoxicity” [82].

The concept of Lipotoxicity

Non-esterified fatty acids and obesity

Non-esterified fatty acids (NEFA), also known as free fatty acids (FFA), are the form in which stored body fat is transported from adipose tissue to its sites of utilization [83]. NEFA transport through the plasma is an essential part of normal physiology, and

regulation of the plasma NEFA concentration is part of the normal pattern of metabolic regulation during feeding and fasting. NEFAs are critical for normal insulin release. However, chronically elevated NEFA and triglyceride (TRL) levels have been implicated in the pathogenesis of both insulin resistance and beta-cell dysfunction [84]. The detrimental effects of NEFAs on beta-cell function have been readily demonstrated *in vitro*. Prolonged exposure of human islets to NEFAs has been shown to result in decreased insulin content [85], blunted glucose-stimulated insulin secretion (GSIS) [86] and increased rates of apoptosis [87].

Plasma NEFAs are released from adipose tissue, and as adipose tissue mass is increased in obesity, it can be postulated that plasma NEFA concentrations may be increased in obese individuals. A significant increase in circulating NEFA levels in obese subjects was found in initial studies (~70% increase compared to lean individuals) [88] and more recent larger studies have also shown significant differences in NEFA concentrations between obese and non-obese groups [89, 90]. This has however, not been the case in all studies with others finding no difference [91].

The exact mechanism by which long-term exposure to NEFAs impairs insulin secretion has not been fully established. It has, however, been shown that prolonged exposure to high concentrations of palmitic and oleic acids induces a pro-apoptotic effect on human pancreatic beta-cells [87, 92]. This effect may be the result of the increased synthesis of toxic metabolites including ceramide from serine [92]. NEFAs can also lead to increased secretion of the free-radical nitric oxide (NO) which is a possible mediator of pancreatic beta-cell damage [93]. Others have shown that reduced insulin secretion is a result of interference with the exit of insulin via the fusion pore

[94]. Another proposed mechanism is that the simultaneous presence of elevated glucose and NEFA results in inhibition of the enzyme responsible for transport of fatty acid into the mitochondria resulting in cytosolic accumulation of long-chain fatty acyl-CoAs which in turn can cause beta-cell damage. All of the studies mentioned above which show significant effects of NEFAs on beta-cell function have been performed *in vitro. In vivo* studies however, have been less consistent in their outcomes. With prolonged NEFA elevation, glucose stimulated insulin secretion was found to be decreased in some studies [95, 96] but not in others [97, 98]. Much of the disagreement relates to how insulin secretion is quantified *in vivo* and in particular whether insulin secretion is assessed in relation to whole body insulin sensitivity, which is clearly reduced by elevated NEFA concentrations [99]. Further problems with comparing the results of *in vivo* studies pertain to a number of facts. First, different protocols are used; second, that the only safe intravenous lipid infusions for human studies which are commercially available do not mimic the same NEFA profiles that are present in obesity [99]; and third, that the *in vivo* studies are conducted over a short period of time (the majority less than 48hrs) which is not a true model of chronic obesity, which may have elevated NEFA levels persisting over many years.

In obesity, the accumulation of excess visceral fat results in adipose tissue surrounding most abdominal organs. Ectopic fat can also become deposited within organs including the pancreas [100]. Even if the plasma concentration of NEFAs is not substantially elevated in obesity, it may be that the accumulated pancreatic lipids may act as a local source of NEFAs and other lipid-derived metabolites, exposing the beta-cell to considerably higher concentrations than maybe predicted from systemic concentrations (**Figure 1-8**).

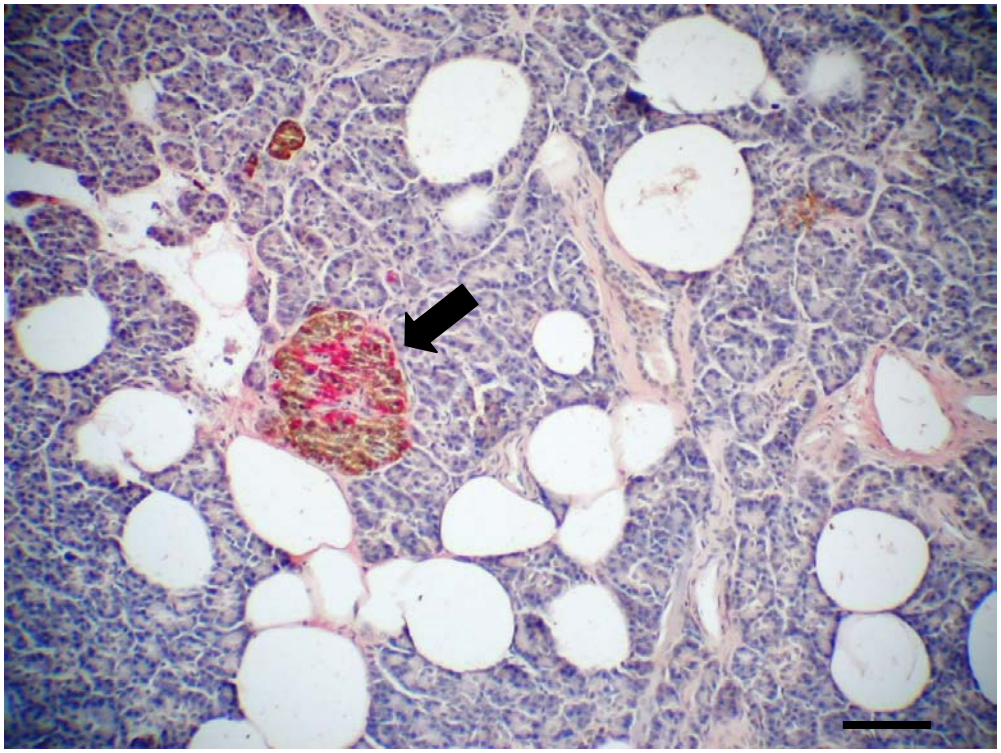


Figure 1-8: Histological specimen of a human pancreas showing high adipocyte content within the exocrine pancreas.
Islet (arrow) stained brown for insulin and red for glucagon. Circular spaces are individual adipocytes within the exocrine pancreas (blue). Scale bar represents 50µm.

The influence of pancreatic fat content on various markers of *in vivo* beta-cell function has been assessed [101]. Patients underwent both a proton magnetic resonance spectroscopy scan to quantify the pancreatic fat content and a modified oral glucose tolerance test to calculate various beta-cell function parameters. The results showed a positive relationship between increased pancreatic fat content and decreased beta-cell function as measured by insulin secretion rate corrected for HOMA-IR (a measure of insulin resistance) and beta-cell glucose sensitivity. As pancreatic fat volume, assessed by pancreatic computerised tomography (CT), has been shown to be higher in obese individuals (~70% higher) (BMI >30 kg/m^2) compared with a lean group (BMI <25

kg/m^2) this would suggest that obese individuals are at risk of lipotoxic mediated impaired beta-cell function [64].

When NEFA supply exceeds utilization, non-adipose tissues, including pancreatic islets and muscle begin to accumulate intracellular triglyceride [102]. This has also been shown to have an inhibitory effect on beta-cell function [103]. Taken together, both increased NEFA and triglyceride concentrations provide potential mechanisms which could induce beta-cell damage.

Pro-inflammatory cytokines and obesity

Adipose tissue, traditionally considered to be a long-term energy storage organ, has more recently been identified as having the ability to secrete numerous proteins which have collectively been termed as 'adipokines'. These include tumor necrosis factor (TNF-alpha), interleukin-6 (IL-6), leptin, adiponectin, visfatin, resistin, apelin, retinol and binding protein (Rbp4). The production of most of these adipokines is upregulated in the obese state [104]. Although IL-6 has been shown to be increased in patients with T2DM, and elevated levels are predictive of the development of T2DM [105], the effects on insulin secretion is mediated through the effects on the hepatocytes rather than a direct effect on beta-cells. However, TNF-alpha, has been shown to have direct effects on beta-cell function. In 1993, TNF-alpha was identified as a pro-inflammatory product of adipose tissue [106]. The levels of TNF in the adipose tissue and plasma of obese individuals are increased [107] and a reduction of body weight in these individuals is associated with a decrease in TNF-alpha expression [108]. TNF-alpha has a well-described role in inflammation, but within beta-cells TNF-alpha has been found

to have multiple effects. In rodent models these include promoting beta-cell apoptosis and inhibiting glucose-induced insulin secretion [109, 110].

Are the lipotoxic effects reversible?

An important question for islet transplantation however, is whether any lipotoxic induced changes in beta-cell function persist when the islets are removed from the obese environment. *In vitro* studies have tried to mimic this by incubating isolated mouse islets and INS-1 beta-cells in NEFA-containing media (oleate and/ or palmitate) for 48 h followed by a 24 h period without NEFA. It has been noted that although the glucose stimulated insulin secretion reduced after the initial period of culture in NEFA-containing media, this significantly improved when the NEFAs were removed from the culture media [111].

Isolating islets from obese donors and assessing their function *in vitro* or following transplantation into non-obese individuals, allows the opportunity to assess function outside the lipotoxic environment. Matsumoto et al [112] have provided one of the few studies aimed at trying to answer this question. They assessed the basic function of islets isolated from obese and non-obese subjects and found there to be little difference in the glucose stimulated insulin secretion between the 2 groups. They also assessed the ability of islets isolated from each of these 2 groups to reverse diabetes in a nude mouse transplant bioassay. Following transplantation of the islets under the left kidney capsule, the cure rate 4 weeks after transplantation of a graft containing 2,000IEQ was 64.7% in the obese group and 77.5% in the non-obese group, but this difference was not significant (P=0.34). However, the mean number of days required to normalize plasma glucose was shorter in the non-obese group (P=0.04). Although

interesting, there are limitations to this study. The pancreas transport and isolation protocols were not consistent throughout the study introducing confounding variables, and the non-obese group contained a significant number of donors with a BMI between 28-30 kg/m^2 and as a result the two groups were not particularly distinct from one another. Although rodent models have been used extensively to study various aspects of islet function, as yet no studies have specifically assessed the function of clinically transplanted islets from obese compared to non-obese donors.

Within clinical whole pancreas transplantation, as mentioned previously, it is difficult to compare recipient outcomes for patients transplanted with organs from obese and non-obese donors, as obese donor recipients tend to experience a higher rate of technical failure. However, the function of grafts from obese donors that were not affected by a surgical complication showed equivalent function to that of lean donors (BMI< 25) at 1 and 3 years post-transplant [63]. Although small, this study none-the-less suggests that if the complications can be overcome in whole pancreas transplantation, graft function may be similar when derived from obese or non-obese donors.

Could obese donors provide optimal functioning islets?

Hyperinsulinaemia is a common finding in obesity [113] and this increase in insulin is predominantly from increased secretion rather than diminished clearance [114]. Within the US, 85% of the obese population, with a BMI between 30-40 kg/m^2, were able to maintain near-normal glycaemic levels with only 15% developing established diabetes [115]. Indeed patients with a BMI as high as 144 kg/m^2 have been noted not to have

diabetes (personal communication, Bariatric Surgery Dept St Richard's Hospital, Chichester).

The main reason why more obese individuals do not develop diabetes is that the beta-cells are remarkably plastic in their ability to regulate insulin release [79]. Fasting insulin secretion has been shown to be up to 550% higher in obese (mean BMI= 43·6kg/m^2) vs lean subjects (mean BMI= 22·8kg/m^2) [89]. A further example is seen in the physiological state of pregnancy. In response to the increased insulin resistance in the last trimester of pregnancy, beta-cell insulin secretion can be increased as much as 400% [116]. The compensatory mechanism to achieve this stimulation of insulin secretion is likely to be a result of up-regulation of beta-cell function (improved secretion from the same number of islets/beta-cells) with some potential contribution from beta-cell expansion.

If obese individuals can up-regulate beta-cell function to a significant degree without developing diabetes it implies that their pancreas contains islets that are robust and flexible - features that are important for islet transplantation. The concern however, may be that chronic overstimulation may lead to functional impairment, for example an inability to adaptively suppress insulin secretion if the stimulus for increased insulin secretion is altered: a number of studies have shown a disproportionately lower reduction in insulin secretion in relation to the increased insulin sensitivity following weight loss [117, 118].

Summary on the use of obese donors for islet transplantation

It is clear from the *in vitro* data presented that the increased levels of NEFA and adipokines, which occur in obesity, can potentially have a detrimental impact on beta-

cell function. What is unclear is whether impairment in beta-cell function, caused by obesity, is enduring following removal from the obese environment. Obesity rates are continuing to increase and the WHO predict that 25.8% of the UK male population and 28.3% of the female population will have a BMI >30kg/m^2 by the year 2015 [119]. Although in 2009, only 10% of the pancreas donors in the UK had a BMI of >30kg/m^2 (NHS blood and transplant service, personal communication), this is likely to increase, as obesity rates continue to increase at a rapid rate. Obese pancreas donors provide an important source of islets for islet transplantation but it is imperative to determine if the function of these islets is appropriate. Detailed *in vitro* and *in vivo* assessments are required to answer this question and these will form the basis of *Chapters 2 and 3.*

1.3.2.3 Obesity and the effect on islet morphology

Obese donors and islet yields

Although there is only limited data on the assessment of islet function from obese donors, significant research has investigated the effect of donor obesity on islet yields. Islet yield, measured by an Islet Equivalent calculation (IEQ), which takes into consideration islet number and volume [120], is an important determinant of islet transplant success [121]. Several studies have demonstrated that islet yields following isolation are correlated positively with increasing BMI of the pancreatic donors [112, 122-124]; this suggests that obese donors may be a preferential choice for maximizing islet isolation yields.

This increase in islet yield could occur for two main reasons; first, the obese donor pancreas may contain a larger islet mass or second, the pancreatic morphology of

obese donors facilitates the release of islets during the islet isolation process, improving islet recovery. The evidence for both these explanations will now be reviewed.

Increased Islet Mass: Evidence in non-primates

In rodents and many other experimental animals beta-cell mass is considered to be dynamic. The half life of beta-cells in rodents <1 year old has been estimated to be 30-60 days [125]. Extra beta-cells are generated primarily from beta-cell replication leading to an increased islet size [126] with additional contribution from new islet formation (neogenesis) [127, 128].

Two well established models in rodents, partial pancreatectomy and partial duct ligation have both shown beta-cell replication and neogenesis can take place in young rodents (<1year) [128, 129]. In rodents a definite compensatory increase of beta-cell mass in response to the insulin-resistant state of obesity has been shown [130-133]. In obese mice, the beta-cell mass was approximately two-fold greater than lean mice by 10 weeks of age. This difference increased progressively so that beta-cell mass was nine fold greater in the obese mice by 40 weeks of age [133]. In older rodents (>1 year) pancreatic damage, obesity and potentially neogenic inducing agents, such as glucagon-like peptide 1 (GLP-1), however have not been shown to produce a regenerative response [134, 135].

Increased Islet Mass: Evidence in humans

The aforementioned studies in rodents, have led to the assumption that endocrine cells in the human pancreas also have capacity for adaptive expansion in response to increased secretory demands. The literature to support this in humans is, however,

limited. Measurement of the adaptive changes in beta-cell mass over time is unavailable as longitudinal studies of pancreatic morphology cannot be performed. Observations can therefore only be made from pancreases obtained at autopsy, pancreas donation, and surgical resection.

Butler et al [136] compared pancreas autopsy biopsies of severely obese subjects (mean BMI≈38 kg/m^2) with lean subjects (BMI ≈22 kg/m^2). Using a morphometric approach on multiple sections they established that the obese group showed an increase in beta-call mass of approx 50%, compared to the lean group. A more recent study [137] again using autopsy specimens, compared overweight/ obese donors (mean BMI=30kg/m^2) and lean donors (mean BMI=22kg/m^2); this showed a more modest 20% increase.

Morphometric analyses of a small number of pancreatic specimens procured from both organ donors and from surgical resection after partial pancreatectomy have also showed that relative beta-cell volume correlated significantly with BMI [138]. The main focus of all these studies was to assess the total beta-cell mass. They have however, provided little evidence as to whether this occurs as a result of increased islet number or islet size.

Evidence of neogenesis in the human pancreas

Although readily accepted in animals the concept of beta-cell/islet neogenesis after birth in humans is controversial [139]. Apart from the obesity studies mentioned above another situation in which beta-cell mass has been shown to increase is during

pregnancy. Only two human studies [140, 141] have been able to assess beta-cell mass during pregnancy, as the ability to obtain post mortem specimens is very limited. One study showed that during pregnancy the fraction of pancreas occupied by beta-cells, in histological specimens, increased 140% compared with non-pregnant women [140]. It was suggested that this was predominantly due to enlargement of the islets of Langerhans and hyperplasia of the beta-cells however, only five cases were studied and the morphological evaluation was limited. The second study [141] on the other hand reported that the pancreatic fractional beta-cell area increased by only 40% in human pregnancy, with no change in mean beta-cell size, islet size or beta-cells per islets. No increase in beta-cell replication or change in beta-cell apoptosis was detected, but duct cells positive for insulin and scattered beta-cells did increased with pregnancy. This indirect evidence in human pregnancy supports the belief that at least some beta-cell formation arises from sources other than beta-cell replication. Other circumstantial evidence for islet regeneration comes from studies on T1DM patients. Studies have reported increased hormone-positive cells within the ducts of recent-onset T1DM patients [142]. A similar appearance has been documented in the adult human pancreas of patients with chronic pancreatitis. Studies have demonstrated that around the ducts, increased numbers of insulin-containing cells, as well as cells containing other endocrine and exocrine markers are seen in this disease process [143].

Despite the evidence mentioned above which suggests the occurrence of neogenesis, other studies are less convincing. Following a 50% pancreatectomy, biopsy specimens where collected and further specimens were then taken from the same pancreas remnant following repeated surgery [144]. The number of islets per pancreatic tissue area was not significantly increased by the partial pancreatectomy nor were there

any differences in the mean beta-cell diameter. It concluded that partial pancreatectomy does not prompt beta-cell regeneration in adult humans. This would explain the high incidence of diabetes following pancreatic resections. A small study [145], comparing pancreas specimens from five obese non-diabetic subjects and five lean non-diabetic subjects showed no significant difference in the percentage of ductal cells positive for insulin or increase in the number of islets around exocrine ducts. Measurement of the lifespan of human beta-cells, estimated by the degree of the intracellular lipofuscin body accumulation [146], indicate that human beta-cells, are long-lived and that little beta-cell neogenesis occurs in adulthood. A subgroup analysis of the obese population showed little change in the lipofuscin body proportions compared to lean controls suggesting that little adaptive change occurs. This is supported by a study using radiocarbon nuclear DNA dating of beta-cells from three samples collected following human cadaveric islet isolation from donors aged 15, 48 and 80 years old. ^{14}C levels indicated that the "birthdate" of cells was in the first three decades of life and that no substantial proportion of cell division occurred subsequently [147].

The regeneration potential in humans is therefore questionable and at best is relatively small. The cell clusters that do occur are also unlikely to survive the isolation process and therefore unlikely to have an impact on the total islet yield.

Morphological changes in the obese pancreas which may facilitate islet recovery following isolation

Obese donors have been noted to have a higher intra-pancreatic fat content [64] (**Figure 1-9**); this increase in fat distribution has been proposed to facilitate the release of islets during the collagenase digestion [123]. Supporting evidence for this comes from studies

showing increased pancreatic interstitial fat content to be associated with improved islet yields [148, 149]. A recent study which performed a multicentre analysis of variables associated with successful human islet transplantation [150] was in agreement with this finding, reporting that pancreases with moderate to heavy fat infiltration increased the likelihood of isolation success.

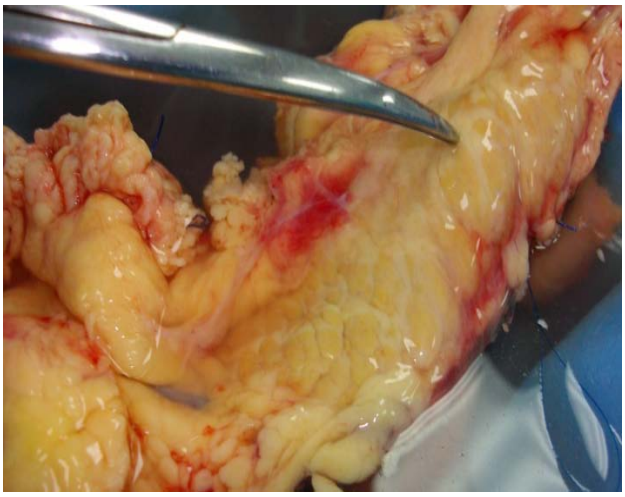


Figure 1-9: Pancreas donor with a BMI of 34kg/m^2.
Scissors are pointing to infiltrative fat in the pancreas parenchyma.

Summary on obesity and the effect on islet morphology

It is generally agreed that obese donor pancreases provide higher islet isolation yields but as yet it is unclear if this occurs as a result of an actual increase in pancreatic islet number and/ or size or alternatively a result of improved islet recovery during the islet isolation procedure. This will form the basis of *Chapter 4.*

1.3.3 Expanding the donor acceptance criteria

1.3.3.1 Increasing the upper age limit for donors

For islet transplantation, older donors (regarded as >45 years) have been shown to provide at least similar, if not higher, islet yields compared to younger donors [124, 148]. As transplanted islet mass is often considered a critical component to the success of islet transplantation [151], it would seem an attractive option to use this group of donors as a source of islets for transplantation. Glucose tolerance has however been shown to decrease with age. The main reason proposed is that older individuals are more insulin-resistant than their younger counterparts [152, 153]. Another contributing factor may be that there is an age-related reduction in beta-cell insulin secretion [154]. This has been supported initially by i*n vitro* studies using isolated rodent islets [155, 156] and later in a study using human islets [122]. The studies in rodents have compared the function of islets isolated from older and younger rodent donors and found a significant decrease in the glucose stimulated insulin secretion (GSIS) in the older group. In 2006 Ihm et al [157] carried out both *in vitro* and *in vivo* studies (in rodent transplant models) using human islets isolated from donors either <40 or >40 year old. These confirmed the previous finding that there was a significant reduction in islet function in the older group, assessed by GSIS, and also showed a reduction in diabetes reversal rates in nude mice, using islets from the older cohort. Clinical studies in both whole pancreas and islet transplant recipients are in agreement with these findings. The risk of developing poor glycaemic control and premature loss in transplanted whole organ pancreas patients has been shown to be higher in recipients from older (>45 years old) compared to younger donors [158, 159]. As a result older donors are therefore generally considered a relative contraindication for whole pancreas transplantation

[160]. Only one study has assessed the influence of donor age on clinical islet transplantation outcomes [161]. This study divided islet transplant recipients into two groups depending on donor age (<45 and >45 years old) and found at one month follow-up all islet graft function parameters were significantly higher in patients who had received islets from younger donors.

The cause of this decline in beta-cell function is much debated but one of the major hypotheses is the mitochondrial theory of ageing. This proposes that reactive oxygen species generated by the mitochondria lead to accumulative oxidative damage to mtDNA and a decline in mitochondrial function with age. Cree et al [162] have shown an age-related decline in mtDNA copy number in isolated human islets which would potentially support this theory in beta-cells.

To achieve a balance between using as many organs as possible without compromising the clinical outcomes, in 2008 the UK Islet Transplantation Consortium (UKITC) initially set an upper age limit of 65 years old for donors. In light of the current research in 2010, this was reduced to 60 years old and likely to reduce further. Increasing this age limit to accept more donors would seem unwise.

1.3.3.2 Increasing Pancreatic Cold Ischaemia Time

The time from the cross clamping of the aorta at organ retrieval to the beginning of islet isolation is termed the pancreas "cold ischaemia time" (CIT). A significant increase in islet yield in pancreases with a CIT <8hrs compared to those > 8hrs has been shown by several groups [163, 164]. The chances of achieving a successful isolated islet yield

(defined as >250,000IEQ and 80% purity) has also been shown to be negatively correlated with increasing duration of cold ischaemia [122]. Functional data on the islets isolated from pancreases with a prolonged CIT is available from rodent [165] and porcine models [166]. Islets isolated from rodent pancreases stored in University of Wisconsin (UW) solution for either 3 hours or 18 hours were compared. Islet cell viability after isolation was significantly reduced in the long-CIT group and reversal of diabetes following transplantation occurred earlier in the short-CIT group compared to the long-CIT [165]. Functional studies on human islets are however scarce.

As a result of the reduced islet yields and potential reduction in function with increasing CIT, an upper limit of 10hrs was set by the UKITC in 2008. However, given the emerging literature above this was reduced further to 8 hrs in 2010.

1.3.3.3 Accepting donors after cardiac death

The majority of organs which are retrieved for transplantation are from brain-stem dead, heart-beating donors [167]. The organs are perfused by assisted cardio-respiratory support until artificial organ perfusion begins at retrieval. Organs retrieved from donors following cardiac death (DCD) rather than brain-stem death have been successfully used in kidney [168] and liver transplantation [169]. One of the concerns in using these donors is that there is an unavoidable delay between the cessation of cardio-respiratory function and the initiation of artificial organ perfusion. This time is termed the warm ischemia time (WIT) and during this the organs are hypoperfused thus leaving them susceptible to damage. This may be a particular concern for the pancreas, because of the

high concentrations of proteases, which may leak from ischaemic exocrine cells and damage the adjacent islets [170].

Markmann et al [171] have demonstrated that large numbers of normally functioning islets can be recovered from DCD donors (median WIT=22.9 mins (15–47)). Islets from these donors performed as well as islets isolated from donors who had died from brain stem death in a panel of *in vitro* and *in vivo* (rodent transplant model) functional assays. There is little data available however regarding the duration of WIT which could compromise the islet quality. Islets isolated from pancreases procured from DCD donors with a warm ischemia time (WIT) of <25min have been compared with islets isolated from donors who had died from brain-stem death [170]. The *in vitro* findings suggest that when islet function was assessed, both the groups had comparable results suggesting these islets would potentially be suitable for clinical use if the warm ischaemia time is <25mins.

These encouraging results have prompted groups to undertake clinical transplants using islets isolated from DCD donors into T1DM patients. This first of these used islets with a WIT of 25 mins. Following this single preparation, the recipient was rendered insulin independent [171]. In Japan, the majority of pancreases are obtained from DCD donors. A report from the Japan Islet Transplantation Registry [167] has shown that islet transplantation using DCD donors, particularly when multiple islet transplantations were used, ameliorated severe hypoglycemic episodes, significantly reduced exogenous insulin requirements and improved HbA1c levels. These studies and others [172] are encouraging and highlight a group of donors who maybe suitable for clinical islet programs. This would potentially help to expand the

donor pool for islet transplantation further. At present however, there is yet to be a detailed study comparing clinical outcomes from recipients receiving islets from brain-stem dead donors as opposed to DCD donors. DCD donors are however, now being accepted for islet transplantation within the UK, providing the WIT is kept to less than 30mins and all other parameters for pancreas acceptance are optimal.

1.3.3.4 Accepting donors in the early stages of type-2 diabetes

Pancreases from donors in the early stages of non-insulin dependent T2DM have been proposed to be a source of islets that could be considered for clinical islet transplantation [173]. Traditionally T2DM was considered to be a disease caused by insulin resistance therefore one could follow the rationale of isolating islets from these donors and transplanting them into a more insulin sensitive patient. It is however becoming more widely accepted that T2DM is a combination of insulin resistance and progressive beta-cell failure [174]. This will be discussed next.

1.3.3.5 Summary on expanding the donor acceptance criteria

A number of studies have aimed to explore the potential to increase the pancreatic islet donor pool. Current evidence suggests that increasing the donor age will likely compromise islet function. Increasing the cold ischaemia time with the present preservation methods, is also likely to lead to a detrimental effect on islet yield and function. The use of non-heart beating donors could significantly increase the donor pool, providing the warm ischaemia time is kept to a minimum, but further *in vivo* studies are required to demonstrate this.

1.3.3.6 Donors with Type 2 diabetes as a source of islets for clinical islet transplantation

Suitability?

Pancreases from T2DM donors have been suggested as a possible option to increase the donor pool for clinical islet transplantation [173]. Evidence from both human and animal studies suggests that T2DM is characterized by a decreased functional beta-cell mass that cannot adapt the insulin secretion to compensate for increasing insulin resistance thus driving the development of overt T2DM [175]. In the early stages of the disease process however normoglycaemia can be restored if significant weight loss occurs, resulting in an improved insulin sensitivity [176]. Data from T2DM patients, who have undergone malabsorptive bariatric surgery, have shown that one month after surgery there was a significant improvement in glycaemic control, that the first phase insulin secretion was restored and the beta-cell glucose sensitivity had fully normalized [176]. Thus, the 'damage' of the beta-cell that precipitates T2DM may be reversible.

Static incubation experiments using islets isolated from donors in the early stages T2DM show that the beta-cells are responsive, and that a significant increase in insulin secretion occurs following incubation in low and then high glucose [177]. When compared to non-diabetic controls at 5.5mM glucose both sets of donors produced similar insulin secretion, although the total insulin secretion was lower in the T2DM donor islets at 16.7mM glucose. The pattern of insulin release from islets isolated from three subjects with T2DM has also been assessed [178]. They concluded that the pulsatile nature of insulin secretion remained intact in all islets from the subjects with T2DM which were assessed (20 islets in total).

Large islet yields (>400,000IEQ) have been possible from T2DM donors in the early stages of the disease [173, 179] however with increasing duration of diabetes the yields are reduced [179].

Only one study has assessed the graft function of recipients transplanted with islets from donors with an abnormal HbA1c [173]. The first of these islet preparations was from a donor with an HbA1c of 6.3%. The recipient had had a previous islet transplant from a donor with a normal HbA1c which had reduced their insulin requirements to 0.27units/kg per day. However, within 1month of their second islet infusion from the abnormal HbA1c donor using 6,062 IEQ/kg the recipient was rendered insulin independent with excellent glycaemic control (HbA1c, 5.3%–5.7%) and remained insulin independent for 4 years. The second preparation isolated was from a donor with a significantly elevated HbA1c 7.9%. The recipient had had a previous islet transplant. Three months after the islet infusion using the islets from the donor with an abnormal HbA1c (5,721 IEQ/kg) a further reduction in his insulin requirements from 0.32units/kg per day to 0.14units/kg per day was observed but the patient did not become insulin independent.

Concerns

Insulin secretion

Despite the encouraging preliminary data regarding islet isolation yields and transplanted islet function there are however a number of concerns regarding the use of T2DM donors. Substantial beta-cell failure is now believed to occur at an early stage in disease progression, i.e. before diagnosis, after which there is an accelerated decline

[175, 180]. In the UKPDS, beta-cell secretory capacity was estimated to be reduced by 50% by the time fasting hyperglycemia was diagnosed [81, 181]. It has been suggested that this reduction in function may commence 10-12 years prior to diagnosis [81] with a progressive decline with increasing duration of diabetes.

In the early stages of T2DM or in patients with IGT, first-phase insulin release is almost invariably lost [182]. The nature of this very specific defect is not well understood but the suppression of this rapid phase of insulin secretion may be causally related to the loss of pulsatile insulin secretion also seen in T2DM. It is also not clear if this represents a primary or acquired alteration of beta-cell function. Both animal and human data however support a crucial physiological role of first-phase insulin secretion in postprandial glucose homeostasis[183]. This effect is primarily achieved in the liver, allowing prompt inhibition of endogenous glucose production and limiting the postprandial rise in plasma glucose level [183].

A reduction in both beta-cell mass [136, 184] and insulin content has been shown in the pancreases of donors with established T2DM [137, 185], both of which are likely to contribute to the reduced secretory capacity. Only one study [177], has measured the insulin content of islets isolated from T2DM donors in the early stages of the disease and found 36% lower insulin content per islet compared to controls. In another study, islet density (islets/mm^2) was shown to be reduced in the pancreases from obese T2DM subjects compared with obese controls [136] indicating both a reduction in the total number of islets and a reduction of beta-cells per islet.

Glucagon dysfunction

Abnormal alpha-cell function in T2DM patients has been noted *in vivo*. In 1975, Unger et al proposed the bihormonal-abnormality hypothesis in diabetes. In this they stated that the absolute or relative glucagon excess which occurs in diabetes is a principal factor in the over-production of glucose [186]. In a number of studies plasma glucagon levels have been found to be elevated in patients with diabetes, both in the fasted state [187] and following a carbohydrate load [188] (**Figure 1-10**), and it is generally agreed that in T2DM the plasma glucagon levels are inappropriately elevated in the context of the hyperglycemia.

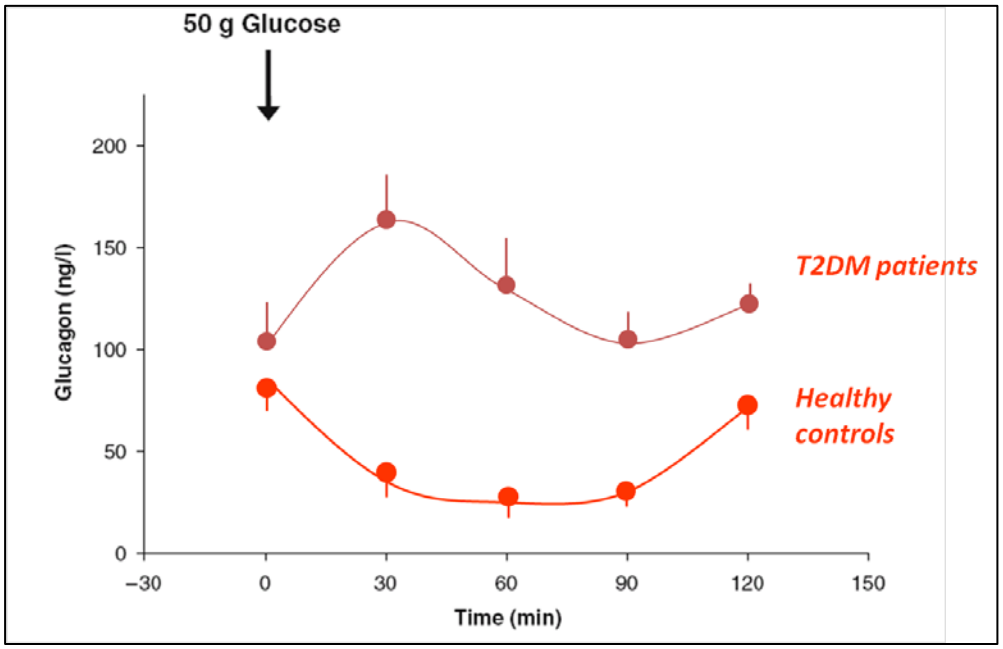


Figure 1-10: Plasma glucagon levels in response to a large carbohydrate meal in subjects with normal glucose tolerance and in patients with T2DM.
Mean±SEM. Adapted from [189].

Local insulin secretion is likely a key regulator of glucagon secretion and the defects in alpha-cell function have been proposed to be a result of beta-cell dysfunction. Other evidence however points to a direct alpha-cell defect resulting in an impairment

of glucose sensing with the alpha-cell, which become hyporesponsive to the suppressive effects of glucose [190].

What is not apparent and requires more evidence is whether the function improves when the islets are taken out of the T2DM environment. One potential way to study this is to assess the function of islets isolated from T2DM donors but unfortunately the opportunity to do this has been very limited in humans.

Amyloid Deposition

Although islet amyloid is infrequently observed in humans who do not have disturbances of glucose metabolism, it is observed at autopsy in the vast majority of individuals (>90%) with T2DM [191-193]. The extent of the amyloid deposition varies with some individuals only having a minimal number of islets affected, but in other patients, the deposits are widespread and affect many islets. Although amyloid deposition has not been identified as the main pathogenesis of T2DM it can be potentially toxic to the islets.

The major component of islet amyloid is islet amyloid polypeptide (IAPP), a 37–amino acid peptide, which is co-stored with insulin in beta-cell secretory granules [194]. This IAPP refolds to a β-conformation and oligomerises to form insoluble fibrils [195]. The most potent cytotoxic effect of human IAPP occurs soon after peptide reconstitution, when IAPP is in small oligomeric aggregates [196, 197]. Mature amyloid deposits are less cytotoxic than the small aggregates and probably represent a space-filling lesion that progressively replaces beta-cell mass [198].

The functional affects of amyloid on the beta-cells is unclear. In one study beta-cells within amyloid-containing islets have been shown to maintain active insulin transcription/ translation and normal insulin storage [199]. However, culturing human islets with intermediate-sized aggregates of human IAPP caused disruption and vesiculation of cell membranes after 24hrs, and both necrotic and apoptotic cells were evident after 48hrs [196]. As amyloidosis develops within the islets (**Figure 1-11**), it is associated with a progressive reduction in the beta-cell volume per islet [198]; this islet amyloid deposition is suggested to be irreversible [195].

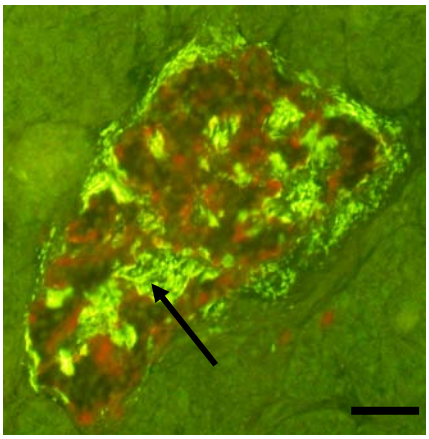


Figure 1-11: Islet amyloid, in a patient with long standing T2DM.
Amyloid labelled with the fluorescent marker thioflavin S (green). Islets labelled for insulin (brown) and glucagon (red). Arrow indicates extensive islet amyloidosis. Scale bar represents 25µm

Human islets from non-diabetic donors transplanted into nude mice have been shown to develop amyloid deposits [200]. If amyloid was to be present in isolated T2DM human islets, which were then transplanted, it is likely that the amyloid process would continue. Although amyloid deposition can potentially occur early in the T2DM process, the extent to which it causes islet damage is unclear.

Summary on the use of T2DM donors

The function of the beta-cells are clearly compromised with long-standing T2DM but it is unclear if this dysfunction is reversible in the early stages of the disease. This will form the basis of Chapter 5

1.3.4 Achieving long-term diabetes stability from a single donor pancreas

Patients receiving multiple donor islet transplants are now experiencing insulin-independence rates similar to that seem in whole organ transplantation [36]. Insulin independence is also becoming achievable from a single donor [21, 201]. Approaches to achieve this on a regular basis include (1) increasing the survival of the transplanted islet graft and (2) maximising the function from the transplanted islets.

1.3.4.1 Improving immunosuppressive regimes

Endogenous insulin secretion and glucose stability have been found to be similar in islet allograft and autograft recipients, despite the latter group receiving less than half as many islets [202]. The reason for this finding is that allograft recipients experience detrimental graft function as a result of autoimmunity, alloimmunity, and immunosuppressive drug toxicity [202]. This highlights the need for refinements in immunosuppression regimes making them less toxic to islets and more effective at suppressing the immune mediated response. Recent advances in immunosuppressive therapies have resulted in graft survival being significantly increased [36, 38]. However ultimately, the aim must be to create an immune-privileged environment at the islet

implantation site. This can be achieved through modulating the immune system (immune-modulation) or alternatively through protecting the islets from the immune system (immune-isolation). One of the most researched areas of immune-isolation is encapsulation. Encapsulation is based on the principle that transplanted tissue is protected for the host immune system by a semi-permeable capsule. These membrane and hydrogel materials allow passage of nutrients and insulin but restrict passage of cellular and humoral components of the immune system. Success has been shown in large animal models [203], however, refinements are required to ensure islet survival is not reduced as a result of the insufficient diffusion of oxygen and nutrients. Uncontrolled foreign body reactions have also been shown to result in excessive formation of scar tissue [204], further limiting the diffusion capacity of the bio-capsules. Recent novel strategies to improve graft survival involve using biodegradable scaffolds to act as a solid support system. The results from these studies in large animals have been encouraging [205], highlighting that manipulation of the microenvironment of transplanted islets may constitute the ideal basis for new approaches to enhance islet engraftment.

1.3.4.2 Using medical therapies to increase the transplanted beta-cell function

A number of therapies are available in the treatment of T2DM to improve beta-cell insulin secretion. These could be adopted to improve the insulin secretion from the transplanted beta-cells. If successful, this could lead to improvement in graft function and potentially reduce the need for a further transplant thereby increasing the number of organs available in the donor pool.

Stimulation of transplanted graft function

For islet transplantation to become a widespread clinical reality, restoration of glycaemic control must be achieved with a single donor, as is currently the case with pancreas transplants [21]. As the islet transplant field continues to look at potential strategies to increase islet yields and alternative sources of islets, various medical therapies could be adopted in the interim to improve insulin secretion from the transplanted beta-cells.

The aim of medical intervention would be to stimulate the insulin secretion of the transplanted beta-cell, thus improving glycaemic control, but not at the detriment of reducing graft longevity. Established oral hypoglycaemic therapies used in T2DM which stimulate insulin secretion include sulphonylureas and meglitinides. Sulphonylureas increase insulin secretion by binding to the SUR1 subunit of the ATP-sensitive K-channels (K_{ATP}-channels). The resultant closure of the K_{ATP}-channels leads to membrane depolarization and the initiation of electrical activity (**Figure 1-6**). Meglitinides e.g. nateglinide and repaglinide, stimulate insulin secretion by a sulphonylurea-like effect of the K_{ATP}-channels but their onset of action and duration of effect is shorter [206]. There are however a number of concerns with using these classes of drugs in islet transplant patients, most notably the undesired side effect of hypoglycaemia. Sulphonylureas trigger insulin secretion in a glucose-independent manner [207], a feature that may explain why sulphonylureas therapy may result in hypoglycaemia. Normally, insulin secretion is under feedback control via the lowering of plasma glucose and reactivation of the K_{ATP}-channels, but this mechanism can no longer operate when the channels are blocked pharmacologically. As the patients have

had the islet transplant to reverse severe hypoglycaemia it would seem counter-intuitive to use these classes of drugs in this group of patients. The additional side effect of weight gain is also unwanted as the increase in insulin resistance would put further stress on the islet graft. A final concern is that *in vitro* studies have shown that prolonged exposure of isolated human islets to sulphonylureas significantly decreases the insulin content of the islets [208] and may induce beta-cell apoptosis [209]. However clinical studies, in particular the UKPDS established glycaemic deterioration occurred regardless of therapy allocation [210].

Using an agent which stimulates beta-cell function in a glucose-dependent manner, ideally combined with weight loss (or at least no weight gain), would be significantly more favourable. These are all features of current incretin-based therapies that result in increased activation of the insulinotropic Glucagon-like peptide-1 receptor (GLP-1R).

1.3.4.3 Glucagon-like peptide-1 receptor agonists

In 1964, it was recognised that the insulin response following oral ingestion of glucose was much greater than the one elicited by a similar intravenous glucose load [211]. This was referred to as the "incretin effect" and is believed to be modulated by intestinally secreted hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). The incretin effect in healthy individuals is responsible for up to 70% of the postprandial insulin secretion [212]. Endogenous GLP-1 is a 30-amino acid peptide produced by intestinal L-cells. It is encoded by the

proglucagon gene [213]; the same gene that in pancreatic islets leads to the production of glucagon. In plasma, GLP-1 has a very short half-life as it undergoes rapid degradation by the enzyme dipeptidyl peptidase 4 (DPP-4). Strategies to increase GLP-1R activation include using modified synthetic forms of GLP-1, which are resistant to inactivation by DPP-4 in the circulation. This class of drugs includes exenatide, a synthetic form of exendin-4 (a polypeptide produced by the salivary glands of the Gila monster) and liraglutide, which is formed by substitution of lysine 34 to arginine and the addition of a 16 carbon fatty acid at position 26 using a glutamic acid spacer on GLP-1. These changes promote aggregation that slows subcutaneous absorption and increases albumin binding, which in turn decreases DPP-4 degradation [214] and extends the half-life compared to exenatide. An alternative strategy is to use a competitive inhibitor of the DPP-4 enzyme (such as sitagliptin) to increase the amount of circulating endogenous GLP-1.

Following activation of the GLP-1R in beta-cells, adenylyl cyclase is activated and cAMP is generated, leading to cAMP-dependent activation of second messenger pathways, such as the protein kinase A and Epac pathways [215]. This results in closure of ATP-sensitive K^+ (K_{ATP}) channels and triggers insulin secretion as illustrated in **Figure 1-6**. The insulin-releasing effect of GLP-1 is glucose-dependent so that blood glucose levels are normalized, without causing hypoglycaemia. As well as enhancement of the acute insulin secretory responses to glucose, there is evidence that long-term stimulation of the GLP-1R may have long-term benefits. These include an increase in insulin content [216] via stimulation of the transcription factor PDX-1 and increased insulin transcription in the beta-cell [217, 218]. In rodents, chronic treatment with GLP-1R agonists increases beta-cell mass due to stimulation of beta-cell proliferation [219],

neogenesis [220] and/or, decreases in beta-cell apoptosis [221]. There have been encouraging results showing increased proliferation in human islets cultured with exenatide [222] and incubation of human islets in liraglutide has also been associated with a preservation of islet mass and reduced apoptosis rates [223]. These studies have however used supra-physiological concentrations of the drugs, significantly higher than that achieved in patients using exenatide or liraglutide therapy in maximal doses [224]. Exenatide has also be shown to have a significant impact on alpha-cells resulting in glucose-dependent reduction in glucagon concentration in both the fasting and post-prandial states in non-diabetic subjects [225] and patients with T2DM [226]. This effect may be therapeutically significant, as diabetes (as discussed above) is a bihormonal disorder in which the hyperglycaemic effect of insulinopenia is exacerbated by hyperglucagonaemia. Recent studies have suggested that the use of these agents in patients with T1DM (who have not undergone transplantation) may help improve glycaemic control potentially through stimulation of residual beta-cells spared from the autoimmune process [227] and/ or also by suppressing glucagon secretion in C-peptide negative individuals [228, 229].

Clinically transplanted human islets have been shown to retain the ability to respond to an infusion of GLP-1, resulting in an increase in insulin secretion [230], and highlights a potential therapeutic strategy to improve graft function.

Summary

GLP-1R analogues could therefore be potential therapeutic agents which may be beneficial for beta-cell replacement therapy (both islet and whole pancreas transplant), not only in terms of improving the transplanted beta-cell insulin secretion but also in stimulation of any residual beta-cell function in the recipient's pancreas, as well as the suppression of alpha-cell glucagon secretion. This will be examined further in *Chapter 6*.

1.4 OVERALL SUMMARY

Although many islet transplant centres worldwide accept obese donors for islet processing, there is limited data on the direct and indirect effects of lipotoxicity on islet function both *in vitro* and *in vivo.* The overall aim of this thesis is to determine if islets from obese donors are a suitable source for islet transplantation in terms of function and then to determine why the islet yields are higher from these donors. The function and integrity of islets from T2DM donors in the early stages of the disease will be assessed to determine if the concerns that have been raised about their use in islet transplantation are valid. Finally, I will aim to determine if the use of GLP-1R agonists could help to improve islet function, thus allowing prolonged graft function and potentially reduce the need for sequential transplants.

1.5 HYPOTHESES

The four overall hypotheses underlying this thesis are as follows:

Hypothesis 1: Although islet yields from obese donor pancreases are increased compared with non-obese donors, these islets are suboptimal in terms of islet function and transplanted islet graft outcome

Hypothesis 2: The higher islet yields obtained from obese donor pancreases, as measured by islet equivalents, are due to obese donors having larger islets rather than having an increased total islet number

Hypothesis 3: Islets from donors in the early stages of T2DM are unsuitable for islet transplantation

Hypothesis 4: GLP-1 receptor agonists offer a therapeutic strategy for improving islet function post-transplantation

CHAPTER 2 THE EFFECTS OF OBESITY ON ISLET FUNCTION

2.1 INTRODUCTION

Obese non-diabetic individuals can maintain euglycaemia and compensate for the increased insulin resistance by increasing plasma insulin concentrations (**Figure 2-1**). Studies have shown that insulin hypersecretion [231] and reduced insulin clearance [232] are the main mechanisms for achieving this. Over the BMI range of 23–37 kg/m^2, insulin requirements to maintain euglycaemia in the fasting state can increase by over 300% [233]. Experiments assessing insulin requirements using euglycaemic hyperinsulinaemic clamp measurements show that lean non-diabetic subjects can require <10 units of insulin in 24hrs whereas obese subjects can require over 240 units to maintain glucose homeostasis [233].

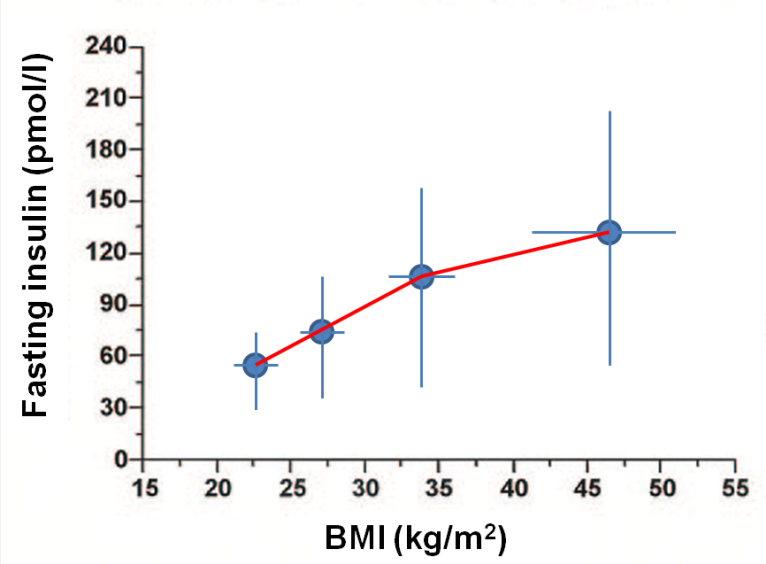


Figure 2-1: Relationship between BMI and fasting plasma insulin concentrations.
Symbols plot the mean and standard deviation for lean subjects (BMI <25 kg/m^2), overweight subjects (BMI <30 kg/m^2), obese subjects (BMI <40 kg/m^2) and morbidly obese subjects (BMI >40 kg/m^2). Adapted from [233]

This is a much larger differential than can be accounted for simply by an increase in beta-cell mass. The 20-50% increase in beta-cell mass proposed in obese non diabetic subjects at autopsy [136] would be insufficient to account for this and this clearly suggests that it is increased function of existing beta-cells, rather than changes in beta-cell mass, which is the major physiological mechanism of adaptation for insulin secretion in humans in response to metabolic demand. This dynamic ability of beta-cells to upregulate their insulin secretion in response to insulin resistance is likely to be one of the main reasons that only a relatively small percentage of obese individuals develop established diabetes [115]. Obese donors, who are normoglycaemic, would therefore seem a reasonable source of islets for islet transplantation, as the beta-cells clearly maintain the ability to respond to an increased insulin demand.

Pancreatic beta-cell dysfunction however, can occur as a consequence of lipid-induced damage [82]. Chronically elevated non-esterified fatty acids (NEFA) [85-87] and triglyceride (TRL) [85] concentrations have been readily demonstrated *in vitro* to cause beta-cell dysfunction. Significantly higher concentrations of circulating NEFA levels have been noted in obese compared to non-obese subjects [88-90]. Obesity has also been suggested to result in increased pancreatic fat accumulation [64] and this may act as a local source of NEFAs and other lipid-derived metabolites, exposing the beta-cell to considerably higher concentrations than maybe predicted from systemic concentrations. Interestingly, it has been reported that a positive relationship was present between increasing pancreatic fat content and decreasing *in vivo* beta-cell function parameters [101]. Islets from obese individuals may therefore be at higher risk of lipid-induced damage compared to non-obese individuals.

A further consequence of the obese environment is that the beta-cells become chronically over stimulated and it is not known if this could ultimately lead to islet dysfunction. Although the beta-cell has been shown to have a large capacity of insulin granules (≈10,000 secretory granules/ beta-cell), with only a relatively small proportion of these being secreted in response to a glucose challenge [15], it has not yet been determined if the insulin granule content continues to be maintained to meet the increased demand in obese subjects.

In addition to insulin, glucagon has an important role in the regulation of glucose metabolism. Relative glucagon hypersecretion has been demonstrated in adults with T2DM both in the fasting state and in response to a meal [186, 234]. A recent study, which predominantly involved adolescent subjects, demonstrated elevated fasting glucagon levels in obese insulin-resistant subjects with normal glucose tolerance and a reduced suppression of glucagon in response to a hyperinsulinemic euglycaemia clamp [235]. The reasons why these abnormalities occur are not clear but isolating islets from obese donors and studying then *in vitro* may help elucidate any potential secretory defects.

A number of cellular components are critical to insulin synthesis and secretion; one of the most important of these is the mitochondria. Islets derive 95% of their energy supply from mitochondrial oxidative phosphorylation with the contribution from glycolysis being only ~2% [236]. The increase in the ratio of adenosine-5'-triphosphate (ATP) to adenosine diphosphate (ADP), which is controlled by the mitochondria is essential for the modulation of insulin secretion [237]. This is supported by the observation that MIN6 beta-cell lines which have been completely depleted of

mitochondrial DNA, and therefore rely solely on glycolysis for ATP production, have been shown to lose their ability to increase insulin secretion in response to a glucose challenge [238].

Mitochondria are also a potential site for cellular damage. The mitochondrial respiratory chain is the major location for the production of reactive oxygen species (ROS) within the beta-cell. Increased oxidative stress and excessive ROS production can lead to cellular damage and apoptosis [239] and it is the mitochondria that are the primary target for this free radical damage. It has been proposed that this damage is implicated in the diabetic state [240]. Being in close proximity to the site of free radical generation, mitochondrial inner membrane components are at a high risk of oxidative injuries, eventually resulting in depolarized mitochondrial membrane and impaired ATP production [241]. Accumulating evidence shows that there is an increase in ROS production when cells are exposed to fatty acids [242]. Long-term exposure of rat islets to long chain fatty acids *in vitro* induced partial uncoupling of beta-cell oxidative phosphorylation that was paralleled by decreased ATP content and decreased mitochondrial membrane potential [243]. The mechanisms by which NEFAs promote ROS generation in mitochondria are currently unclear but it is established that ROS elevation will eventually interrupt the transduction of signals normally coupling glucose metabolism to insulin secretion [244].

Several groups have used the level of intra-islet ATP as a marker of islet viability/function [245, 246]. It is not known if the concentration of intra-islet ATP is increased in obesity, which could reflect increased mitochondrial activity as a result of the increased insulin secretion demand, or alternatively decreased as a result of

increased ROS production. Measuring the concentration of intra-islet ATP content under stimulated conditions may therefore be a useful indicator in determining mitochondrial activity.

It is also likely that the level of ATP, and therefore the capacity for insulin secretion, is regulated by the number and efficiency of mitochondria in beta-cells. This could be related to the level of insulin requirement in obesity. As mentioned previously eukaryotic cells usually contain one nuclear genome (NucDNA) and up to several thousand identical copies of the mitochondrial DNA (MtDNA) with each mitochondrion estimated to contain 1-3 mtDNA copies. This MtDNA is inherited maternally and independently of genomic DNA [247] (**Figure 2-2**). Between tissues there is variation in the mitochondrial number, function, protein composition and morphology. The levels of mtDNA per cell have been shown to be different depending on the tissue type which suggests that this parameter plays an important role in establishing the differences in the oxidative phosphorylation capacity between cell types. For example, cardiac and skeletal muscle have been shown to have significantly higher mtDNA/nuclear DNA levels than the brain and kidney [248]. As there is no published literature comparing the islet mtDNA to other tissues it is difficult to know what the relative concentration would be in comparison. However, given the dependency on ATP production for insulin secretion, it may be hypothesized that this would be relatively high.

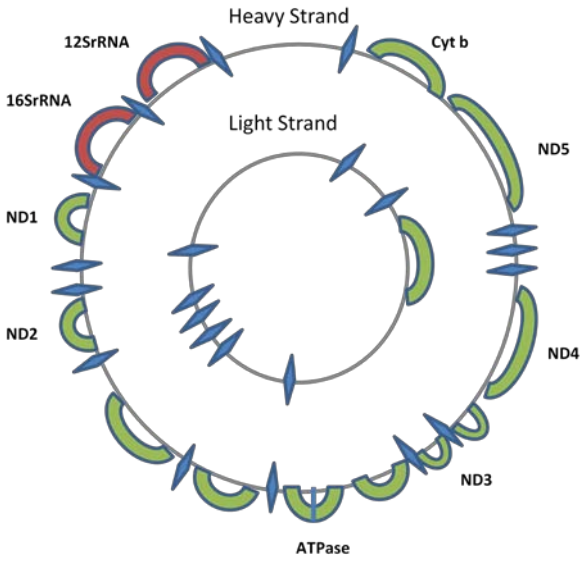


Figure 2-2: Mitochondrial genome.
mtDNA is circular and double-stranded. It has a highly compacted structure consisting almost entirely of coding regions. The two strands of mtDNA are differentiated by their nucleotide content with the guanine rich strand referred to as the heavy strand, and the cytosine rich strand referred to as the light strand. The heavy strand encodes 28 genes and the light strand encodes 9 genes. Of the 37 genes, 13 are for proteins (polypeptides) (green), 22 are for transfer RNA (tRNA) (blue diamonds), and two are for the small and large subunits of ribosomal RNA (rRNA) (red). Adapted from [249]

The mtDNA copy number can also vary in different physiological and pathological situations [250-252]. The relationship between mtDNA content in skeletal muscle and its oxidative capacity has prompted the "gene dosage" theory. This postulates that mtDNA replication is an integral mechanism for increased mitochondrial biogenesis and that amplification of the mitochondrial genome relative to chromosomal DNA is an important feature underlying enhanced expression of mitochondrial genes in highly oxidative tissues[253]. Consistent with this concept are recent reports, which have shown that a moderate-intensity walking intervention in elderly men and women induced amplification of muscle mtDNA that was proportional to increases in oxidative capacity [254].

In most cases, mtDNA levels seem to be linked to organelle number, possibly because of the coordinated expression of structural and regulatory genes [255]. To date no studies have assessed if beta-cells have the ability to increase mitochondrial number as a compensatory mechanism in response to an increased insulin secretory demand. On the other hand, mtDNA is more vulnerable to oxidative stress and damage is more extensive than that occurring for nuclear DNA due to the lack of protective histones and low repair mechanisms [256]. It is therefore possible that as a result of the chronic exposure to a lipotoxic environment, when triglyceride levels are high and beta-cell oxidation of lipids is occurring, the amount of mtDNA per beta-cell may be reduced as a result of damage. As the mitochondria have such an important role in production of ATP for insulin secretion, if the number of mitochondria was to be reduced within beta-cells this could signal as an ominous marker, potentially indicating future beta-cell dysfunction. Assessment of mtDNA in comparison to the total nuclear DNA is regarded as a quantitative index of mitochondrial content in tissues and it would be of interest to determine how this is affected by obesity.

Assessing functional characteristics of human islets isolated from donors with known anthropometric measurements gives a unique opportunity to better understand the effects that obesity may have on islet function.

Aims of this Chapter:

1. To determine if pancreatic fat content and intra-islet triglyceride content are increased in obese compared to non-obese pancreatic donors

2. To determine whether the islet hormonal content and basal/ stimulated hormonal secretion in response to a glucose challenge are altered in islets isolated from obese compared to non-obese donors

3. To determine if mitochondrial function and number is altered by increasing BMI

2.2 METHODS

Donors were considered obese if their BMI was ≥30kg/m^2 and non-obese if their BMI was ≤28kg/m^2. **Table 2-1** shows the inclusion criteria for accepted donor pancreases.

Table 2-1: Inclusion Criteria for Islet Transplantation

Donor Criteria	
Age (years)	>18 and <65
No history of Diabetes	
No history of Alcoholism	
No history of Chronic Pancreatitis	
Procured Pancreas Criteria	
Cold Ischaemia Time (hours)	<10
Heart beating donors only	
No Pancreatic Capsular damage (which would preclude adequate distension)	
No significant fatty infiltration (which would preclude adequate distension)	
Well perfused	
Pancreas weight (grams)	≥69

2.2.1 Islet isolation procedure

Pancreases from human donors (not known to have diabetes) were procured by regional organ retrieval teams following appropriate consent for islet research and local ethics approval (ref 09/H0605/2). The pancreases were stored and transported in University of Wisconsin solution at 4^{o}C. Cold ischaemia time was <10hrs in all cases. Human islet isolation was performed in the Oxford Diabetes Research and Wellness Foundation, Human Islet Isolation Facility using principles of the semi-automated method described by Ricordi [28]. Surface fat was trimmed and the duodenum and spleen were removed. The pancreatic duct was cannulated and infused with a commercially available blend of

collagenase and neutral protease (SERVA, Germany) using controlled syringe loading [257]. This delivers enzymes to the islet-exocrine interface. The islets are then separated from the exocrine and ductal tissues by enzymatic breakdown of the peri-islet collagen matrix. The cannula was removed and the pancreas dissected into blocks (10-14 pieces) and placed in a Ricordi chamber with a digestion circuit. During the digestion phase HBSS buffered with 20mM HEPES and supplemented with 3mM $CaCl_2$ was pumped around the circuit at 37°C (Phase 1). This temperature is optimal for collagenase activity and in combination with gentle mechanical dissociation the pancreas becomes digested [28]. When islets become liberated, the circuit is opened for collection and the temperature reduced to 34^{o}C to reduce enzymatic activity (Phase 2). The digest is collected in 250ml conical tubes containing human albumin (Bio Products Laboratory, UK). In order to wash the debris from the islets the digest was centrifuged at 1000rpm at 4^{o}C for 2 min to sediment the islets. The supernatant was aspirated and the digest pellets were re-suspended in wash media which contained HBSS (Lonza, Belgium) supplemented with 20 mM HEPES (Lonza, Belgium), 20 U/ml Trasylol (Nordic Pharma, UK), 100 U/ml penicillin (Lonza, Belgium), 10 µg/ml streptomycin (Lonza, Belgium) and 0.6 % human albumin (Bio Products Laboratory, UK). The washes were repeated until no debris was observed in the digest. The digested pancreatic tissue was then top loaded on a continuous Ficoll gradient with a density from 1.077 to 1.100 (Biochrom, Germany) and centrifuged in a refrigerated cell separation apheresis system (COBE 2991; Lakewood, CO). Dithizone (DTZ) (Sigma, UK) was used to identify the islets to assess islet purity. DTZ binds to zinc ions within beta-cells (insulin is stored as Zn_2-$insulin_6$ complex within the secretory granules) and thereby selectively stains all the islets red to facilitate counting.

Selected fractions were centrifuged at 1000rpm for 5 min at room temperature to sediment the islets. The supernatants were discarded and islet tissue was pooled; the islets were washed twice in CMRL medium (PAA Laboratories, Austria) (5.5 mM (99 mg/dl) glucose). The islets were then cultured in CMRL medium overnight prior to experiments.

Cell viability was established using fluorescein diacetate and ethidium bromide (Sigma, UK). Live cells will actively convert the non-fluorescent fluorescein diacetate into the green fluorescent compound "fluorescin" (emission wavelength 518nm), a sign of viability; while nuclei of membrane-compromised cells will appear red as a result of ethidium bromide binding (emission wavelength 595nm), and taken as a sign of cell death (**Figure 2-3**).

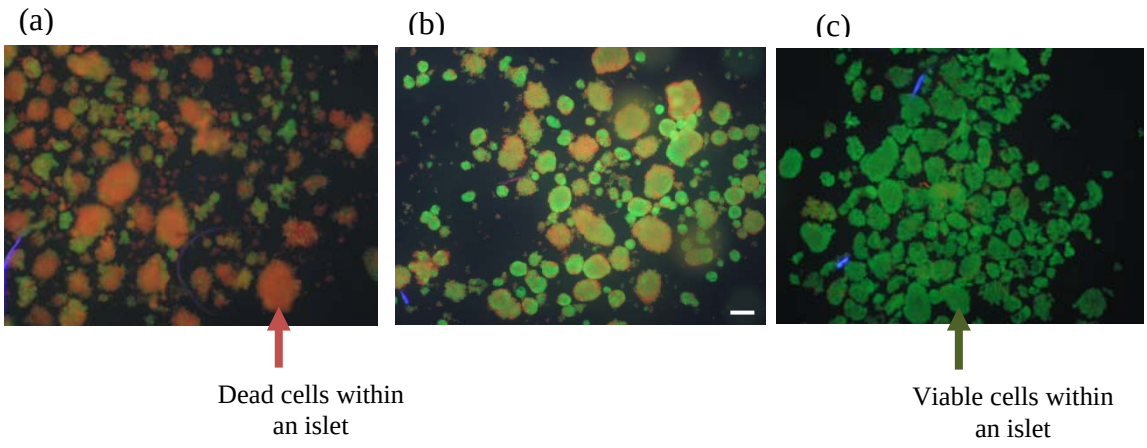


Figure 2-3: Viability stain of human islets in the digest.
Living cells emit green light after addition of fluorescein diacetate (converted to fluorescent fluorescein in viable cells). The nuclei of membrane-compromised cells will emit red as a result of ethidium bromide binding, a sign of cell death. (a) viability ~10%, (b)~50% (c)~95%. Scale bar represents 50µm.

2.2.2 Adipocyte quantification in human pancreatic tissue blocks

Lipomatosis has previously been shown to be the same in the various parts of the dorsal pancreatic anlage (anterior part of the head, the body and the tail) [258]. A $1cm^3$ tissue biopsy was taken from the body of each pancreas before introduction of collagenase and fixed in 10% formal saline (Sigma, UK). Tissues were processed into wax and five micron thick sections cut for immunohistochemistry. The sections were de-waxed in xylene (VWR, UK) and then rehydrated in descending concentrations of alcohol. The slides then underwent an immune staining process so that the islets could be identified. Endogenous peroxidise activity was blocked using methanol/ 0.3% H_2O_2 (Fisher Scientific, UK). The slides were then washed thoroughly in TRIS buffered Saline (0.1M tris in normal saline (9.0% sodium chloride) (Sigma, UK). Swine serum (Vector Laboratories, UK) 1/20 dilution was added to the specimens for 20mins to block non-

specific binding antisera. Primary antibodies were then applied and incubated for 24hrs at 4^{o}C. The following primary antibodies were used: guinea pig anti-insulin (in-house) 1 in 500 dilution with TRIS saline and mouse anti- glucagon (DAKO Denmark) 1 in 500 dilution with TRIS buffered saline. Each primary antibody binding site was identified with secondary antibodies conjugated to peroxidase (DAKO, Denmark) or alkaline phosphatase (DAKO, Denmark) to allow visualization of insulin and glucagon respectively. Sections were then counterstained in Haematoxylin (Sigma, UK) for 30secs and mounted in aqueous mountant and a coverslip applied which was sealed with nitrocellulose dissolved in ethyl acetate. Since pancreatic fat is extracted during specimen dehydration, intrapancreatic fat appears in histological sections as lobulated spaces within the pancreas. If the histological samples had undergone autolysis the cases were excluded.

For morphometric analyses, one labelled section from each donor was used, and sequential fields of the specimens were photographed at 100x magnification (**Figure 2-4**). The median total parenchymal area examined was 20.7mm^2 (range= 11.7-50.5 mm^2) per donor. AxioVision 3.1 (Carl Zeiss, Feldbach, Switzerland) imaging software was then used for quantification. The total parenchyma tissue area and the intra-pancreatic adipocyte area within the exocrine tissue were measured. Interlobular adipocytes were not included in the measurements, only adipocytes in the exocrine parenchyma. The percentage of pancreatic fat relative to total pancreatic area was calculated.

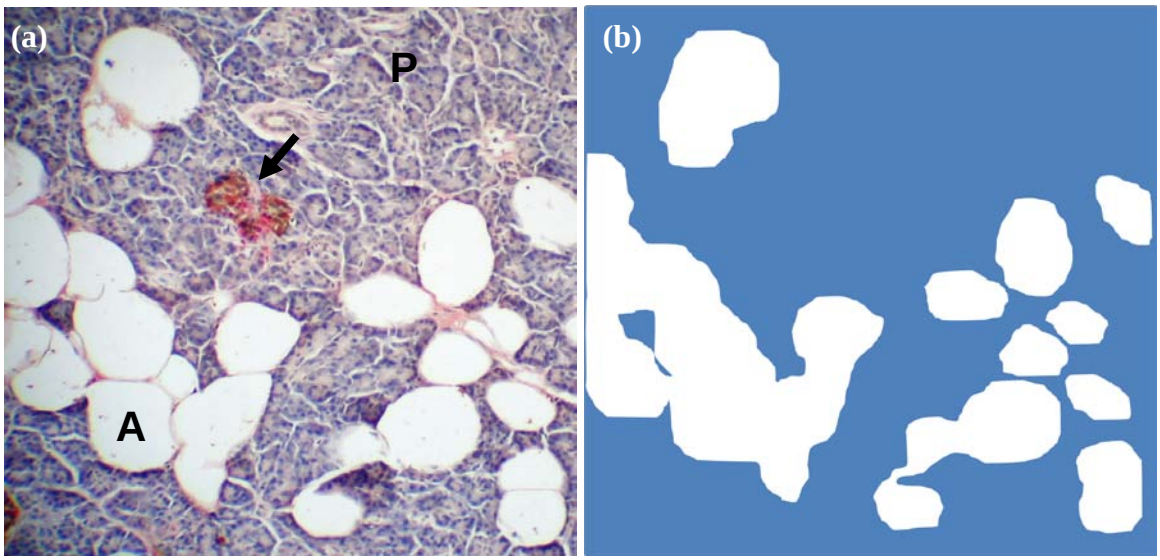


Figure 2-4: Histological quantification.
(a) Arrow indicates an islet (brown/red), (P) parenchyma tissue, (A) adipocytes. (b) Schematic of the adipocytes (white) and parenchyma (blue) showing area used for calculations.

2.2.3 Intra-islet triglyceride and Fatty acid analysis

A 0.5cm^3 sample of peri-pancreatic fat was removed from the outer surface of the pancreas and stored in phosphate buffered saline. As the peri-pancreatic fat is the closest adipose tissue to the islet cells this therefore acts as a good marker to establish if the fat content in the islets is similar in composition to that deposited in and around the pancreas. 300 purified islets of similar size were picked immediately after isolation into a 0.5ml eppendorf tube with phosphate buffer saline and stored at -20^{o}C prior to analysis.

Adipose tissue samples were homogenized in 5 ml 2:1 (v/v) chloroform/methanol with 100 mg/l butylated hydroxytoluene (Sigma, UK). 100 µl of adipose tissue homogenate was then used for fatty acid composition.

JNW was involved in isolating the islets and collecting the islet and peri-pancreaic fat samples for the triglyceride and fatty acid analysis. The lipid extraction and quantification was undertaken by Dr Leanne Hodson (Senior Scientist, OCDEM) - see A*ppendix 8.1* for description of methods used.

2.2.4 Hormone secretion assay

Hormone secretion (insulin, glucagon) was measured from batches of 10 similar sized islets (150± 10µm in diameter) in quadruplicate, which were handpicked using a 10µl pipette from the purified digest. Previous studies have used islets of differing diameter and standardized the islet mass by conversion into equivalent number of islets with a diameter 150 µm (IEQ) [120], alternatively standardized to protein content or to DNA content. Although initial reports have shown that insulin secretion rates are directly related to islet size [259] more recent work on both rodent [260] and human islets [261] has disputed this. Human islet studies have shown that smaller isolated islets (<150µm) are superior to large islets in their *in vitro* function and viability. Smaller islets have been shown to release double the amount of insulin under both basal and stimulated conditions compared to larger islets [261]. A standard islet size of 150µm was therefore chosen for all the secretion studies to eliminate any bias that could be introduced by varying islet size.

The islets were picked into 0.5 ml eppendorf tubes and washed in RPMI-1640 (Sigma, UK) supplemented with 100 U/ml penicillin (Lonza, Belgium), 10 µg/ml streptomycin (Lonza, Belgium) and 10% Fetal Calf Serum (Sigma, UK) to remove cell debris. The islets were pre-incubated for 1 h in a wet chamber at 37°C (5 % CO2 / 95 %

air) in 300 µl of Krebs-Ringer buffer (KRB) which contained the following (in mM) 140 NaCl, 3.6 KCl, 2.6 $CaCl_2$, 0.5 $MgSO_4.7H_2O$, 0.5 NaH_2PO_4, 2 $NaHCO_3$, 5 HEPES and 2 mg/ml Bovine serum albumin (BSA) (Sigma, UK) (pH adjusted to 7.4 with NaOH). The KRB was supplemented with a 1mM (18 mg/dl) glucose concentration. The pre-incubation buffer was discarded. Islets were incubated for 1 h in a wet chamber at 37°C (5 % CO_2 / 95 % air) in 300 µl of KRB supplemented with the test conditions as indicated. The test buffer contained 1 mM (18 mg/dl) or 20 mM (360 mg/dl) glucose. Samples of the supernatant (290µl) were collected at the end of each test period and immediately frozen at -20°C. The remaining supernatant was discarded. The islets were then lysed in 100µl ice-cold acid-ethanol solution (containing 90% ethanol, H_2O and 12M HCl in a ratio of 52:17:1) to release their hormone content. The lysed islet pellets were immediately frozen at -20°C.

The supernatants and pellets from the human hormone secretion experiments were assayed using human insulin (Millipore, UK) and glucagon radioimmunoassay (RIA) kits (Millipore, UK) according to the manufacturer's instructions (For principles see *Appendix 8.2*). Prior to RIA, the supernatants and pellets were diluted in Dilution Buffer which contained HBSS supplemented with 0.25 % RIA grade BSA and 0.008 % sodium azide. The dilutions used for the insulin assays were 1 in 4 for supernatant and 1 in 1600 for the pellet. For glucagon assays the supernatant was assayed undiluted and the pellet 1 in 800.

A Stimulation Index (SI), to enable comparison between samples, was calculated by dividing insulin secretion at 20mM (360 mg/dl) glucose by insulin secretion at 1mM (18 mg/dl) glucose.

2.2.5 Intra-islet ATP content

Intra-islet ATP content was measured under stimulated conditions, as a marker of mitochondrial function to evaluate the energy status of the human islets. Islets with a diameter of 150± 10µm were hand-picked from the purified digest using a 10µl pipette. Measurement of intra-islet ATP was undertaken using bioluminescence measurements. Bioluminescence is now the most widely used method for the assay of ATP due to its very high sensitivity, wide dynamic range, and ease of use [262]. Luciferase is used, which generates light from ATP and luciferin according to the two step reaction below:

Luciferase

ATP + Luciferin + O2 ——> luciferyl adenylate + PPi

luciferyl adenylate + O2 ——> Oxyluciferin + AMP + CO_2+Mg^{2+}+ **LIGHT**

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer. The assay is conducted at ambient temperature (18°C-22°C), the optimal temperature for luciferase. ADP is measured by its conversion to ATP, which is subsequently detected using luciferase.

Islets were cultured for a median of 24hrs (range=20-28hrs) in CMRL culture media prior to assessment. Batches of 10 islets (in triplicates) were washed in 300µl RPMI, then pre-incubated for 1hr in 300µl KRB medium containing 2mg/ml BSA and 1mM (18 mg/dl) glucose at 37^{o}C. This was followed by 1hr incubation in 300µl of KRB solution supplemented with 20mM (360 mg/dl). The supernatant was completely removed and the pellet was mixed with 200µL of nucleotide-releasing reagent (ApoGlow™ kit (Cambrex Bio Science, UK)), vortexed and then frozen at -80^{0}C. The

sample was thawed and homogenised. 100µl of each sample was placed in a 96 well plate. 40µL of nucleotide-monitoring reagent (ApoGlow, ™ kit (Cambrex Bio Science, UK)) was added to the solution, and the ATP levels were measured using a luminometer (Veritas™ Microplate Luminometer, Turner BioSystems, Sunnyvale CA) and expressed as the number of relative light units (RLU). A standard curve was produced using known concentration of ATP. ADP was measured by its conversion to ATP, using an ADP Converting Reagent (ApoGlow, ™ kit (Cambrex Bio Science, UK)), which was subsequently detected using luciferase.

Rotenone (Sigma, UK) in 1µm concentration was added to the incubation media of two islet preparations to determine what proportion of ATP production was dependent on the mitochondria. Rotenone interferes with the electron transport chain in mitochondria. Specifically, it inhibits the transfer of electrons from iron-sulfur centers in complex I to ubiquinone. This prevents NADH from being converted into usable cellular energy (ATP).

2.2.6 Mitochondrial DNA

Human islets with a diameter of 100-200µm were hand-picked from the purified digest using a 10µl pipette (20 per sample) into an eppendorf containing 0.1ml CMRL culture media. The samples were then frozen at -80^{o}C. DNA was extracted as per the Nucleon BACC2 Genomic DNA extraction protocol (**Table 2-2**) (GE Healthcare, UK) and then diluted to a 1/50 concentration with highly purified H_2O.

Table 2-2: Principle steps in the Nucleon™ BACC protocol
cell lysis ▼
deproteinisation with sodium perchlorate ▼
extraction with chloroform and Nucleon™ resin ▼
DNA recovery ▼
DNA washing

Quantitation of mtDNA content using Q-RT PCR

A SYBR Green system based on real-time detection of accumulated fluorescence was used. Triplicate single colour molecular beacon PCRs were used to quantify mtDNA levels relative to nuclear DNA. The SensiMix SYBR mix (Bioline, UK) included SYBR Green dye, Deoxyribonucleotide triphosphate (dNTPs), stabilisers and enhancers. The RT-PCR mix included 12.5μL SensiMix solution, 7.7μl H_2O, 15μL each of the forward and reverse, primers (100μM) (Invitrogen, UK) and 4μL of the DNA sample. Corbett© Robotic hardware and RotorgeneTM 6000 series software were used to pipette the reaction reagents into 100-well rings and to perform the qRT-PCR. Samples were measured on the green channel 470nm. Each well contained 21μl of reaction mix and 4μL of the DNA sample. The amplification conditions used were one cycle at 95^{o}C for 10 minutes, followed by 40 cycles of 95^{o}C for 10 seconds, 55^{o}C for 20 seconds and 72^{o}C for 20 seconds. Samples were analysed in triplicate and averaged for both assays.

Primer sequences were: forward primer, MitHu3130F, (DNA sequence AGGACAAGAGAAATAAGGCC), reverse primer MitHu3301R (TAAGAAGAGGAATTGAACCTCTGACTGTAA) for the mitochondrial fragment;

forward primer 18SrRNA 1546F (DNA sequence: TAGAGGGACAAGTGGCGTTC) and reverse primer 18sRNA 1650R (DNA sequence: CGCTGAGCCAGTCAGTGT) for the nuclear fragment. Each probe was labelled with FAM at the 5' end and TAMRA at the 3' end. The probe oligo sequences were: mit3153T, TTCACA AAGCGCCTTCCCCCGTAAATG, targeting the mitochondrial fragment; and APP161T, CCTGAACTGCAGATCACCAATGTGGTAG, targeting the nuclear fragment.

Melt curves are produced on completion of the amplification reaction and are generated by monitoring the fluorescent signal produced when temperature is increased at small increments. They indicate the number of reaction products generated during the amplification. **Figure 2-5a** shows the mitochondrial and **(b)** nuclear melt curves for the qRT-PCR runs using SensimixTM and SYBR Green® indicating only one product is being formed.

(a) (b)

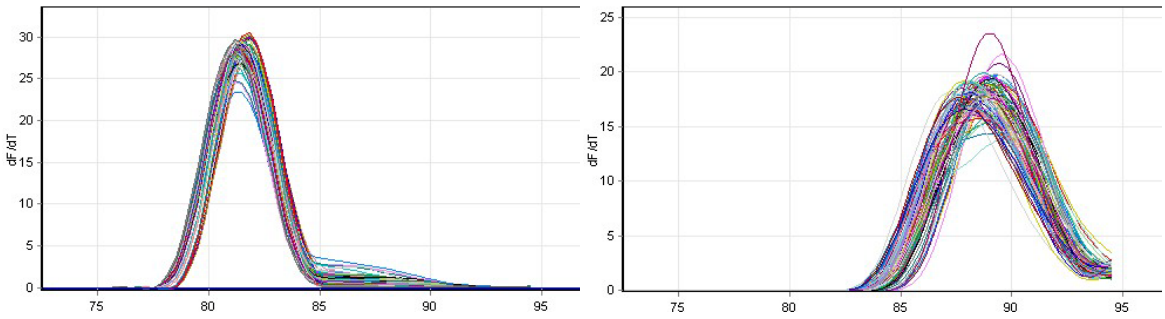


Figure 2-5: Melt curves

(a) Mitochondrial Melt Curves obtained following qRT-PCR using Sybr Green, Sensimix and MitHu/APP primers. (b) Nuclear melt curve after qRT-PCR using 18S rRNA primer with human islet samples.

Calculation of relative mtDNA copy number

As there were relatively low levels of DNA in the human islet samples, it was not possible to accurately work out the DNA concentration. A standard curve containing a 10-fold dilution series of a reference DNA was used to plot a standard curve for mtDNA and for nuclear DNA on each run (R^2 usually 0.99).

The RotorgeneTM 6000 Series software automatically calculated the relative concentrations of the mtDNA and nuclear DNA based on the standards. A threshold line on the graph determined the Critical Threshold (Ct) value of each sample. This line is above the background noise present on the graph but below the point where the amplification curves begin to plateau i.e. the point where nucleotides and primers become limiting factors in the amplification. Its position is based on the optimal line of best fit through the standards and on the threshold line that would give the best R^2 reading (an efficient value was regarded as an R^2 >0.95). The Ct value is the fractional cycle number at the point where the amplification of the DNA reaches a critical fluorescence level, i.e. where the amplification curve intersects the threshold. The standard curve is produced based on the Ct values and concentrations of the standards; from this curve the concentrations of the unknown samples can be obtained. The relative amounts of mtDNA/nuclearDNA were then determined. These assays were carried out with the supervision of Dr Karl Morten (Senior Scientist, John Radcliffe Hospital) and Ms Sarah Wheeldon (Graduate student, John Radcliffe Hospital).

2.2.7 Data analysis

Statistics were analysed using GraphPad prism software (Graphpad, USA). A Student's t-test was used to compare the data if normally distributed and a Mann-Whitney's test was used if the data was not. Spearman Rank Correlation coefficient was used to examine the correlation between mtDNA copy number and islet donor BMI. A p-value <0.05 was considered statistically significant. All data are expressed as mean values ± SE.

2.3 RESULTS

2.3.1 Donor BMI and Adipocyte density

Pancreatic biopsies were available from 21 human pancreases; n=11 non-obese donors (mean BMI 22.54 ± 0.4kg/m^2 (range= 20-24.2 kg/m^2) vs n=10 obese (mean BMI 35.1 ± 0.7 kg/m^2 (range= 31.9-37 kg/m^2)) ($p<0.0001$). Other donor variables including age and CIT were similar.

A significant difference was observed in the % fat content relative to the parenchymal tissue (non-obese mean 2.9% ±1.4 vs obese 5.39%± 1.1) ($p=0.03$) (**Figure 2-6**).

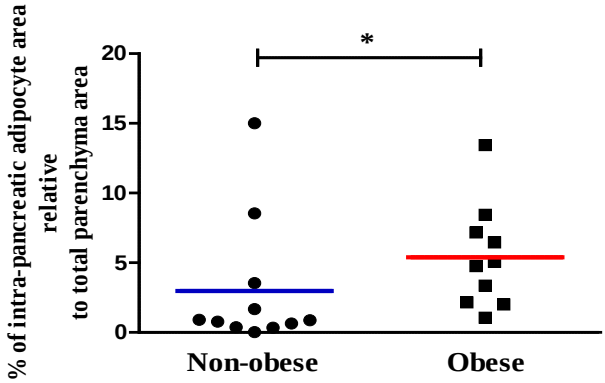


Figure 2-6: Percentage of intra-pancreatic adipocyte area relative to total parenchyma area on histological sections.
Non-obese donors (circles), obese donors (squares). Bar =Mean. $p<0.05$ *

Figure 2-7 shows examples of the differences observed in adipocyte density between a donor with a BMI of 20 kg/m^2 **(a)** and a donor with a BMI of 35 kg/m^2 **(b).** In the obese

cohort intra-pancreatic adipocytes where noted in close proximity to the islets (**Figure 2-7b, c).**

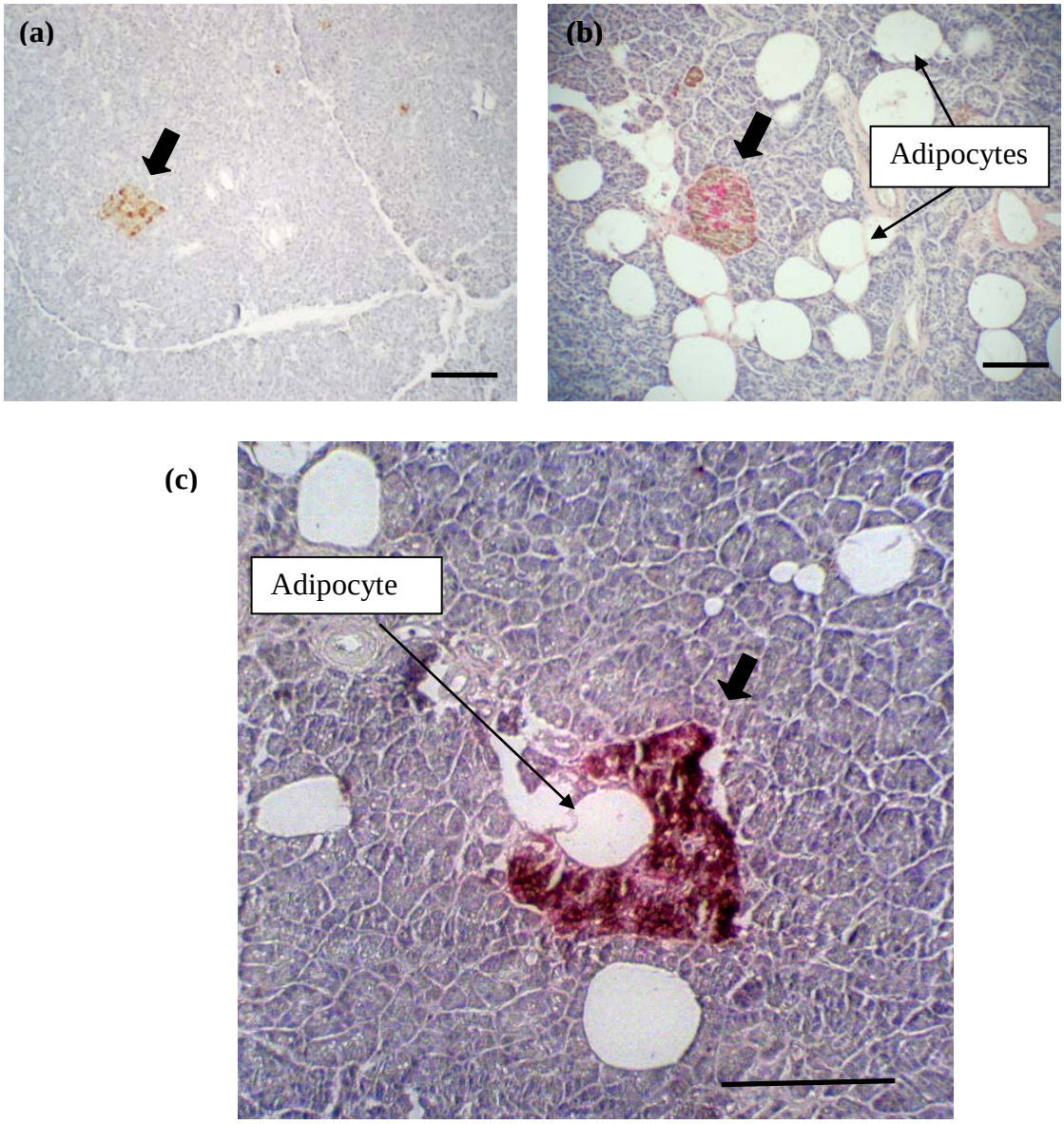


Figure 2-7: Histology sections of human pancreatic biopsies immune-labelled for islet hormones and haematoxylin.
Samples from donors with (a) 20 kg/m^2 and (b) 35 kg/m^2. Islet labelled brown for insulin and red for glucagon (black arrow head). White vacuoles indicate adipocytes within the exocrine parenchyma. (c) Pancreas from a donor with high BMI showing an adipocyte adjacent to an islet. Scale bars represent 100µm.

2.3.2 Donor BMI and Intra-islet triglyceride content

The initial aim was to measure the triglyceride content in the same pancreases used for morphometric intra-pancreatic adipocyte analysis. This however was not possible as not all pancreases proceeded to the purification stage.

Islets isolated from 17 donor pancreases were used for intra-islet triglyceride quantification. n=8 non-obese (mean BMI 26.2± 0.9 kg/m^2 (range=21-28 kg/m^2) vs n=9 obese, (mean BMI 34.2± 0.6 kg/m^2) (range=31-37 kg/m^2) ($p<0.0001$). Other donor variables including age and CIT were similar.

Intra-islet triglyceride content was significantly higher in the obese group ($p=0.036$)) (**Figure 2-8)**.

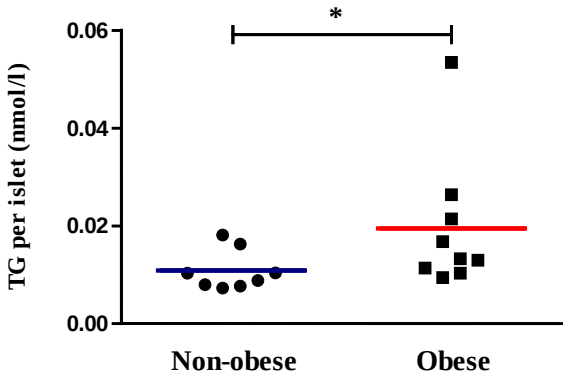


Figure 2-8: Triglyceride content in isolated islets.
Non-obese donors (circles), obese donors (squares). Bar= Mean. p<0.05 *.

The islet triglyceride fatty acid composition was compared to that of the peri-pancreatic visceral fat. The fatty acid composition profiles were not significantly different (**Figure**

2-9). There was a high proportion (~30%) of saturated palmitic acid (16:00) and similar proportions of unsaturated oleic acid (18:01).

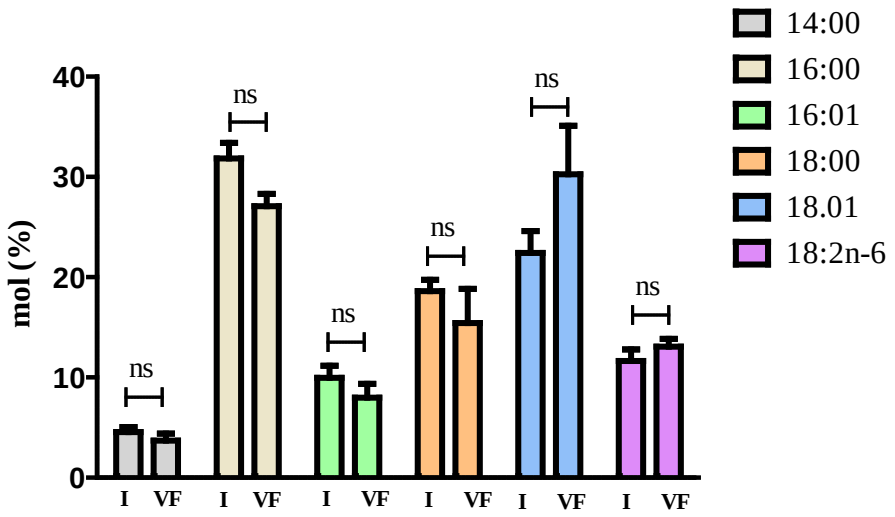


Figure 2-9: Triglyceride fatty acid composition in islets (I) and peri-pancreatic visceral fat (VF).

2.3.3 Donor BMI and Hormone content and secretion

(a) Insulin content/ secretion

Islets isolated from 52 donor pancreases were used for the insulin content and secretion experiments. Non-obese donor pancreases n=25; mean BMI 25.9 ± 0.48 kg/m^2. Obese donor pancreases n=27; mean BMI of 32.8± 0.41kg/m^2. Other donor variables including age and CIT were similar. For donor characteristics, see **Table 2-3.**

Table 2-3: Donor characteristics for *in vitro* insulin content and secretion experiments

	Non-obese	Obese	p-value
N	25	27	
Gender (male: female)	11: 14	14: 13	
BMI (kg/m^2)	25.62± 0.5	32.8± 0.4	< 0.0001***
Age (years)	48.5± 2.5	47.07± 1.9	NS
CIT (hours)	6.9± 0.3	6.3± 0.3	NS
Cause of Death			
ICH	17	19	
CVA	2	0	
Trauma	0	1	
Hypoxic Brain injury	2	4	
RTA	2	1	
Other	2	2	

ICH: Intracranial Haemorrhage; CVA: Cerebral Vascular Accident; RTA Road Traffic Accident

The islet insulin content was not significantly different between the groups **(Figure 2-10a)**. Insulin secretion in 1mM glucose was similar (0.088±0.02 ng/hr/islet (non-obese) vs 0.10±0.02 ng/hr/islet (obese)) and the fold increase in insulin secretion

from low to high glucose (stimulation index) was again similar in both groups (3.93± 0.48 (non-obese) vs 4.37± 0.46 (obese); p=0.4) **(Figure 2-10b).**

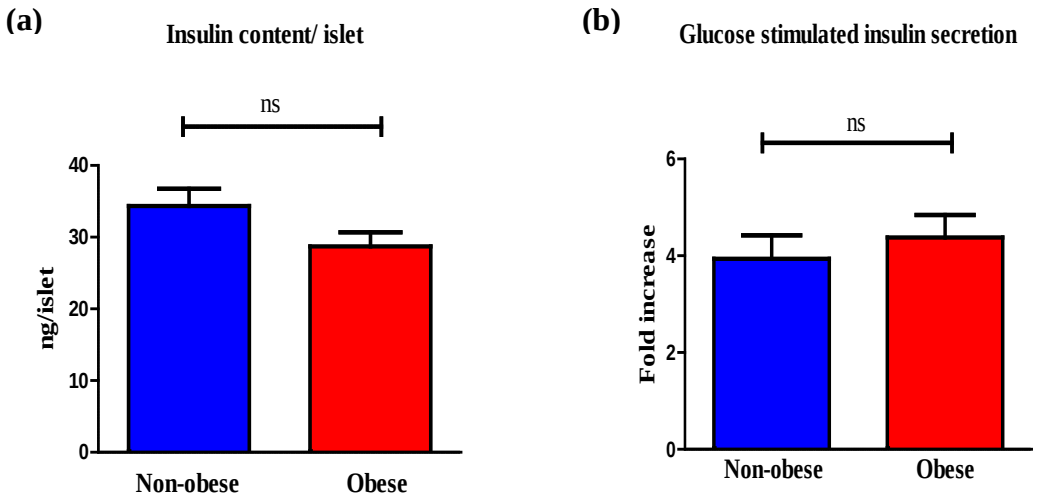


Figure 2-10: Insulin content and secretion for non-obese and obese donors.
Islets isolated from 25 non- obese and 27 obese donor preparations (a) mean insulin content (b) mean fold increase in glucose stimulated insulin secretion (1mM to 20mM glucose).Mean+SEM; p<0.05 *.

(b) Glucagon content and secretion

The number of islets used initially (10 per sample) resulted in hormone secretion values which were below the standard curve. As the standard curve is non-linear (polynomial) this had a significant impact on the value so in those cases the results were excluded. 15 islets per sample were used thereafter. As a result functional data was only available on 19 rather than 54 donors.

Non-obese donor pancreases n=7; (mean BMI 26.2± 0.6 kg/m^2) and obese donor pancreases n=12; (mean BMI 32.9± 0.5 kg/m^2). Other donor variables including age and CIT were similar. The glucagon content per islet (679± 72 pg/islet (non-obese) vs

633.5± 78.5 pg/islet (obese)) and the islet insulin: glucagon ratio **(Figure 2-11)** for these subjects were not significantly different.

Two donors from each group failed to suppress glucagon secretion from low to high glucose. The mean percentage suppression was not significantly different 34± 6% (non-obese) vs 36± 6% (obese). Glucagon secretion in 20mM glucose was also similar (4.2± 1.2 (non-obese) vs 3.2± 0.9 pg/islet/hr (obese); p=0.54).

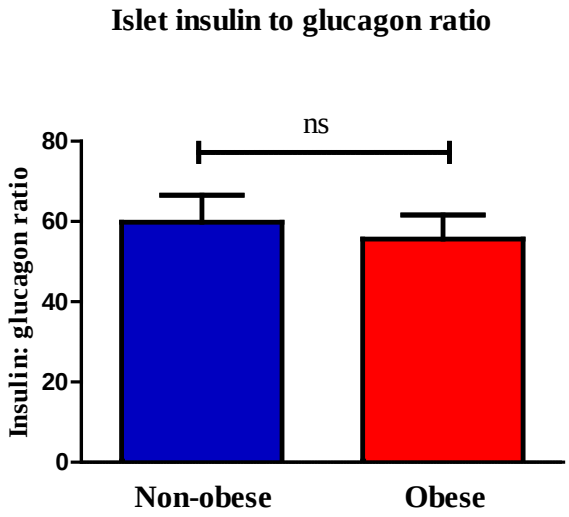


Figure 2-11: Mean ratio of insulin to glucagon content per islet for non-obese and obese donors.
Islets isolated from non-obese (blue) and obese (red) donor preparations Mean +SEM

Donor BMI and intra-islet ATP content

Due to the high demand for human islets for research within Oxford, only islets from 35 of the 54 donor pancreases were collected for ATP measurement (n=17 non-obese vs n=18 obese) **(Table 2-4)**.

Table 2-4: Donor characteristics for *in vitro* intra-islet ATP content

	Non-obese	Obese	p-value
N	17	18	
Gender (male: female)	8/9	8/10	
BMI (kg/m^2)	26.5± 0.5	32.7± 0.5	< 0.0001***
Age (years)	49.4± 2.8	48.1± 2.3	NS
CIT (hours)	6.6± 0.3	6.4 ± 0.3	NS
Cause of Death			
ICH	11	15	
CVA	1	0	
Trauma	2	1	
Hypoxic Brain injury	1	2	
RTA	1	0	
Other	1	0	

ICH: Intracranial Haemorrhage; CVA: Cerebral Vascular Accident; RTA Road Traffic Accident

ADP levels were found to be highly variable within batches of islets from the same preparation. Therefore, the intra-islet ATP content per islet was used rather than ATP/ADP ratio. The mean intra-islet ATP content was similar in both groups (4.2± 0.4 pmol/islet (non-obese) vs 4.3± 0.3 pmol/islet (obese)) **(Figure 2-12a).** The percentage increase in ATP when islets were incubated in either low or high glucose was measured. In 2 preparations no change in ATP was seen, overall the mean increase was similar (24.1± 3.9% (non-obese) vs 26.1± 6.9% (obese)).

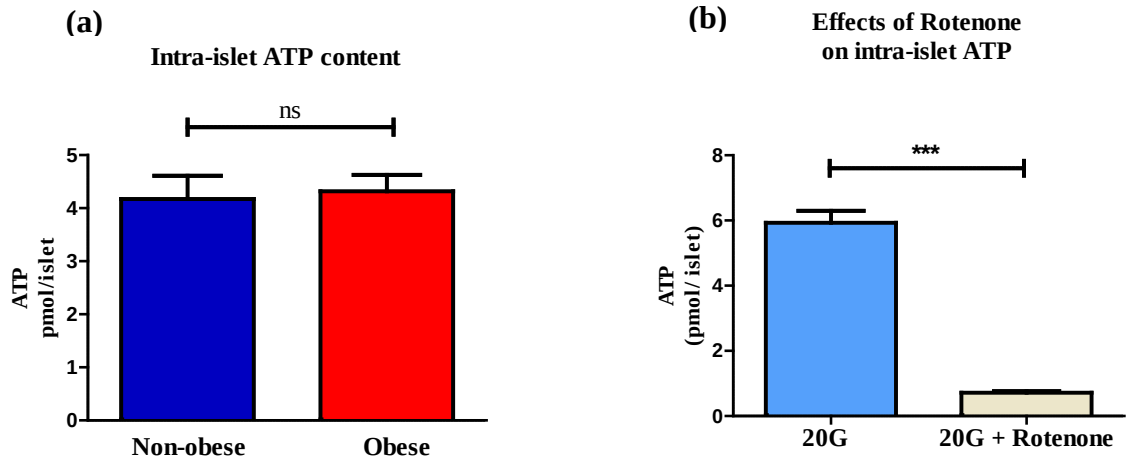


Figure 2-12: Intra-islet ATP concentration.
(a) Mean Intra-islet ATP concentration for islets isolated from 17 non-obese and 18 obese donor preparations. (b) Effects of Rotenone, an inhibitor of the mitochondrial electron transport chain, on intra-islet ATP concentration.

The addition of rotenone had a clear inhibitory effect on ATP production. In stimulated conditions (20mM glucose) the addition of rotenone reduced the ATP concentration by 88± 2% **[Figure 2-12(b)].**

2.3.4 Donor BMI and Mitochondrial copy number

Islets aliquots from 39 of the 54 donor pancreases were available for mtDNA copy number quantification. The donor BMI ranged from 21.7-38.1 kg/m^2. No significant correlation was found between the relative mtDNA copy number and donor BMI on univariate analysis (Spearman Rank r^2=0.001, p=0.81) **(Figure 2-13)**. As age has been shown previously to affect the mtDNA copy number [162] the age of the donor was included in a multivariate analysis. Taking this into consideration the r^2 value remained

similar, indicating a lack of correlation between mtDNA copy number and donor BMI independent of age.

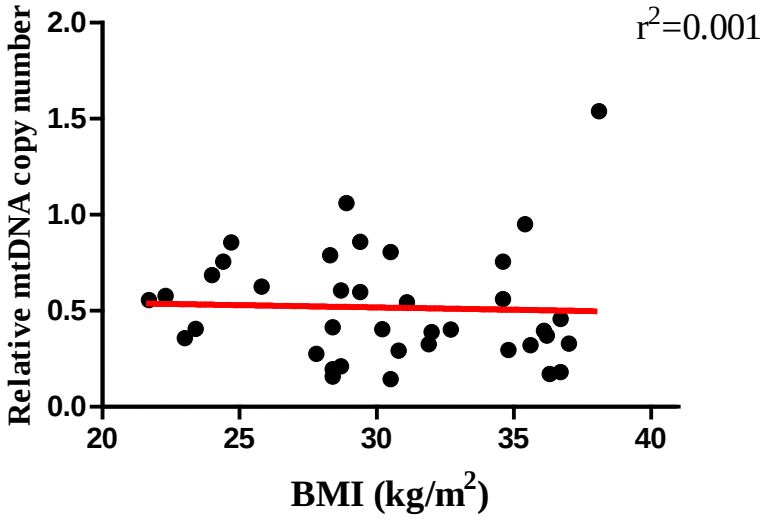


Figure 2-13: Correlation between donor BMI and relative mtDNA copy number.

2.4 DISCUSSION

Studies in humans have used various modes of imaging to estimate pancreatic fat density *in vivo* which has then been correlated with BMI. Pancreatic Magnetic Resonance Imaging (MRI) has shown that mean pancreatic fat content was positively associated with BMI (p= 0.0001) [263] and abdominal computed tomography scanning has shown an increase of 68% in pancreatic fat content in obese patients compared to lean controls [64]. In my study pancreatic biopsies where taken from freshly procured pancreases and immediately fixed in formalin. Morphometric analysis showed an 83% higher mean fat content in the obese group compared to non-obese donors.

Pancreatic TG content in lean and overweight/obese normoglycaemic subjects has been quantified using magnetic resonance spectroscopy (MRS) [264]. Obese donors were found to have a pancreatic TG content 7-fold higher compared to lean subjects. In the current study triglyceride was extracted from hand-picked islets isolated from obese and non-obese donors and was found to be significantly higher in the obese group (~2 fold). The fatty acid composition within the islets was shown to be similar to the surrounding peri-pancreatic fat indicating that the fat infiltrates not only into the pancreas, but also into the islets.

The increase in pancreatic adipocyte content seen in obesity could potentially lead to high local NEFA concentrations around the islets. This combined with the raised intra-islet triglyceride content would raise concerns of potential islet dysfunction. In rodent models, an association has been shown between pancreatic lipid accumulation and deterioration of beta-cell function [8]. In non-diabetic men pancreatic fat content

(measured by MRS) was inversely associated with various aspects of beta-cell function [101] but this was not the case in all studies [263]. It was therefore important to determine if islets isolated from obese donors showed any functional defects. The Stimulation Index in response to a glucose challenge was similar in islets isolated from obese and non-obese donors; this demonstrates that the glucose sensing and secretory apparatus of islets from obese donors remained intact when the islets were extracted and placed in a new environment. Insulin release in basal conditions (1mM glucose) was also similar in both groups, indicating that islets from the obese donors maintain the ability to sense low glucose and proportionately reduce insulin secretion despite the chronic upregulation in insulin secretion. In addition, there was no evidence of a reduction in the islet insulin content implying that the beta-cells are able to upregulate insulin production as well as insulin secretion in response to demand.

For this study islets were in culture for a 20-28 hr period prior to commencing the experiments. One potential criticism of this would be that this culture period could have allowed a chance for islet recovery. A study by Pinnick et al [111] showed that chronic exposure of mouse islets to NEFAs, over a 48hr period *in vitro* had a detrimental effect on insulin secretion. The islets were then cultured for a further 24hrs in media with NEFAs present or absent. The insulin secretion significantly improved in the islets cultured for 24 h in NEFA-'free' media compared to those continuously exposed. For clinical islet transplantation however, the islets are routinely cultured for 24-36 hrs, and thus the *in vitro* culture setting in these experimental conditions reflects that of the clinical situation. As the primary end-point is to determine if islets from obese donors have any functional changes prior to transplantation, a culture time similar to that used for transplantation is appropriate.

Fasting plasma glucagon levels have been shown to be higher in both obese adolescent and adults subjects with normal glucose tolerance (~30% higher in obese subjects) [235, 265]. In obese adolescents, the fasting glucagon level tended to be elevated in association with decreasing insulin sensitivity. Whether this occurs as a result of the alpha-cells developing resistance to a paracrine suppression of insulin or alternatively an intrinsic defect in the function of alpha-cells is unclear. Isolating islets from obese donors to study glucagon secretory responses has not been studied previously. Although the numbers in each group were small, this preliminary data would suggest that the secretion of glucagon in islets isolated from obese subjects is suppressed appropriately in elevated glucose conditions. An intrinsic defect in the alpha-cell function in obese subjects would therefore seem unlikely, but a larger study is required to confirm this. The glucagon content and insulin to glucagon ratio was also similar in both the obese and non-obese group.

Mitochondrial ATP production is required to maintain cell homeostasis and function, and its content in beta-cells can be indicative of secretory capacity [266]. This relationship is supported by previous studies of both whole pancreas and liver transplantation, that have shown that graft function and graft survival correlate significantly with the ATP content of donor tissue [267, 268]. The intra-islet ATP level has also been shown to be an important test that correlates with islet transplant outcome [269]. The ATP/ADP ratio, the mitochondrial membrane potential and *in vitro* glucose-stimulated insulin secretion have also been shown to have an 86% accuracy in predicting in vivo functional potency when combined into a single composite scoring formula [270].

Initially the ATP/ADP ratio was chosen as a marker of mitochondrial function. A luminometric method for determination of ATP and ADP (Luciferin-Luciferase-reaction) was used for these experiments. However, the ADP results were highly variable within batches of islets from the same preparation. Interestingly a recent publication has also found this [246]. ATP content per islet was therefore used as a more reliable indicator. Brandhorst et al [271] have previously commented that they noted a large variability of the intra-islet ATP content of human islets isolated from different donors. In their study, they showed a coefficient of variation (CV) ~ 110% suggesting that the test should be used with caution. Their study however, was in a small group of 10 donors with a large range in age and BMI. The CV values in our study although not ideal, where significantly better ~30% likely as a result of the more stringent inclusion criteria. The time in culture before testing should be similar for all the islet samples as recent evidence has shown that a prolongation of the culture time can have a significant influence on the islet ATP content [266]. Median time of 24 hrs in culture was established to give the islets the opportunity to settle after the isolation procedure and is similar to that used in our clinical transplant regime.

When rotenone was added to the incubation media, the ATP content per islet showed a clear reduction. This demonstrates that ATP measurements were sensitive to changes in mitochondrial activity.

ATP production between the groups was similar. The percentage increase in ATP from basal to stimulated conditions was measured to determine the increase in ATP that occurs in glucose metabolism. The mean increase was 25.25% with no significant difference between the groups. Our data are consistent with measurements

from other groups which have used INS-1 cell lines (~18% rise in ATP content from 6.6 to 12.8mM glucose) [272]. Studies which have measured the ATP:ADP ratio in mouse beta-cells have found a dramatic decrease in free ADP with only modestly increased ATP following glucose metabolism and suggest that ADP changes are in fact the main determinate for changes in the ATP/ADP ratio [237]. It may be therefore that intracellular ATP concentration increases only modestly with elevated glucose concentration in human islets with too little variation to allow detection of any differences between obese and non-obese subjects. Measuring ADP levels by alternative means (e.g. Mass Spectroscopy or High-Performance Liquid Chromatography) would seem prudent in future experiments to identify any changes in the ATP/ADP ratio rather than using ATP levels alone.

On the basis of the proposed "gene dosage" theory where the relationship between mtDNA content in tissues is directly related to its oxidative capacity, it was likely that mtDNA copy number would be increased in obesity as a result of the extra demands placed upon insulin secretion and therefore the mitochondria, in the insulin resistant environment. The results of the current study however do not support this hypothesis and it would seem there is no relationship between the mtDNA copy number and BMI within the beta-cell. There may however be several reasons why the oxidative capacity increases yet the mtDNA content does not change. Cytochrome C Oxidase (COX) is an enzyme (complex IV) that constitutes the last step in the respiratory chain [273]. Previous research has used the level of COX activity as a reference for the oxidative phosphorylation system in mitochondria [248]. Organs which have a high COX level have been shown to also have the highest cellular mtDNA levels. However, when each organ was analyzed separately (heart, skeletal muscle, kidney, brain and

liver), there was no correlation for any of the tissues except for skeletal muscle. The authors proposed that this indicates that in skeletal muscle, the gene dosage must be more relevant in determining the observed COX activity than in the other tissues where other levels of regulation must be playing a more important role. It maybe that the beta-cells are a tissue where the gene dosage is not in direct correlation with the oxidative phosphorylation system and that increases in energy production might be achieved by an overall increase in mitochondrial biogenesis, rather than specific increases in mtDNA copy number. An example of an increase in mitochondrial biogenesis without an increase in mtDNA content is seen in the skeletal muscle of obese subjects who underwent moderate-intensity exercise [251]. After a 4-month period there was a significant increase in oxidative enzyme activity in skeletal muscle yet there was no increase in the muscle mtDNA content. It was proposed that in this situation the mitochondria may adapt by increasing their inner mitochondrial membrane, as an increase in cardiolipin levels (a phospholipid that is unique to the inner mitochondrial membrane) was noted, rather than by increasing the mtDNA content. These studies and others show that mitochondrial activity in a tissue can increase without a proportional increase in mtDNA. Cardiolipin levels were not measured in the current studies but this would be something I would be keen to measure in future work.

2.4.1 Conclusion

Although islets from obese donors are regularly used worldwide for islet transplantation it has been unclear if their function was potentially suboptimal compared to non-obese donors. The experiments in this chapter have demonstrated that the pancreatic adipocyte content and islet TG content is elevated in obese donors indicating increased lipid storage in this environment. Despite these findings the islets isolated from obese

individuals were found to have a similar content and secretory capacity, for both insulin and glucagon to that of non-obese donors. The intra-islet ATP concentration and mtDNA copy number were also similar. The results have also shown that when islets from obese donors are removed from their native environment they are able to adaptively reduce their upregulated basal insulin secretion. These *in vitro* data therefore show that islets from obese donors are not suboptimal compared to those from non-obese donors. It is important however, that these *in vitro* findings are also confirmed *in vivo*. This will be the focus of the next chapter.

CHAPTER 3 CLINICAL OUTCOMES FOR OBESE DONOR ISLET TRANSPLANT RECIPIENTS

3.1 INTRODUCTION

Obesity can create a lipotoxic environment which can potentially impair beta-cell function (see previous chapters). It however, remains unclear whether this impairment is irreversible or not. Experiments *in vitro* argue that the effects may be reversible and that normal function can be restored within a few days [111]. Nevertheless, transplantation of islets from obese donors could potentially be detrimental, as the islets being transplanted could have undergone lipotoxic damage. Determining the *in vivo* function of islets following removal from an obese environment is therefore important.

In vivo islet function can potentially be measured in a number of ways. An indirect way to assess islet function in relation to changes in insulin demand would be to monitor beta-cell function before and after significant weight loss or alternatively a more direct method would be to assess graft outcome following transplantation of islets from an obese donor into a lean recipient. In obesity, significant weight loss improves insulin sensitivity which in turn would be expected to cause a reduction in insulin secretion. Whether this reduction in insulin secretion is appropriate [274] is however debatable and a number of studies have shown a disproportionately lower reduction in insulin secretion in relation to the increased insulin sensitivity following weight loss [117, 118]. A clinically significant consequence of excessive insulin production following gastric banding (GBS) is hyperinsulinaemic hypoglycaemia. Although the incidence is low [275], continuous glucose monitoring system (CGMS) studies have shown that this is more frequent than commonly recognized [276]. Pancreatic

specimens from patients with post-GBS hyperinsulinemic hypoglycaemia have shown no difference in the beta-cell area compared to controls [145]. It has therefore been proposed that the insulin secretory function per beta-cell was inappropriately high in these patients potentially, as a failure to adaptively decrease insulin secretion following surgery in proportion to decreased insulin demand. Although this hypothesis has been refuted by others [276] and it is of course possible that there is something in the environment of the obese individual that continues to stimulate insulin secretion, this however, does raise the question as to whether there is a failure of adaptive change after prolonged oversecretion of insulin.

Comparing transplant outcomes from obese and lean islet donors offers an opportunity to assess beta-cell function more directly. De Freitas Mathias et al [277] isolated islets from adult obese rats and lean controls. Streptozotocin-induced diabetic rats then either received an islet transplant from the obese or the lean rats to evaluate the capacity of islets in regulating blood glucose concentration. The results showed the transplanted obese islets produced a mean fasting insulin concentration which was actually lower than controls; however the difference was not significant. They concluded that the islets from obese rats did not continue to oversecrete insulin suggesting that their function was not permanently altered by the onset of obesity. Matsumoto et al [112] transplanted streptozotocin induced athymic nude mice with a similar islet mass of human islets from either low (<30kg/m^2) or high BMI (>30kg/m^2) donors. They followed the mice for 4 weeks to determine if diabetes could be reversed at which stage the animal underwent nephrectomy of the kidney-bearing graft to demonstrate the return of hyperglycaemia. Their data showed that islet function was

similar in the two groups and that high BMI donor islets could reverse hyperglycaemia effectively in a mouse model.

Data from whole pancreas transplantation has been of little to help to determine if obesity affects beta-cell function in a transplanted graft as the post-operative complication rates are significantly higher and thus confound the data. Transplanting islets from human obese donors into lower BMI T1DM recipients allows the opportunity to assess the *in vivo* secretory potential of these beta-cells in a more appropriate environment than a rodent model can provide.

Aim of this chapter

1. To determine if transplanted islet graft function from obese donors is suboptimal compared to non-obese donors.

3.2 METHODS

The number of patients transplanted in our Oxford cohort during the study period was not large enough to enable statistically robust comparisons to be made between patients transplanted with islets from obese and non-obese donors. Five of the eight patients transplanted during the study period, had a donor BMI over 30kg/m^2. Of the three lower BMI patients, two received a 2nd transplant during the study period so the results from a single graft could not be attained. A collaboration with the University of Alberta, Edmonton was therefore established to enable more robust data to be collected.

The aim of the first set of studies presented below was therefore to assess the obese donor recipients in the Oxford cohort, to determine if significant metabolic benefits could be attained by transplanting islets from obese donors into lower BMI T1DM recipients.

The aim of the second set of studies was to directly compare the graft outcome following transplantation of a marginal islet mass from low vs high BMI donors in a larger cohort of patients. This study was part of a collaboration with the University of Alberta, Edmonton, Canada. As part of this collaboration I spent time in Edmonton and collected and analysed the data for this part of the study.

3.2.1 Islet isolation

For methods, refer to *Chapter 2*.

Following isolation, a small aliquot of islets (100µl) from each of the final purified preparations was used to assess islet yield. Islets were stained with dithizone, counted under a light microscope and expressed as total islet number and islet equivalents (IEQ), that is, islets normalized to a diameter of 150µm. Purity and viability of the samples were also assessed; this was required to be over 50% and 80% respectively before transplantation could take place.

3.2.2 Islet recipients

All islet recipients were T1DM patients (>5 years) who were C-peptide negative (< 20 pmol/L). Patient selection criteria included both male and female patients over the age of 18. All had demonstrated severe recurrent hypoglycaemia with impaired hypoglycaemia awareness. Evidence of compliance with expert medical advice, formal diabetes self-management re-education and intensified insulin therapy which had been optimized at a tertiary referral centre was a pre-requisite to inclusion.

Exclusion criteria included patients with, impaired renal function (isotopic GFR <60 mls/min/1.73m^2 or macroalbuminuria (AER > 300 mg/24hr), malignancy, desire for pregnancy, active infection, unstable heart disease, known portal hypertension, gall stones or liver haemangioma, daily insulin requirements > 0.7units/kg and those with potential for bleeding (coagulopathy, active duodenal ulcer and untreated proliferative retinopathy) .

3.2.3 Islet transplantation

Under local anaesthetic and conscious sedation, using a transhepatic approach, a peripheral portal vein branch was punctured using standard ultrasound guidance. A 4F sheath (11-20cm) is placed into the portal vein. A pigtail catheter is then inserted and a portogram obtained with pump injection of contrast to define the anatomy. Portal venous pressure was measured at base line and was required to be less than 12mmHg. The pre-prepared infusion of islet cells (cells suspended in Dextran 40 (Sigma, UK) in 0.9% sodium chloride and 35 units Heparin per kg body weight – maximum volume of 10ml diluted to a total volume of 100ml) was administered. The portal pressure was repeated intermittently during the transplant and if it exceeded 12mmHg and this was sustained, the infusion was stopped. The islet infusion took approximately 10-15 minutes and was by gravity only. After completion of the transplant the transhepatic tract was embolised using coils. Adjunctive Gelatin-sponge (Gelfoam) particles were used if required. Doppler ultrasonography of the portal vein and liver function tests were performed within 24 hours after transplantation.

Oxford/ Newcastle

All transplants were islet transplant alone (ITA) and were performed in the Oxford Radcliffe Trust, Oxford or the Transplant Institute, Freeman Hospital, Newcastle. Identical protocols were used at both sites. Each islet preparation from a donor was matched to the recipient's blood type and cross-matched for lymphocytotoxic antibodies, but HLA matching was not required.

Immunosuppressive treatments were based on the UK Islet Transplant Consortium agreed protocol. This consisted of Alemtuzumab (Campath, ILEX Pharmaceuticals) [humanized CD-52 monoclonal antibody] induction with Mycophenolate Mofetil (CellCept, Roche) [reversible inhibitor of inosine monophosphate dehydrogenase in purine biosynthesis] and Tacrolimus (Prograf, Astellas) [calcineurin inhibitor] for maintenance therapy. Patients were seen weekly for 1 month after the islet transplant, then fortnightly for a month, then monthly thereafter. I actively reviewed all the Oxford patients.

Edmonton

All transplants were islet transplant alone (ITA) and took place in the Department of Surgery, University of Alberta. Immunosuppressive treatments in all cases consisted of Daclizumab (humanized monoclonal antibody to the alpha subunit of the IL-2 receptor), Sirolimus (mammalian target of rapamycin (mTOR) pathway) and Tacrolimus (calcineurin inhibitor) for maintenance therapy. Patients were seen weekly for 1 month after the islet transplant, then fortnightly for a month, then monthly thereafter. The consulting diabetes physician was part of the Edmonton islet transplant team.

3.2.4 Assessment of Islet Graft Function

Recipient glycaemic control was assessed before transplantation and 3 months after the transplant. All recipients had only undergone one islet transplant during the assessment period.

Oxford/ Newcastle

Metabolic outcomes were assessed using the following parameters: HbA1c, mean glucose over 1 week, time in normoglycaemia monitored by Continuous Glucose Monitoring System (CGMS) ± home glucose downloads (*Appendix 8.3* for method). CGMS has been shown to be a useful tool in the assessment of glycaemic variability before and after transplantation [278]. The number of severe hypoglycaemic episodes was also recorded by the patient. Adverse events were documented. Graft function was assessed by exogenous insulin reduction. Insulin independence was defined as no further requirement for exogenous insulin administration for a duration of >30days, with a fasting capillary glucose level not exceeding 7.0mmol/L more than three times per week; and 2-hr postprandial capillary glucose not exceeding 10.0 mmol/L on more than three occasions in a week. The HbA1c was also required to be <6.5%.

Insulin and C-peptide are secreted in equimolar amounts by the pancreas [279]. C-peptide levels can be a useful in demonstrating endogenous insulin secretion in a subject receiving exogenous insulin. C-peptide levels however, vary according to blood glucose and a random measurement is therefore of little quantitative value. The measurement of C-peptide and glucose in response to a stimulus however, provides a direct measure of beta-cell function [280]. Which stimulus to use is debatable, and a number of tests have been proposed. These can involve a glucose challenge such as the Mixed Meal Tolerance Test (MMTT), Oral Glucose Tolerance Test (OGTT) or Intra Venous Glucose Tolerance Test (IVGTT), or others that avoid a carbohydrate challenge for example the arginine-stimulation test. The benefit of the arginine-stimulation test is that it avoids the hyperglycaemia which can occur following a carbohydrate challenge,

which could be potentially damaging to the graft. An oral carbohydrate challenge however takes into consideration the incretin effect in which the secretion of GIP and GLP-1 occurs, whereas IV tests do not. Given the documented reproducibility of the test, the previous experience of using a MMTT in islet transplant patients worldwide, and the reduced carbohydrate load compared to the OGTT (37grams compared to 75grams), this was selected as the test of choice [280] (*Appendix 8.4* for method).

Assessment of graft function can also be determined using empirical scoring systems. The Beta Score [281] is a previously validated score which provides an integrated measure of beta-cell function and this takes into account fasting glucose, HbA1c, stimulated C-peptide, and absence of insulin or oral hypoglycaemic agent use. The beta-score ranges between 0 (no function) and 8 (perfect function).A potential of two points are awarded for 4 different parameters. (**1**) Fasting glucose; 2 points (≤5.5 mmol/l), 1 point (5.6- 6.9mmol/l) 0 points for (≥7 mmol/l) (**2**) HbA1C; two points (≤6.1%), 1 point (6.2-6.9%), 0 points HbA1C (≥7%) (**3**) Stimulated and/or basal C-peptide; 2 points (>0.3 nmol/l), 1 point (0.1- 0.29nmol/l), 0 points (<0.1nmol/l) (**4**) Insulin requirements and oral hypoglycaemic agents; 2 points (absence of use of both), 1 point (OHA use or insulin requirements 0.01-0.24 units/kg/day, 0 points if insulin requirements ≥0.25 units/kg/day (**Table 3-1**).

Table 3-1: Edmonton Beta score

Components	Score of 2	Score of 1	Score of 0
Fasting plasma glucose (mmol/l)	≤5.5	5.6–6.9	≥7.0
HbA1c (%)	≤6.1	6.2–6.9	≥7.0
Daily insulin (units/kg) or OHA use	nil	0.01–0.24 and/or OHA use	≥0.25
Stimulated C-peptide (nmol/l)	≥0.3	0.1–0.29	<0.1

If fasting C-peptide was ≥0.3nmol/l, then the stimulated C -peptide level was assumed to be ≥0.3nmol/l. N.B If stimulated C-peptide was <0.1nmol/l, then an overall score of 0 was awarded. Adapted from [281]

Graft function was considered optimal when the beta-score was 7 or 8, suboptimal when the beta-score was 4 to 6 or poor when the beta-score was 3 or less. The Secretory Units of Islets in Transplantation (SUIT) index [282] uses the formula 250 x Fasting C-peptide (nmol/l)/ (Fasting blood glucose (mmol/l) -3.43) to provide a score relative to the percentage of beta-cell function of a normal subject younger than 40 years, who would have a SUIT index score of 100.

Edmonton

Metabolic control outcomes were HbA1c and the number of severe hypoglycaemic episodes 3 months after transplant. Graft function was assessed at 3 month using patient exogenous insulin reduction, the beta score described above and the SUIT index. The islet transplants and follow-up care was carried out by the clinical islet transplant team in Edmonton.

Data Analysis

Variables were expressed as mean ± SE and were compared using a paired or unpaired student t-test were appropriate. All calculations were made using GraphPad Prism 5 software. P values <0.05 were considered significant.

3.3 RESULTS

3.3.1 Oxford/ Newcastle: Metabolic outcomes of recipients receiving obese donor islets

The characteristics of the obese donors used for islet transplantation are shown in **Table 3-2**. All donor pancreases had a cold ischaemia time (CIT) of <10hrs (mean 6.8± 0.44hrs), and mean donor age of 45± 4.4y. No donors had a history of diabetes.

Table 3-2: Donor characteristics (Oxford/ Newcastle)

N	5	
Gender (Male: Female)	2: 3	
History of Diabetes	0	
BMI (kg/m^2)	35± 1	(32- 38.1)
Age (years)	45± 4.4	(37- 57)
Heart beating/ Non Heart beating	5/0	
Cause of death		
Intracranial Haemorrhage	2	
Hypoxic Brain Injury	3	
Cold Ischaemia time (hours)	6.8± 0.44	(6- 8)

Data are mean± SE. Range in brackets

A total of five patients (all female) received a single graft from an obese donors (**Table 3-3**). The mean graft size used for transplantation was 6,997± 1544 IEQ/kg.

Table 3-3: Recipient patient characteristics (Oxford/Newcastle)

N	5	
Gender (Male: Female)	0/5	
Age (years)	48.8±5.2	(31-60)
Weight (kg)	59.6±4.7	(50-76)
Duration of Diabetes (years)	32±2.8	(25-39)
Transplanted Islets (IEQ/kg)	6,997± 1,544	(3,358-12,000)

Data are mean± SE. Range in brackets

Metabolic outcomes

All metabolic parameters were significantly improved at 3 months. The mean HbA1c had significantly improved in all cases from a pre-transplant level of 8.2± 0.5% to 5.62± 0.22% ($p<0.05$) (**Figure 3-1a**) and was in the non diabetic range (<6.1%) for 4/5 of the patients. CGMS/ home glucose readings confirmed this reduction in HbA1c was not due to increased time spent in hypoglycaemia. The stability of the glucose control was assessed using a CGMS in four patients and from home glucose devices in one patient. All showed a significant increase in the time spent in normoglycaemia (**Figure 3-1b**) and an improved 7 day mean glucose (<0.05). **Figure 3-2** shows examples of CGMS tracings before and after transplant demonstrating the significant improvement in glycaemic control. During the 3 month period no patients had a severe hypoglycaemic event compared with an average of 5.1 events in the same period pre-transplant (**Figure 3-1c**).

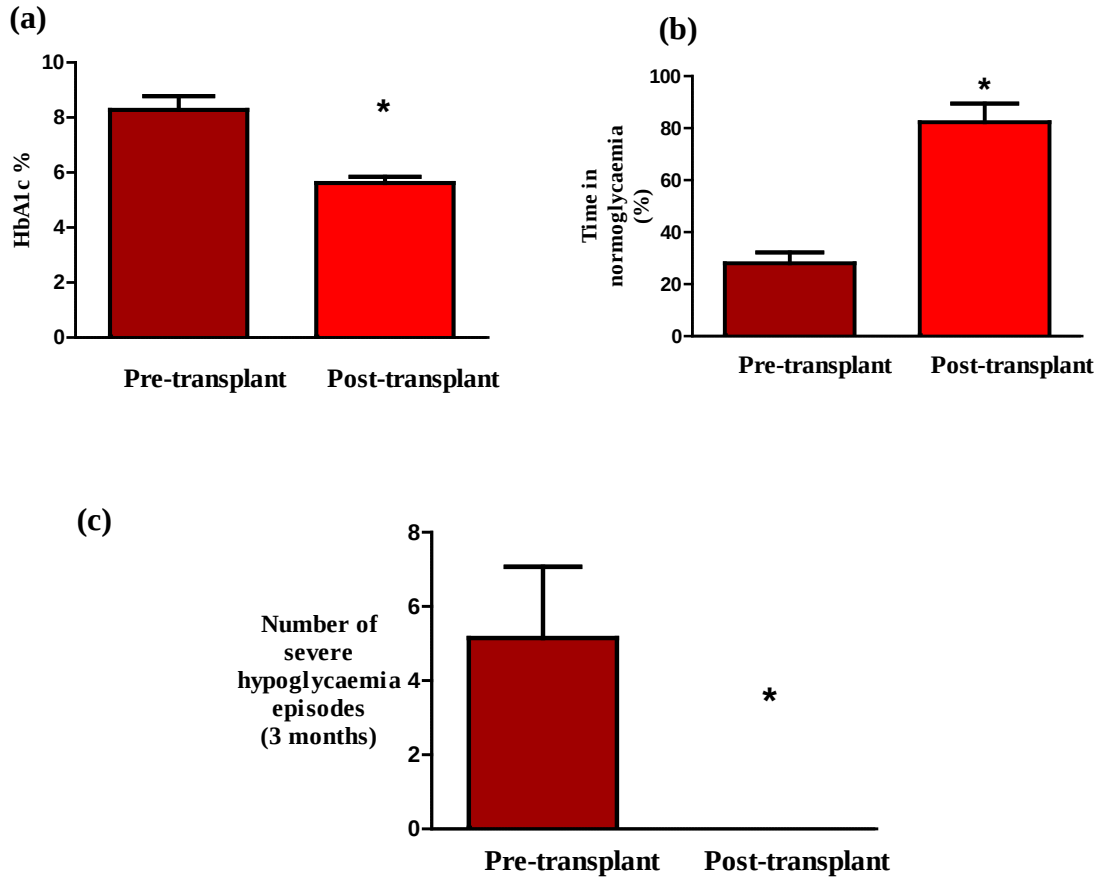


Figure 3-1: Metabolic outcomes pre and 3 months post-transplantation (Oxford).
(a) HbA1c (b) Time spent in normoglycaemia during 7 day CGMS (c) Number of severe hypoglycaemia episodes in a 3 month period

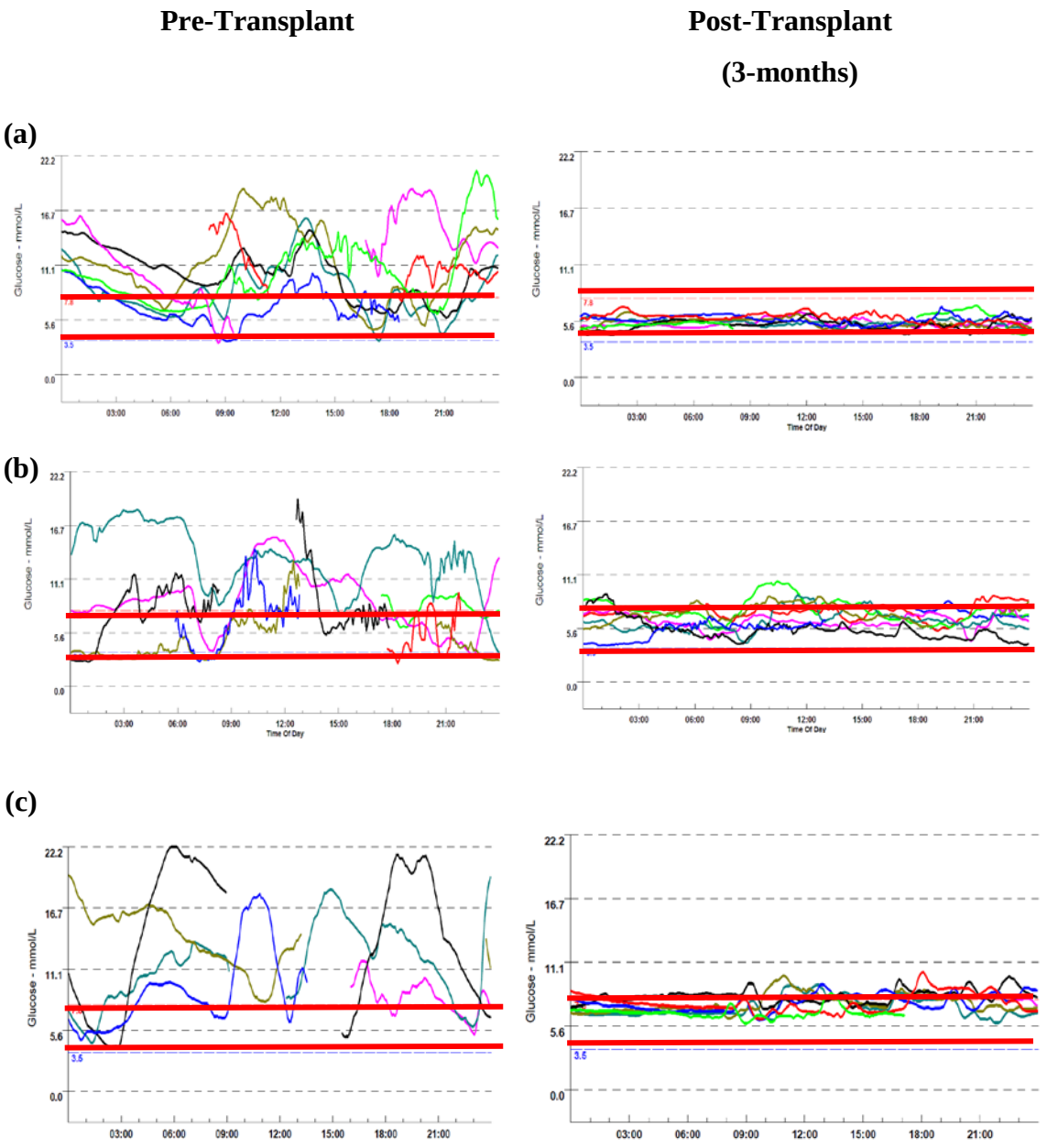


Figure 3-2: Continuous glucose monitoring over a 7 day period pre-transplant and 3 months post-transplant.

Red lines indicate normoglycaemia range. Highlighting the significant improvement in glycaemic control that can be achieved following transplantation with islets from high BMI donors

Graft function

Graft function was achieved in all cases and all grafts were still functional at the time of assessment at 3 months. Exogenous insulin requirements dropped substantially following the transplant from a pre-transplant dose of 0.466± 0.06 to 0.12± 0.04 units/kg/day (p=0.01) (**Figure 3-3a**). Two recipients had become insulin independent following a single transplant.

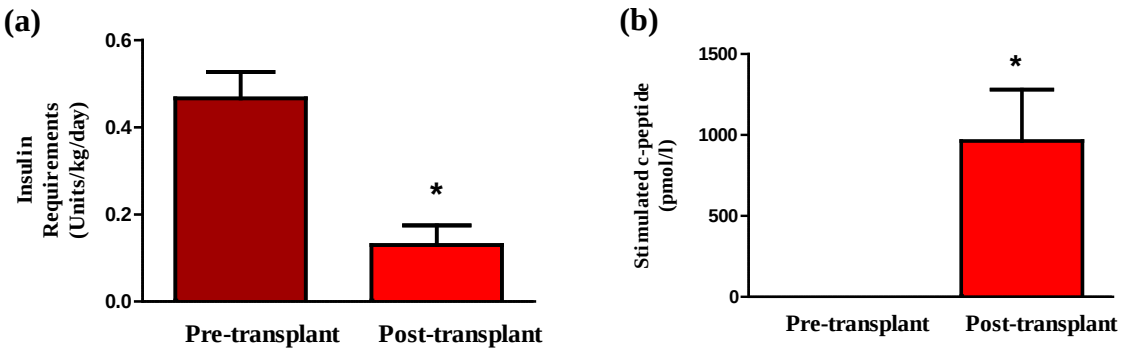


Figure 3-3: (a) Exogenous insulin requirements pre and 3 months post transplant (b) Stimulated C-peptide, undetectable pre-transplant and increased in all cases at 3 months
Data are mean +SE. p<0.05 *

The Mixed Meal Tolerance Test (MMTT) was used to assess the stimulated beta-cell response to meals. A normal response to the MMTT in control subjects shows a stimulated C-peptide level of 1000 to 1500 pmol/l [283]. In all patients there was a large incremental rise in C-peptide at 90mins (**Figure 3-3b**) with 2/5 patients achieving a c-peptide in the normal range following a marginal islet mass transplant.

The Beta Score has been used previously to give an overall measure of clinical outcome. At 3 months the mean Beta Score in the group was 5.6/8 with only 1 recipient

achieving a score ≥ 7 indicating optimal graft function. No patients had a score <3 indicating poor function. The SUIT scores following transplantation reflect the functioning islet mass. The combined mean score was 25.8/ 100 indicating the minimal islet mass achieved following a single islet transplant.

Acute complications

In one case general anaesthesia was required due to excessive pain. A transient elevation in liver transaminases was seen in 4/5 patients which had settled by 3 months. This transient rise in liver enzymes is well documented following islet transplantation [284]. No patients had experienced complications as a result of the immunosuppression.

3.3.2 Edmonton: Metabolic outcomes of recipients receiving islets from non-obese vs obese donors

A total of 35 patients had undergone a single transplant from either an obese donor (>30kg/m^2) (mean BMI 33.4± 0.87kg/m^2) n=16; or a non-obese donor (<28kg/m^2) (mean BMI 23.6± 0.69kg/m^2) n=19 (**Table 3-4** for donor characteristics). The donor age was similar in both groups (46.12± 2.5y vs 43.5± 3.1y (p=0.54)). No donors had a history of diabetes and all pancreases where from heart-beating donors

Table 3-4: Donor characteristics (Edmonton)

	Non-obese	**Obese**	***p value***
n	19	16	
Gender (Male/Female)	7/13	6/10	
BMI (kg/m^2)	23.62±0.69	33.41±0.87	*<0.0001**
Age (years)	43.58±3.1	46.12±2.5	*0.54*
Cold Ischaemia time (hours)	6.76±0.70	5.98±0.74	*0.45*

Data are mean± SE. p<0.05 considered significant *

Both groups of recipients received islets of a similar mass 5,612± 359.8IEQ/kg vs 5,756± 267IEQ/kg (p=0.74) (**Table 3-5** for recipient characteristics). All recipients received the same immunosuppression regime.

Table 3-5: Recipient patient characteristics (Edmonton)

	Non-obese	**Obese**	***p value***
n	19	16	
Gender (Male: Female)	8/ 11	4/ 12	
Age (years)	45.7±2.3	44.2±2.2	*0.65*
Weight (kg)	67.06± 2.7	68.55± 2.7	*0.70*
Insulin (Units/kg/day)	0.58±0.03	0.64±0.05	*0.34*
HbA_{1C} (%)	8.43±0.29	7.86±0.32	*0.14*
Transplanted Islets (IEQ/kg)	5,756±267	5,612±359	*0.74*

Data are mean± SE. $p<0.05$ considered significant *

Glycaemic Control

At 3 months all metabolic parameters were significantly improved in both groups. The mean HbA1c had reduced significantly from 7.86± 0.32 to 6.3± 0.15%; ($p <0.0001$) (Obese) and 8.4± 0.29 to 6.02± 0.17%; ($p <0.0001$) (Non-obese) but the difference between the two groups was not significant (**Figure 3-4**). 43% of the obese group and 47% of the non-obese group had an HbA1c in the non-diabetic range. No patient in either group had a severe hypoglycaemic episode in the 3 months following transplant.

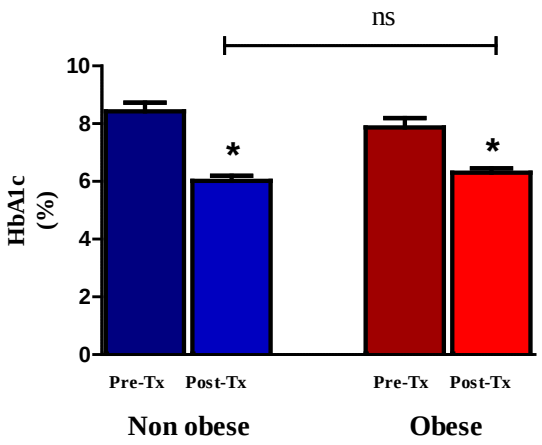


Figure 3-4: HbA1c before and after transplant (Edmonton).
Data are mean+ SE. p<0.05 *

Graft function

All patients achieved primary graft function. Insulin requirements had reduced significantly in both groups (0.64± 0.05U/kg to 0.30± 0.05U/kg (obese donors), (p<0.0001); 0.58± 0.04U/kg to 0.24± 0.05U/kg (non-obese donors); (p<0.0001)) however the difference between the groups was similar (**Figure 3-5a**). Two patients in each group had become insulin independent.

The Beta Score was similar in both groups (mean 4.5± 0.42 (obese) vs 4.63± 0.31 (non-obese) (p=0.80)) (**Figure 3-5b**). Two subjects in the obese group achieved an optimal score of ≥7 and 1in the non-obese group. The SUIT score was highly variable within each group and no significant difference was seen between the groups (40.77± 8.2 (obese) vs 28.79± 4.6 (non-obese); p=0.19) (**Figure 3-5c**).

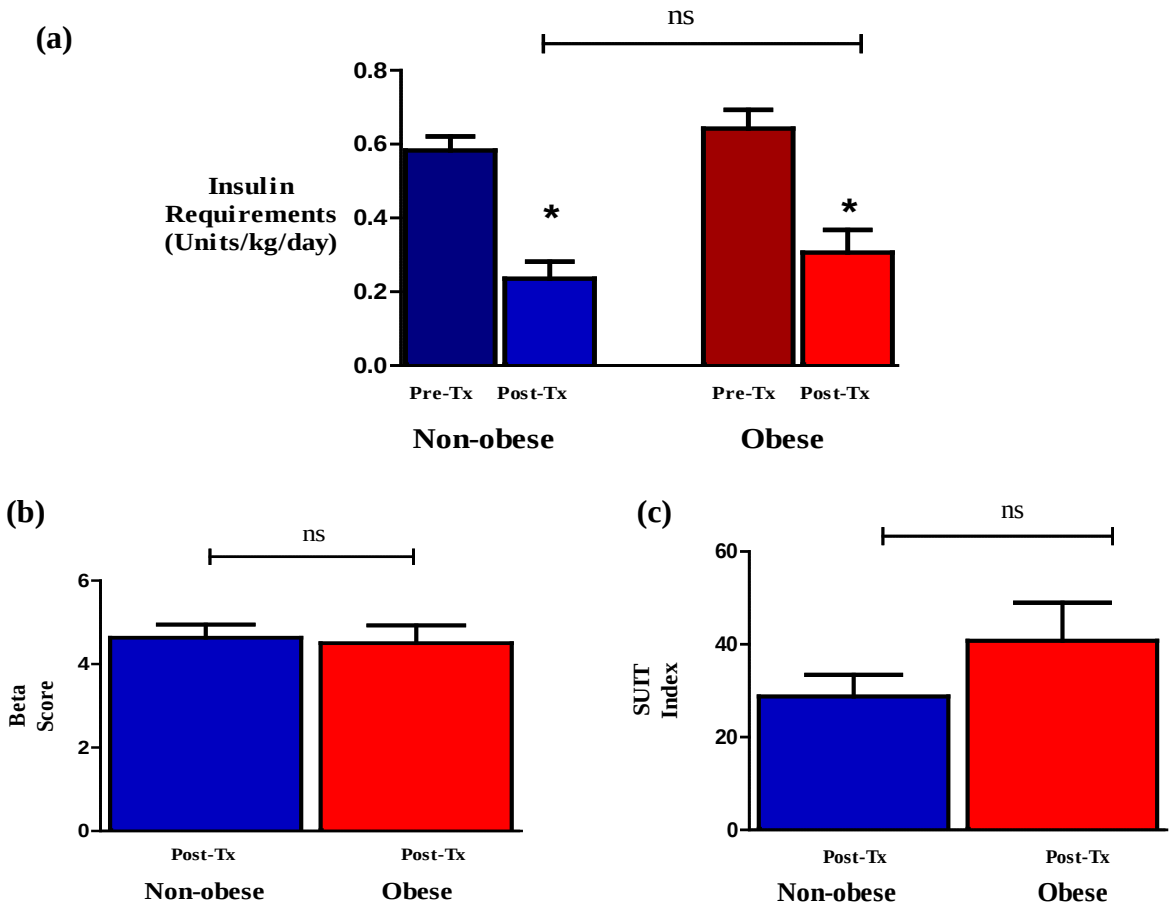


Figure 3-5: Metabolic outcomes pre and 3 months post-islet transplantation (Edmonton). Recipients of islets from non-obese donors (blue bars) or obese donor (red). (a) HbA1c (b) Exogenous insulin requirements(c) Beta score (d) SUIT Index

3.4 DISCUSSION

The results from the first part of this study clearly demonstrate the significant metabolic benefits that can be obtained in T1DM recipients receiving islets from obese donors. In all five cases, the patients' labile diabetes had been resolved and no episodes of severe hypoglycaemia had been reported. This improvement in glycaemic control after a 3-month period is reflected by the significant improvement in HbA1c that was seen in all cases, with 80% of the patients attaining an HbA1c in the non-diabetic range. CGMS recordings and to a lesser degree, regular home glucose monitoring provide a more accurate reflection of glucose stability. CGMS recordings before and 3 months after transplantation were available for 4 of the 5 patients. In these cases after transplantation the percentage of time spent in hypoglycaemia during the 7 day period was <1%, the time in normoglycaemia was significantly increased and a significant improvement in the mean glucose was noted.

A general overall marker of graft function is the reduction in insulin requirements the recipient can attain whilst maintaining a stable HbA1c. In this study all recipients had a significant reduction in their insulin requirement with 2 patients achieving insulin independence. The other three patients have diabetes which is now characterized by stable blood glucose levels, no problems with severe hypoglycaemia, persisting endogenous insulin production and the capability of being readily managed with low-dose insulin. More specific assessment of graft function following a mixed meal tolerance test showed all patients had a large incremental rise in C-peptide at 90mins.

The calculated SUIT score is useful in demonstrating the minimal functional islet mass that is available following a single islet transplant. When shown in conjunction with the metabolic outcome data, it however highlights that a minimal islet mass can still achieve significant metabolic benefits. This low engrafted functioning islet mass is undoubtedly the major factor explaining the fact that reversal of diabetes with islets isolated from a single donor is still relatively uncommon. It has been shown previously [285] that insulin independence tended to be obtained when the SUIT index was more than 28 which it has been proposed may indicate that only 28% of the beta-cell mass of a normal subject is required for insulin independence.

Following the results from the first part of this study the question that then needed to be addressed is whether islets from obese donors could provide a similar, inferior or potentially superior metabolic benefit compared to non-obese donors. The initial objective was to collect data from the other UK centres to help generate a meaningful number of patients in each group to allow comparisons to be made. Within the UK however, each centre was using different islet isolation protocols and immunosuppression regimes, which would have introduced significant variables.

A collaboration was therefore established with the islet transplant group in Edmonton, Canada. Patients were selected who had been transplanted with a single donor with a BMI <28 or >30kg/m^2. The outcomes following transplantation of a marginal islet mass from obese and non-obese donors were therefore directly compared. The results once again showed the significant metabolic benefits that can be attained from islet transplantation. Both groups achieved significant improvements in HbA1c

and also a reduction in insulin requirements. Using these determinants of metabolic outcome there was however, no difference between the groups at 3 months following transplantation. It could be argued that the parameters at this stage are artificially improved as a result of tight clinical management and frequent clinical reviews early after transplantation, rather than more directly due to islet graft function. However this should not account for any differences between the groups as both received follow-up at the same intervals by the same clinical team. Choosing a later time point would have been more preferable, as it has the benefits of assessing graft longevity. However, as the majority of patients received a further transplant the results become confounded. In view of the observation that early islet graft function, as assessed by the Beta Score, was a major determinant of long-term graft function [286] our data are still useful in potentially predicting longer term clinical outcomes. The mean Beta Score was similar in each group, and the proportion of patients achieving a score of ≥ 7, indicating optimal graft function, was not significantly different.

As the outcome markers of graft function are similar between the groups, the function of the beta-cells would seem to be preserved and not compromised as a result of the donor's obese environment. The insulin requirements and rates of insulin independence after the marginal islet mass transplanted were similar in each group, and it would therefore appear that the beta-cells do not continue to hypersecrete insulin and can adaptively reduce their insulin secretion. A final concern would be that other variables could account for the lack of observed differences seen between the groups. When the donor characteristics were compared both groups had a similar age, CIT, islet transplant mass and immunosuppressive regime. All donors were heart beating donors

and the isolations and transplants were all performed in the same centre by the same isolation and transplant teams using the same protocols. The variability of these parameters was thus kept to a minimum to avoid confounding the data and the only significant difference between the groups was BMI.

3.4.1 Conclusion

These clinical studies show that islets transplanted from obese donors can be successfully used to improve metabolic function in unstable T1DM recipients. In addition, this study shows that when short-term metabolic outcomes between obese and non-obese donors are compared, similar outcomes can be achieved.

The finding from this chapter and the previous chapter therefore support the use of these donors for islet transplantation. Next, I was keen to determine the reasons behind the increased islet yield (IEQ) observed in high BMI donors.

CHAPTER 4 MORPHOLOGICAL STUDIES ON THE EFFECT OF OBESITY ON ISLET STRUCTURE AND ISOLATION YIELDS

4.1 INTRODUCTION

High donor BMI has been demonstrated to have a positive correlation with islet yields when expressed as Islet Equivalents (IEQ) [122-124, 148], and also to be a predictor of successful islet isolation outcomes (islet yield≥250,000 IEQ) [124]. The reason for this relationship is at present unclear, but there are two possible explanations: First, that pancreatic tissue from obese donors contains an increased islet mass- either in terms of an increase in total islet number or an increase in islet size; or second, islet mass is the same, but the ultra-structure of obese pancreases, facilitates the increased release of islets during the islet isolation.

Although studies using CT imaging of the pancreas have suggested a strong positive correlation between total pancreatic volume and increasing BMI [64], it cannot be presumed that islet volume also increases to the same extent. An increase in islet number within the pancreas of obese compared with lean rodents, has been shown [287]. In addition, some researchers have demonstrated an increase in islet size [126]. However, the situation in humans has been less convincing. One of the first studies to assess islet number and density within human pancreatic autopsy specimens, showed a ~50% higher beta-cell mass in very obese (mean BMI~35kg/m^2) compared to lean (mean BMI~22 kg/m^2) non-diabetic subjects [136]. However, there was no difference in mean islet size, or islet density between the two groups. A more recent study of pancreas post-mortem specimens [137] however, has suggested that the increase in beta-cell mass in moderately obese subjects (mean BMI~30 kg/m^2) was only in the region of ~20% higher. Suggested mechanisms behind this expansion of beta-cell mass have been 1) hypertrophy of individual differentiated beta-cells cells [140]; 2) replication of pre-

existing differentiated beta-cells [288]; or 3) neogenesis from stem / progenitor cells thought to be located in the pancreatic ducts [143]. The ability of human beta-cells to replicate or develop from neogenesis in adulthood has however been questioned [146, 147] and where an increase in replication has been demonstrated, the amount has been relatively minimal [289].

The studies in this chapter investigate the cause of the higher donor yields (IEQ) seen in obese donors.

Aims of this chapter

1. To determine if pancreatic weight is directly correlated with BMI.

2. To histologically analyse pancreatic biopsy specimens (taken from a proportion of the donor pancreases prior enzymatic digestion) to determine islet size and density in relation to the exocrine parenchymal tissue and BMI.

3. To examine the relationship between isolation yields, both in terms of the total isolated islet number and the islet size distribution of the purified islets, with donor BMI.

4.2 METHODS

Pancreases which met the agreed 2008 UK Islet Transplant Consortium (UKITC) Inclusion Criteria were used for the isolation outcome studies (see **Table 2-1**). A total of 75 pancreases were analysed.

4.2.1 Pancreatic weight

Pancreases were retrieved as detailed in *Chapter 2*. On arrival at the Oxford Islet Isolation Facility, the duodenum and spleen were first removed and the peri-pancreatic fat was trimmed without damaging the pancreatic capsule. The trimmed pancreas was then weighed before collagenase perfusion. The donor characteristics are shown below **(Table 4-1)**.

Table 4-1: Donor characteristics for pancreatic weight relationship. Median (range).

n	**75**
BMI (kg/m^2)	**28.9 (20- 38.1)**
Body Weight (kg)	**85.0 (54- 120)**
Age (years)	**48 (21- 60)**
Gender (M:F)	**44: 31**
Pancreatic weight (grams)	**115 (66-199)**

4.2.2 Pancreas and islet morphometry

Islet size has been shown to be similar in all parts of the pancreas originating from the dorsal primordial bud (body and tail) [192], with a slightly higher density in the tail [137]. A 1cm^3 biopsy was taken from the body of the pancreas in a subgroup of the 75

pancreas (n=20 pancreases). Histological identification of islets by immunohistochemistry was performed as described in *Chapter 2*. Donor characteristics for the non-obese and obese groups are shown in (**Table 4-2**).

Table 4-2: Donors Characteristics for histology samples.
Mean ±SEM; range shown in brackets

	Non-obese	**Obese**	**p- value**
n	**10**	**10**	
BMI (kg/m^2)	**22.5 ±0.4 (20- 24.2)**	**35.1± 0.7 (31.9- 37)**	**< 0.0001****
Age (years)	**57.8 ±2.3**	**50.8 ±2.6**	**ns**
Gender (M: F)	**7: 3**	**7: 3**	

For morphometric analyses, one labelled section from each donor was used, and sequential fields of the specimens were photographed at 100x magnification. The median total parenchymal area (A_p) examined was 20mm^2 (range= 11.7-50 mm^2) per donor. Ductal tissue, interlobular tissue, localised fibrotic areas and areas of nodular pancreatitis were excluded. Only islets in the exocrine parenchyma were included in the morphometry. The individual islets area was measured for a median of 67 islets (range= 48-102) per donor. AxioVision 3.1 imaging software (Carl Zeiss, Feldbach, Switzerland) was used for quantification. Total adipocyte area within the exocrine parenchyma was also recorded (A_a). (**Figure 4-1**)

As infiltrating pancreatic fat has been shown not to replace the parenchymal area, the islet numerical density (ρ_N), relative to the parenchyma tissue, was calculated using **Equation 1i**. The percentage of the total islet area (A_i) relative to the parenchymal density was also calculated (**Equation 1ii)**. The median islet size was also determined for each preparation.

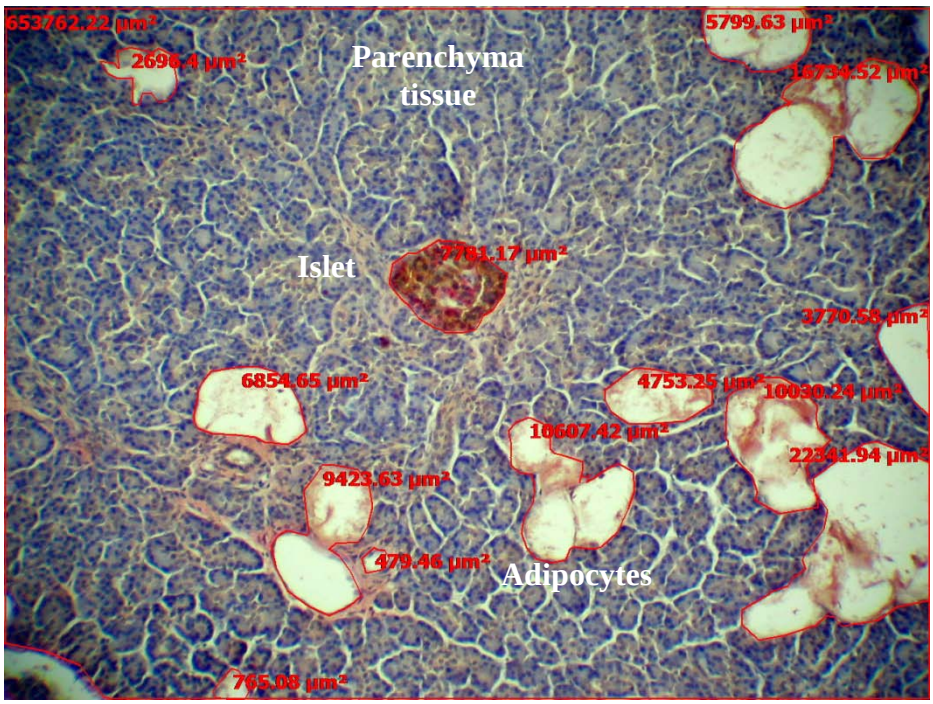


Figure 4-1: Histological quantification.
Parenchyma tissue (blue), islet (brown/red), adipocytes (white). Red lines drawn around region of interest. Area shown in µm^2.

Equation 1: Islet numerical and area density calculation

(i) Islet numerical density (ρ_N) = $N/(A_p-A_a)$

(ii) Percentage of islet area = $A_i/(A_p-A_a)$ *100

where A_i, A_p, A_a and N denote islet area, exocrine parenchymal areas, adipocyte area and islet number, respectively

4.2.3 Assessment of isolated islet yield and islet size

4.2.3.1 Islet Yield

Human Islets were isolated as described in *Chapter 2.*

The final purified islet preparation was agitated to ensure islet re-suspension then a 1ml sample was collected aseptically using a fixed 1ml pipette and transferred to a 15ml centrifuge tube. Nine millilitres of CMRL media was added to the 1ml aliquot. Five drops of dithizone (DTZ) solution (Sigma, UK) was added to the islets. DTZ binds to zinc ions within beta-cells (insulin is stored as Zn_2-$insulin_6$ complex within the secretory granules) and thereby selectively stains all the islets red to facilitate counting. The islets were allowed to stain for 2-3 minutes (**Figure 4-2)**, before being re-suspended, and 4 x 100µl samples of the 10ml suspension collected and streaked onto a 150mm Petri dish. Islets were counted at 20x magnification with a measurement graticule in the eyepiece. The number of islets was counted, and the diameter of each islet was measured. Calculation of the total islet number in the preparation was then calculated using the number counted, the dilution of the sample and total volume of the pooled purified fractions. Using a recognised scoring system [120], a total Islet Equivalent (IEQ) score which takes into account both the islet number and islet size was calculated for each preparation **(Table: 4-3**). A percentage of the purity (for example, equal volume of islets and acinar tissue count as 50% purity) and viability (using fluorescein diacetate/ethidium bromide) were also estimated (see Chapter 2 for methods).

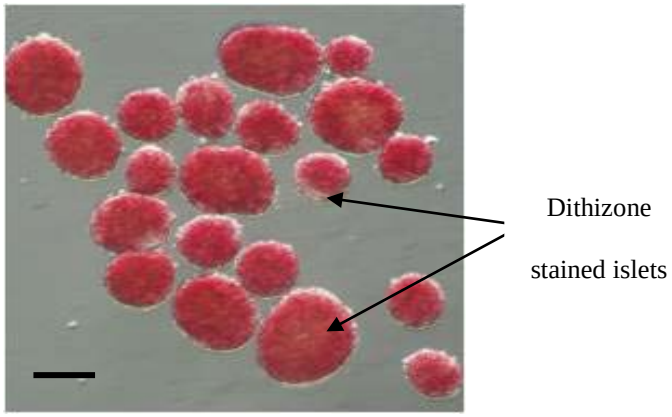


Figure 4-2: Dithizone staining of an islet aliquot from the purified digest.
Dithizone binds to the zinc in the beta-cells staining the islets red. Scale bar represents 50µm

Table: 4-3: IEQ quantification.
A 400µl sample was collected from a purified islet preparation. Below is a worked example to demonstrate how IEQ is calculated.

Islet diameter (µm)	**<50**	**50-100**	**100-150**	**150-200**	**200-250**	**250-300**	**300-350**	**350-400**
Mean volume (μm^2)	**0**	**294,525**	**1,145,373**	**2,977,968**	**6,185,010**	**11,159,198**	**18,293,231**	**27,979,808**
Number of islets (n)		**28**	**18**	**11**	**12**	**10**	**0**	**6**
Islet Equivalent number (IEQ)	**0**	**x0.16 6**	**x0.65**	**x1.7**	**x3.5**	**x6.3**	**x10.4**	**x15.8**
Total IEQ per category	**0**	**4.6**	**11.7**	**18.7**	**42**	**63**	**0**	**94.8**

Total number of islets= 85 *dilution factor (x1600)=136,000 islets
Total IEQ= 234.8 * dilution factor (x1600)= 375,680 IEQ

Worked example showing the impact of larger diameter islets on the IEQ

IEQ: conversion factor which approximates the whole islet sample as if all islets were 150µm diameter. Adapted from [290]

4.2.3.2 Isolated islet size measurement

An aliquot of each preparation was removed from the final purified digest which contained > 50 islets. These were stained with DTZ (as described previously), individual islet size was measured using an eyepiece graticule. As islets with a diameter of <50µm are difficult to distinguish from islet fragments, only islets >50µm were included in the analysis. Each islet measurement was stratified into either 50- 150µm (small); or >150µm (large). Preparations were defined as coming from non-obese (donor BMI (<28kg/m^2) or obese donors (donor BMI >30kg/m^2).

4.2.4 Data Analysis

Variables were expressed as mean ± SE or median with range. Results were compared using a unpaired Student t-test or Mann Whitney's test were appropriate. A p-value <0.05 was considered statistically significant. For continuous variables a Spearman rank correlation was used. Kolmogorov-Smirnov test was used to compare cumulative frequency. A p-value <0.05 indicates a significant correlation. All calculations were made using GraphPad Prism 5 software.

4.3 RESULTS

4.3.1 Donor BMI and pancreatic weight

To identify factors which were associated with pancreatic weight, linear regression analysis between continuous variables and pancreatic weight was performed. There was a moderate positive correlation between pancreatic weight and BMI (r^2=0.105; p=0.004)) (**Figure 4-3**). However, the strongest correlation was noted between donor body weight and pancreatic weight (r^2=0.29; p<0.0001) (**Figure 4-4**). No correlation was seen with donor age (r^2=0.001; p=0.99).

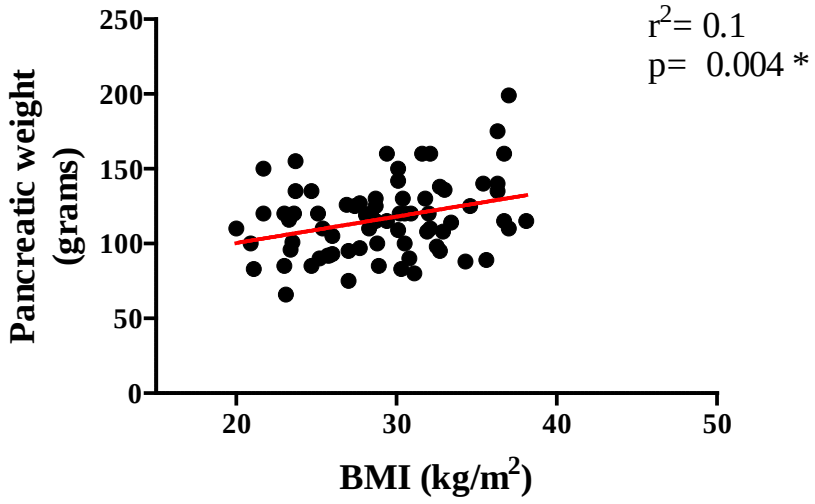


Figure 4-3: Positive correlation between donor BMI and pancreatic weight.

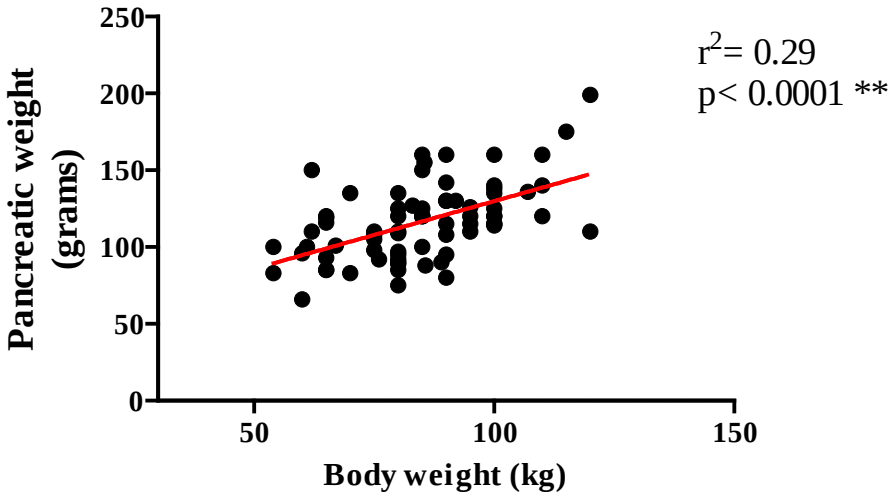


Figure 4-4: Positive correlation between donor body weight and pancreatic weight

4.3.2 Donor BMI and islet size / density from pancreatic biopsy specimens

The median islet size was similar in the two groups (4056 μm^2 (614- 44093) (non-obese) vs 4117 μm^2 (382- 63574) (obese) (**Figure 4-5**). Cumulative frequency distribution of the islet area was also found to be similar between the groups (**Figure 4-6**).

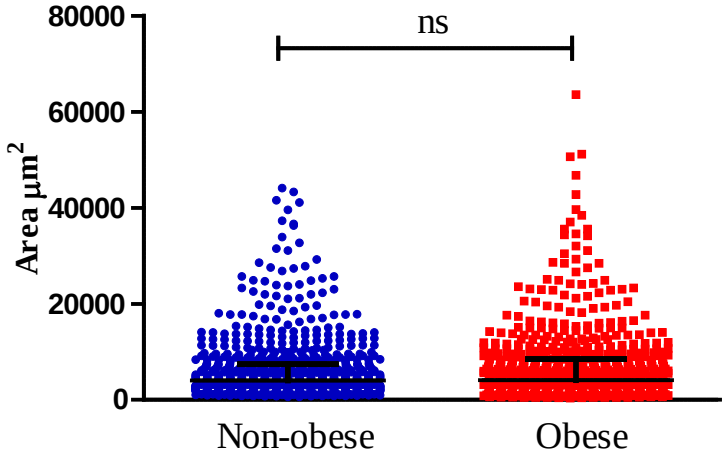


Figure 4-5: Size distributions for islets within pancreatic tissue specimens from non-obese and obese donors.
Combined data from 10 donors in each group. Black bar represents median with IQR

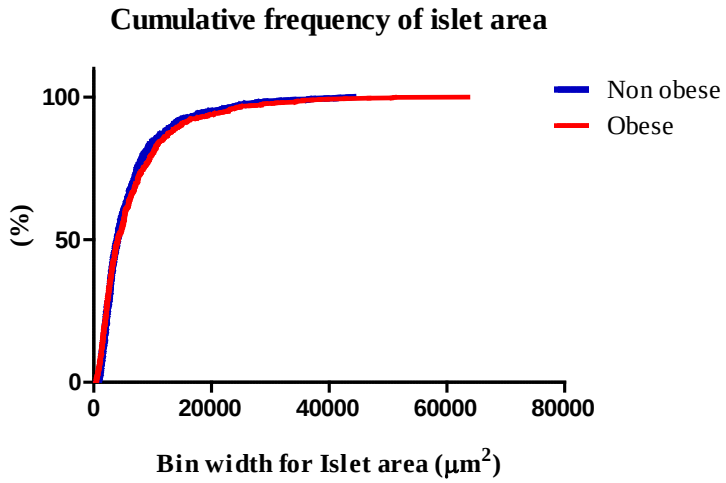


Figure 4-6: Cumulative frequency distribution of islet area.
Non-obese donors (blue), obese donors (red). p=0.37

Both the islet numerical density and the percentage islet area relative to the total parenchymal tissue was similar in the two groups (**Figure 4-7 (a), (b)**).

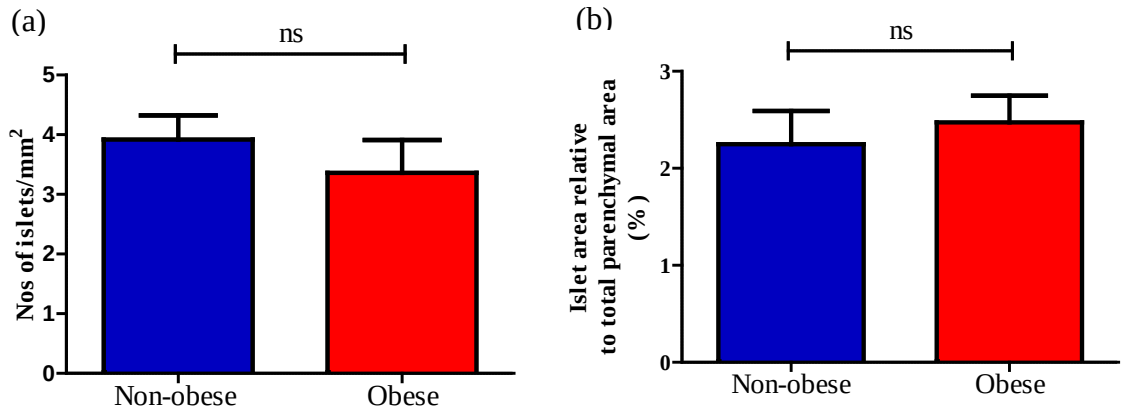


Figure 4-7: Numerical islet density (Number of islets per mm^2 of parenchymal tissue) (a) and percentage islet area relative to total parenchymal tissue (b); measured from histology specimens. Non- obese donors (blue), obese donors (red)

4.3.3 Donor BMI and Islet Isolation outcomes

4.3.3.1 Islet Yields

Of the 75 pancreases which were processed, 11 preparations were not purified into islet fractions since the pre-purification counts indicated a non-transplantable yield (digest count <250,000IEQ) and these pancreases also did not have specific research consent to proceed. Twenty-two preparations failed to purify adequately and were also excluded from the study. Forty-two pancreases gave a purified islet yields of >50,000 IEQ, with a purity of >50% and a viability >80%. All these were included for analysis. There was a positive correlation of donor BMI with islet yield (IEQ) (r^2=0.09; p=0.049) (**Figure 4-8**). However, this was not statistically significant when islet yield was measured as islet number (r^2= 0.01; p=0.57).

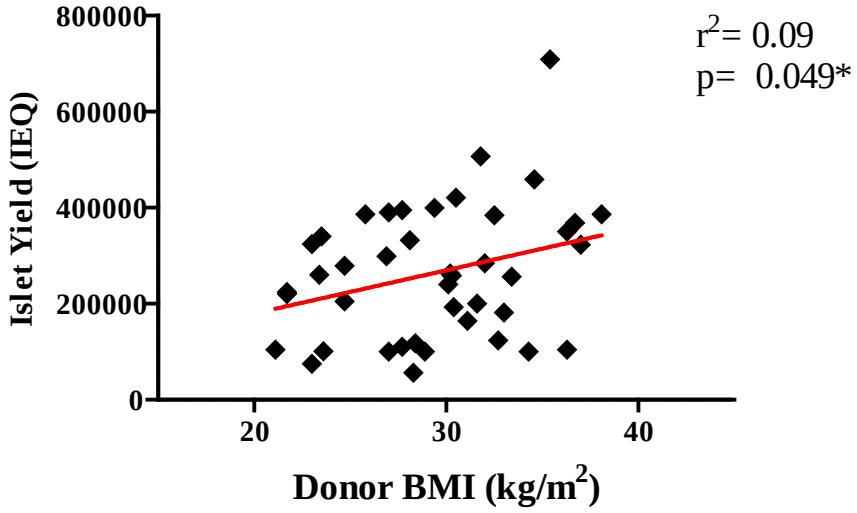


Figure 4-8: Positive correlation between islet yield (IEQ) and donor BMI

4.3.3.2 Donor BMI and isolated islet size distribution

The relative proportions of small (50-150µm in diameter) and large (>150µm) islets were calculated for each preparation. Donor mean BMI 24.5± 0.6 kg/m^2 (21.1-27.7) (non-obese) vs 33.3± 0.5 kg/m^2 (30.1- 38.1) (obese). No significant difference in age or CIT between the groups. The proportion of larger sized islets (150µm) was greater in the group with higher BMI (34.8± 2.4% (obese) vs 26.8± 3.1% (non-obese) $p<0.05$) (see **Figure 4-9**).

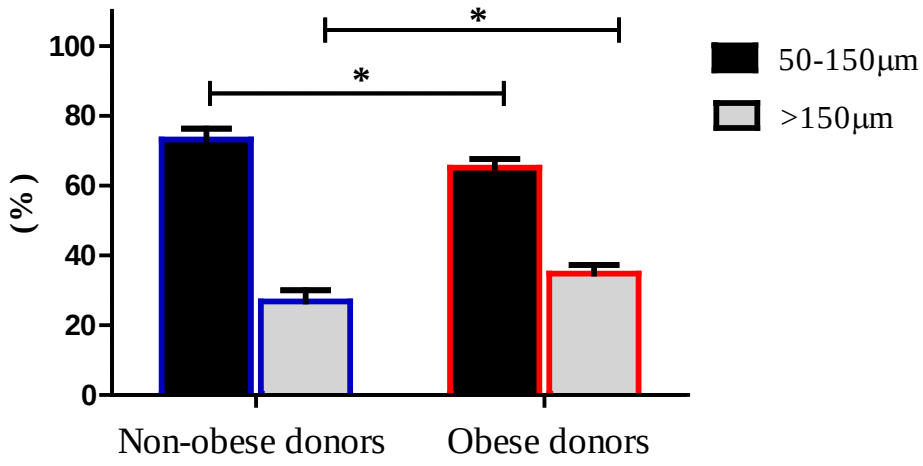


Figure 4-9: Size distributions (50-150µm; >150µm) for isolated islets from non-obese (blue border) and obese donors (red border).
Black bar represents the mean % of the total islets counted with a diameter of 50-150µm, gray bar represents mean % of islets >150µm in diameter. p<0.05 *

4.4 DISCUSSION

The weight of autopsy specimens from the liver, spleen and kidneys have been shown to be positively correlated with donor BMI [291]. A positive correlation has been found between pancreas volume (measured by CT imaging) and subject BMI [64]. However, the correlation with the weight of autopsy pancreatic specimens and donor BMI, although strong in some studies [137], has been less conclusive in others [291]. There are however, limitations to using hospital autopsy records, especially with regards to pancreatic specimens. If there is a prolonged period prior to pancreas procurement, which is often the case with autopsy specimens, the pancreas becomes prone to autolysis and oedema. Both of these factors are likely to cause bias in the assessment of pancreatic weight. Only one previous study has compared donor BMI and pancreas weight using cadaveric donors [292]. This also showed a positive correlation, the findings being more pronounced in male donors. In the present study, using freshly procured pancreases from cadaveric donors, a significant positive correlation was found between donor BMI and pancreas weight. Donor body weight however, was found to have the strongest correlation. This correlation was independent of donor gender or age. Pancreas volume has previously been shown to plateau after the age of 20 with little change in adulthood [64], and our study was in agreement with this finding. The first part of this study therefore, confirms that donor BMI is indeed associated with an increase in pancreatic weight, indicating that obese donors tended to have larger pancreases. The increase in pancreatic weight may be due to increased parenchymal mass and/or an increased fat mass. In the study of Saisho et al [64], parenchymal volume measured by CT was increased by only 13% in the obese group (mean BMI 33.6 kg/m^2 ±3.4) compared with the lean group (mean BMI 22.1 kg/m^2 ± 1.8) however,

pancreatic fat volume was 68% greater in the obese groups compared with the lean group. Previous morphological studies of autopsy specimens have shown no increase in pancreatic exocrine parenchyma with increasing subject body weight and the authors proposed that any potential increase in pancreatic weight which occurs, is related to an increase in adipose tissue content [258].

Donor BMI was found to be positively correlated with islet yield after collagenase digestion. This is also in agreement with previous studies [123]. In the present study however, the selection of pancreases was within relatively tight guidelines which meant that other factors potentially affecting outcome were kept to a minimum (eg. variation in cold ischaemia time). The limitations however, are that donors with very high BMI (>40kg/m^2) were excluded, as they do not fulfil the current clinical acceptance criteria, and donors with very low BMI (<20kg/m^2) were not available as these pancreases are routinely offered to the whole pancreas transplant programme in the UK. Final isolation yields were not available for all cases since a number of pancreases were excluded. Retrospective analysis showed that the proportion of pancreases from obese and non-obese donors that failed to produce adequate purified islet fractions was however similar.

An increase in the mean ratio of IEQ/ islet count in obese vs non-obese donors has been used previously to suggest that obese donors may contain a higher proportion of larger islets [112]. This has led to the assumption that pancreases from obese donors contain a higher proportion of larger islets. To assess this in more detail the size of individual islets, isolated from obese and non-obese donors was stratified, as either small (50-100µm) or large (>150µm). This showed a significant increase in the number

of large islets isolated from obese, as opposed to non-obese donors. Pancreatic histological sections taken from obese and non-obese donors, however, showed that when all islets were measured there was no difference in the median islet area or the cumulative frequency between the groups. These results suggest that there is little difference in the islet size distribution within the pancreas of obese and non-obese donors. The differences between the isolation and the histological data imply that it may be easier to liberate larger islets from obese donors or alternatively that there is a reduction in the incidence of digestion related fragmentation in larger islets from obese donors. One hypothesis would be that the pattern of collagenase distribution to the islet-exocrine interface is affected by the increased content of adipocytes in obese donors and hence intact larger islets may be easier to liberate. Indeed, previous reports have shown that pancreatic interstitial fat content may be a critical determinant for isolation success [149]. Previous studies have demonstrated that the mean digestion period for effective islet isolation is significantly shorter for pancreases from high BMI (>30kg/m^2) compared to lower BMI donors (<30kg/m^2) [112]. As collagenase has been shown to penetrate inside the islets after distension [293], this reduction in digestion time could result in larger islets being less susceptible to fragmentation, hence a larger proportion are present in the final digest. One final possibility is whether the obese donor islets are larger because of cell swelling secondary to injury. If this was the case however, one would expect the function of obese donor islets to be impaired, and the studies described in the earlier chapters of this thesis have shown this not to be the case.

In theory, it is beneficial for islet transplantation that obese donors provide a higher proportion of larger isolated islets, as this will increase the IEQ and thus help attain yields deemed suitable for transplantation. There may however, be potential

problems with using preparations with high proportions of larger islets. After the isolation process, islets are no longer attached to the vascular system until revascularization takes place. Rodent models suggest it can take 7–10 days following transplantation to re-establish a vascular supply [294]. During the initial period in culture and the days following the transplant islet metabolism therefore depends solely on oxygen diffusion. Even after full revascularization transplanted rodent islets have been found to have a pO_2 level of only 3–8 mmHg, independent of the implantation site [295]. It has been proposed that oxygen diffusion to the centre of a larger islet may be inferior to that of a smaller islet resulting in central necrosis. It is for these reasons that studies have investigated whether the size of isolated islets may be a key factor in determining human islet transplantation outcome [261]. Initially demonstrated in rodent [260], and later in human islets [261], small islets have been shown to be superior to large islets with regard to *in vitro* insulin secretion and show a higher survival rate during both normoxic and hypoxic culture [261]. The same study also showed that in human islet transplantation, the total islet number rather than total islet volume, proved to be more reliable to predict stimulated C-peptide response in the recipient.

4.4.1 Conclusion

These data confirm previous findings that a positive correlation exists between islet yield and BMI. This study has in addition, demonstrated that the reason for this increased yield is a result of increased total islet volume as opposed to total increased islet number. Histological specimens however infer that islet size distribution is similar within the pancreases of obese and non-obese subjects and it would appear the increase

in the proportion of larger islets seen in the isolate from obese donors is a result of the isolation process rather than a true morphological variation in islet size.

Given the literature mentioned above which suggests that small islets may be superior in function, the finding that obese donor islet preparations consist of high proportions of large islets may raise concerns as to whether these donors are optimal for clinical transplantation. *Chapter 3* has shown that comparable short-term graft outcomes (at 3 months) were achieved, when transplanting islets from obese and non-obese donors. Whilst this is reassuring, this does highlight the need for long-term monitoring of graft function in these recipients.

The last three chapters have confirmed that obese donors can provide a source of suitable islets for clinical islet transplantation. The next chapter addresses the question of whether islets from donors with early T2DM might also be suitable.

CHAPTER 5 ISLET FUNCTION AND MORPHOLOGY IN TYPE-2 DIABETES

5.1 INTRODUCTION

In an attempt to expand the donor pool for clinical islet transplantation, it has been suggested that donors in the early stages of T2DM, may provide a potential source of islets [173]. This may seem paradoxical; however, the islet function in the early stages of the disease process is unclear.

Insulin secretion defects in T2DM have been demonstrated *in vivo* and several alterations have been noted. These include a reduced or absent first-phase insulin response to glucose [183], alteration in the insulin secretion patterns after ingestion of a meal [296] and changes in basal oscillations of secretion [297, 298]. Data from T2DM patients, who have undergone malabsorptive bariatric surgery, have however shown that one month after surgery there was a significant improvement in glycaemic control, that the first phase insulin secretion was restored and the beta-cell glucose sensitivity had fully normalized [176]. Large islet yields (>400,000IEQ) have also been possible from T2DM donors in the early stages of the disease [173, 179].

Only limited data is available on the sub-cellular function of islets isolated from patients with T2DM. The first of these studies was performed in 1994 on islets isolated from two T2DM donors aiming to assess insulin secretion during glucose stimulation [299]. This showed a marked decrease in insulin secretion compared to controls. Two other studies using isolated islets from the pancreases of eight [179] and thirteen [300] T2DM donors have since been published, which have shown similar findings. In addition, the ability of islets, isolated from T2DM donors, to restore normoglycaemia in

immunodeficient diabetic mice following transplantation was reduced, compared to islets from non-diabetic subjects [179]. In the majority of these studies however, the T2DM donors had long-standing diabetes or were on insulin therapy.

As T2DM is a complex disorder involving islet dysfunction, feedback loop dysregulation and changes in peripheral tissue sensitivity to insulin [301], clinical studies in T2DM patients make use of modelling systems (e.g. HOMA-B [301] and the disposition index [302]) to help determine abnormalities in islet function. The ability to isolate human islets from the native pancreas however, can provide a direct insight into any alpha and beta-cell defects that may be present.

T2DM is considered a bihormonal disorder involving impaired secretion of both insulin and glucagon. A number of clinical studies have shown an impaired suppression of the glucagon response following a meal [186] and in addition, elevated fasting glucagon levels have been noted in these patients [303]. This hyperglucagonaemia potentially contributes to elevated blood glucose levels compounding the already problematic hyperglycaemia caused by a relative insulin deficiency. In addition an impaired glucagon response to hypoglycaemia has been noted in both T1DM [304] and T2DM [305] representing a limiting factor for insulin treatment in both conditions.

To date only one study has tried to assess the glucagon secretion from isolated human islets [179]. In this study a perifusion secretion method was used, but found neither the control nor the diabetic islets demonstrated evidence of glucagon inhibition

after a glucose challenge. As the glucagon secretion in the control islets was not suppressed by elevated glucose, it is difficult to interrupt the results.

Amyloid deposition in pancreatic islets is a common pathological feature of T2DM, and found in at least one islet at post-mortem in more than 90% of T2DM subjects [192, 193]. As amyloid deposits are cytotoxic and are associated with decreased islet beta-cell mass, the deposits will thereby reduce the capacity for insulin release. It has been proposed that amyloid may play a role in the insufficient insulin output observed in T2DM [198]. Although amyloid deposition is unrelated to the duration of diabetes, the extent to which it can affect the pancreatic islet function in the early stages of the disease is unclear [195].

There is a significant need to increase the pancreas donor pool, however, the factors mentioned above, highlight that further studies are required to determine if islets from T2DM donors are suitable for clinical islet transplantation.

Aims of this Chapter

1. To isolate islets from donors known to have T2DM and from age-matched non-diabetic (ND) controls, to determine insulin and glucagon content, secretory response to low and high glucose and depolarization-induced alpha- and beta-cell exocytosis.

2. To examine histological specimens from T2DM donors, diagnosed <5 years, to determine if amyloid deposition can be extensive early in the disease process.

5.2 METHODS

5.2.1 Patient Characteristics

The main clinical characteristics of the T2DM and age matched ND donors are shown in (**Table 5-1)**

Table 5-1: Donor characteristics for Diabetic and non-diabetic subjects

	Age (years)	Sex (M/F)	BMI (kg/m^2)	Cause of death	CIT (hrs)	Duration of Diabetes (years)	HbA1c (%)	Diabetes Treatment
T2DM donors								
1	57	F	27.7	ICH	8	4.8	8.1	Metformin
2	34	F	31.3	ICH	6	5	6.0	Metformin
3	59	F	26.7	ICH	6	4	5.4	Diet only
4	52	M	44.1	ICH	4.5	2.5	6.9	Metformin
5	67	F	21.5	HBI	10	2	6.1	Diet only
6	48	M	23.1	ICH	6.5	4.1	NK	Gliclazide
Mean ± SE	**52.8± 4.6**	**_**	**29.1± 3.3**	**_**	**6.8± 0.7**	**3.7± 0.5**	**_**	**_**
ND donors								
1	53	F	28.4	ICH	4.5	N/A	N/A	N/A
2	36	M	36.3	Trau	4	N/A	N/A	N/A
3	58	M	32.2	Trau	6.5	N/A	N/A	N/A
4	49	M	36.3	ICH	5.5	N/A	N/A	N/A
5	60	M	27.7	Trau	9.5	N/A	N/A	N/A
6	41	M	30.5	ICH	4.5	N/A	N/A	N/A
Mean ± SE	**49.5± 3.8**	**_**	**31.9± 1.5**	**_**	**5.7± 0.8**	**_**	**_**	**_**

CIT: Cold Ischaemia Time; HBI: Hypoxic brain injury; ICH: Intracranial Haemorrhage;

N/A: not applicable, NK: not known

5.2.2 Islet isolation

Human pancreatic islets were isolated in the Oxford DRWF islet isolation facility using methods described previously (*Chapter 2).*

5.2.3 Functional studies

Islet hormone content and secretion measurements were performed as described previously (*Chapter 2).* Exocytosis was studied by capacitance measurements in isolated beta and alpha-cells. JNW was involved in each stage of the islet isolation process, as well as preparing the cells for the electrophysiology experiments however, capacitance measurements were completed by Dr Matthias Braun (OCDEM, Oxford) – (*see Appendix 8.5 for methods).*

5.2.4 Morphometric identification of amyloid in pancreatic tissue specimens

Pancreatic samples from T2DM donors, known to have diabetes for <5 years and controls, matched for age and BMI, were obtained with local ethical consent. Specimens were fixed in; 1) 10% formal saline (Sigma, UK) and processed for light microscopy (see ***Chapter 4 methods***), and 2) 2.5% glutaraldehyde (Sigma, UK) and processed for electron microscopy (EM) (see *Appendix 8.6*). Five-micron thick sections were immunolabelled for the islet hormones insulin and glucagon as described in ***Chapter 4***. Thioflavine S 1.0% (mg/ml) (Sigma, UK) (which binds to proteins in amyloid beta-sheet) was then added to the sections for 5 minutes. Sections were then washed in

distilled water x3 and then in 70% alcohol for 5 minutes. Following a further wash in distilled water they were mounted in aqueous mounting medium and the coverslip sealed with nitrocellulose dissolved in ethyl acetate. The sections were viewed using a microscope with a fluorescein isothiocyanate (FITC) filter. Thioflavine S fluoresces green/yellow in colour if amyloid is present (emission wavelength, 550nm). Each islet in the section was viewed and the presence or absence of amyloid noted.

5.2.5 Fixation and embedding of specimens for electron microscopy

JNW collected pancreatic samples for electron microscopy from T2DM donors and assisted in the fixation process. Dr Anne Clark (OCDEM, Oxford) processed the samples and produced the EM photographs.

5.3 RESULTS

For the T2DM pancreases the mean islet purity was 60% with viability of 80% which this was not significantly different to the controls.

5.3.1 Secretion studies

Insulin and glucagon secretion in response to a glucose challenge was measured for islets from six T2DM donors and six ND donors.

5.3.1.1 Insulin content and secretion

The mean insulin content of islets from the T2DM donors was less than that of the controls (23.3± 4ng/islet (T2DM) (mean ±SEM) vs 32±5 ng/islets (ND) but this did not reach significance (**Figure 5-1a**). No significant difference between the T2DM and control islets was observed for insulin release at a basal level of 1mM glucose (0.13± 0.04 vs 0.12±0.03 ng/islet/hr; p=0.15). There were significant increases in secretion in both groups when challenged with 20mM glucose **(Figure 5-1b)**. The fold increase in insulin stimulation from the T2DM islets was however significantly less than ND islets (2.2±0.4-fold vs 4.6±0.9-fold stimulation $p<0.05$) **(Figure 5-1c)**.

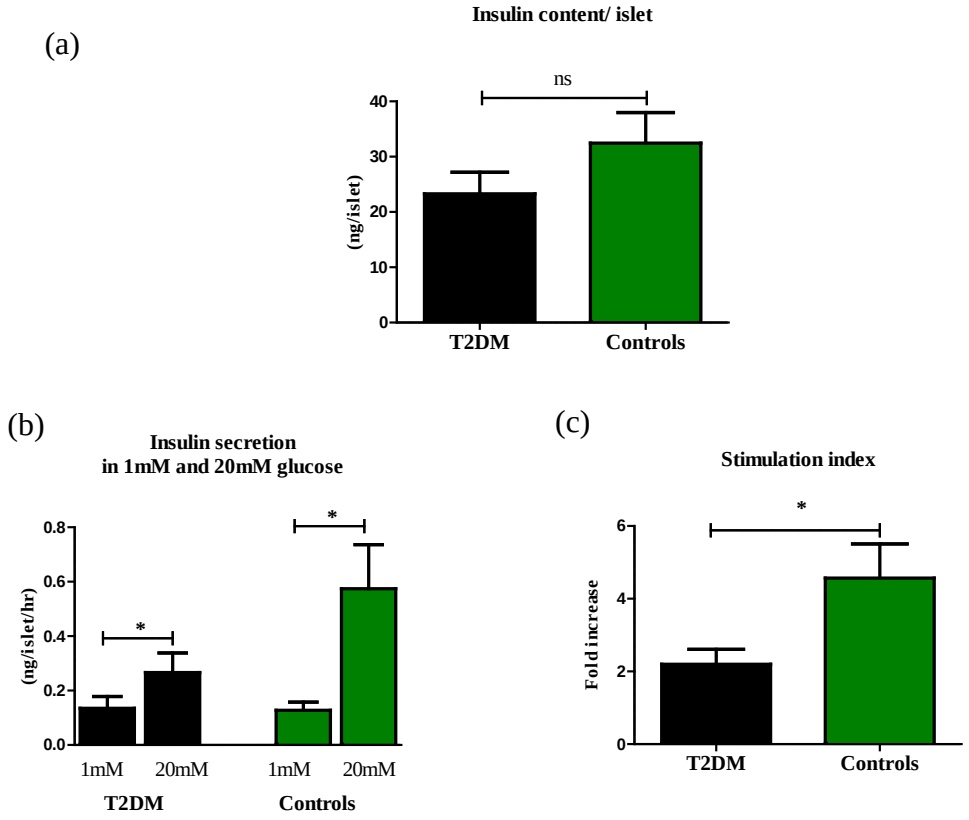


Figure 5-1: Insulin content and secretion; islets isolated from 6 T2DM donor and 6 non-diabetic donor preparations.

(a) Mean insulin content (b) mean insulin secretion in 1mM and 20mM glucose (c) mean fold increase in glucose stimulated insulin secretion (1mM to 20mM glucose). Mean +SEM; * p<0.05

5.3.1.2 Glucagon content and secretion

Glucagon content was highly variable in the T2DM group; Two of the donors contained over 250% more glucagon than the highest in the controls **(Figure 5-2a)**. The ratio of islet insulin: glucagon contents was significantly lower (~ 3 fold) in the islets from T2DM compared to islets from ND subjects (19.5±6.2 vs 64.1±10.3; p=0.004) (**Figure 5-2b).** In the ND donors islets glucagon secretion at 20 mM glucose was reduced from that secreted at 1mM (mean 34±8%). Each sample showed a reduction in secretion. In five of the six preparations the T2DM alpha-cells responded with a stimulation rather

than inhibition of glucagon secretion when glucose was increased from 1 to 20 mM (**Figure 5-2c**). The mean stimulation of glucagon secretion in response to 20mM glucose was 68±26% for the preps that showed a paradoxical rise. The mean glucagon secretion in low glucose was 7.7 ±2.7pg/islet/hr and 6.2 ±1.8 pg/islet/hr (p=0.6) in the diabetic and non-diabetic islets, respectively.

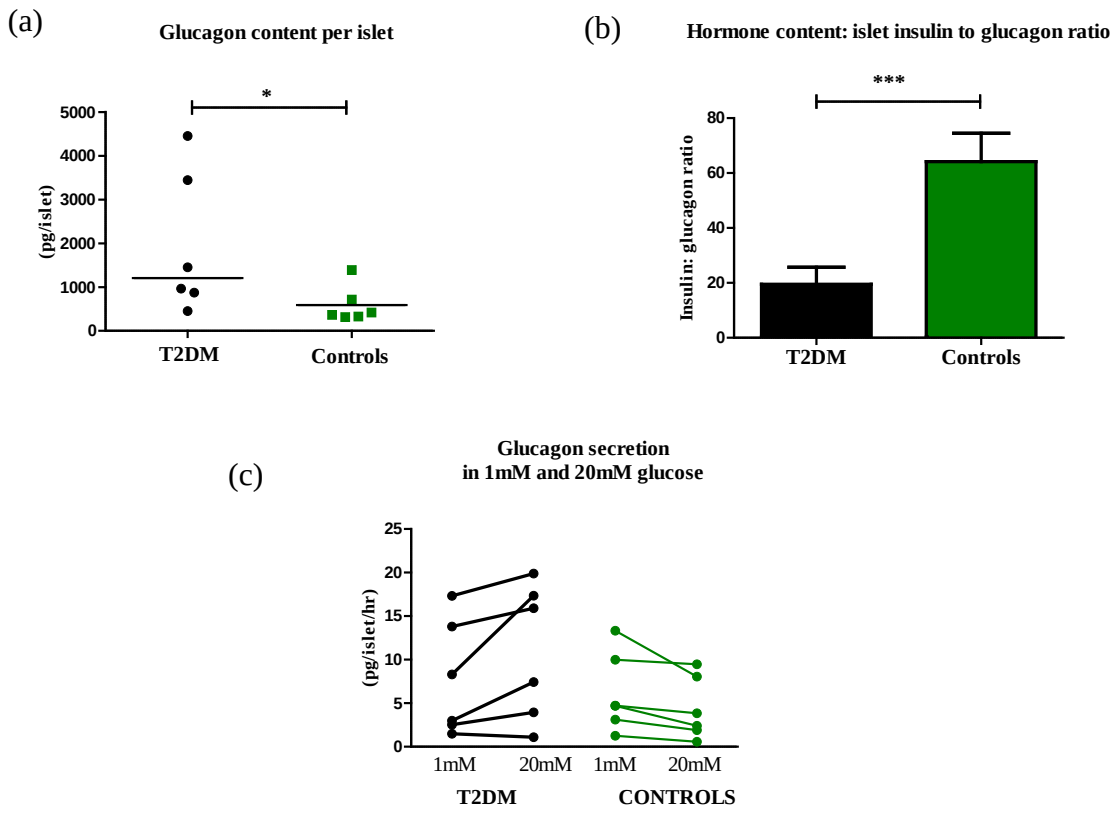


Figure 5-2: Glucagon content and secretion in islets isolated from 6 T2DM donor and 6 non-diabetic donor preparations.

(a) Glucagon content; bar represents the group median (b) Ratio of insulin to glucagon content. Islets from controls had ≈3 fold higher ratio compared to islets from T2DM donors; mean+ SEM (c) 1mM and 20mM glucose, highlighting the paradoxical rise in glucagon secretion which was observed in most of the T2DM islets. Mean ±SEM; * p<0.05

5.3.2 Capacitance measurements

Exocytosis was studied by capacitance measurements in isolated beta-cells from each of the 6 T2DM donors and age-matched ND controls (52.8±4.6 vs 53.1±3.3 years). A median of 8 cells (range= 4-17) per donor was studied in the T2DM group and 7 cells (range=5-10) in the ND group. Following a 500ms depolarisation from -70 to 0 mV, the capacitance response was 24.7± 5.5 vs 22.4±4.7 fF/pF (p=0.75) for the T2DM and non-diabetic donors respectively (**Figure 5-3a)**. The responses were normalized to cell capacitance (proportional to cell surface area) to compensate for any differences in cell size.

Alpha-cell capacitance recordings were available for 5 of the 6 T2DM donors. A median of 6 cells (range= 3-10) per donor was studied in the T2DM group and 4 (range= 2-6) in the ND group. There was no difference in the exocytotic responses between the groups 10.4±1.2 vs 12.2±1.2 fF/pF (p=0.58) for the T2DM and non-diabetic donors respectively (**Figure 5-3b)**.

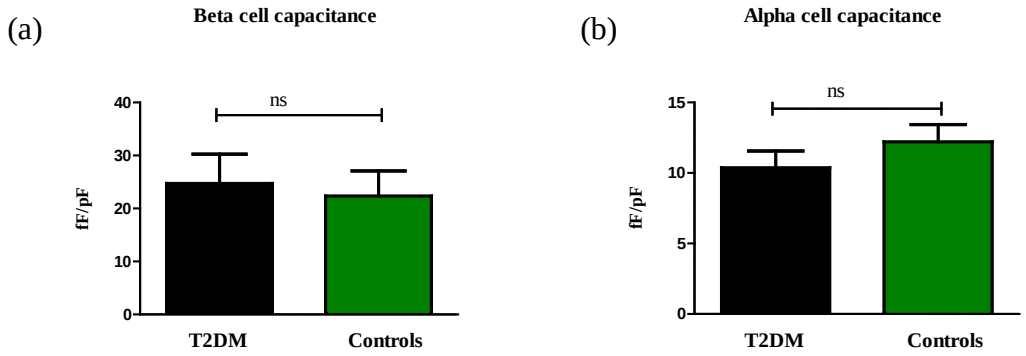


Figure 5-3: Capacitance measurements for (a) beta-cells from 6 T2DM donor and 6 non-diabetic donor preparations and (b) alpha-cells from 5 T2DM donor and 5 non-diabetic donor preparations. Data are presented as measured increases in cell capacitance (measured in fF; 10-15F) elicited by 500-ms depolarizations from -70 mV to zero mV. The responses are normalized to cell capacitance (measured in pF; 10-12 F) to compensate for any variations of cell size. Mean ±SEM; * p<0.05

5.3.3 Islet cell morphology

5.3.3.1 Structure

The morphology of the islets examined in the T2DM donor pancreases appeared similar to that of controls (**Figure 5-4a,b)** with large number of granules present within both the alpha and beta-cells when examined by electron microscopy (**Figure 5-4c**).

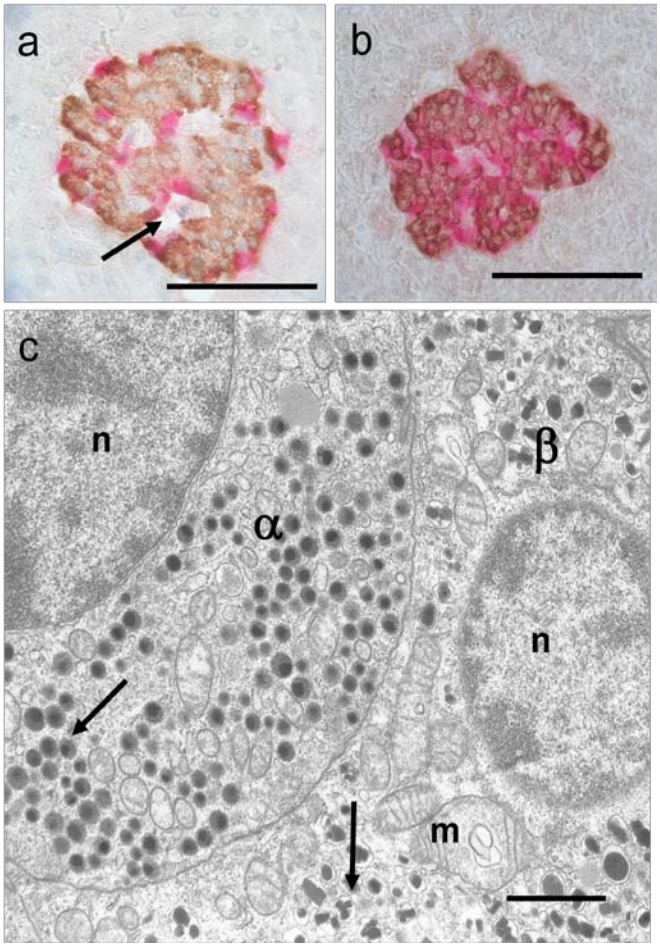


Figure 5-4: Islet structure in ND and T2DM subjects.
Islets labelled for insulin (brown) and glucagon (red). (a) In non-diabetic islets glucagon positive cells are largely adjacent to the capillaries at the islet periphery and penetrating the central area (arrow). (b) Islet structure and cell distribution is not different in islets in T2DM. (c) Islet alpha (α) and beta (β) cell from pancreas of a T2DM subject. The cytoplasm of the cells are filled with granules (arrows). n=nucleus; m=mitochondrion; scale bars (a) and (b) 50microns (c) 1µm

5.3.3.2 Amyloid

The presence of intra-islet amyloid was determined in nine donors in the early stages of T2DM, median duration of diabetes 4 years (range= 1-5 years), all controlled with diet or oral hypoglycaemic agents. The median number of islets assessed per donor was 70 (range= 24-100). Six out of nine donors (66%) had islets containing amyloid. In these

donors the median percentage of islets affected was 9.4% (range=2.8-53%). In some cases only a small area of the islet was affected by amyloid and the islet architecture was retained (**Figure 5-5a**) but in others, >50% of the islet contained amyloid with severe loss of islet cells (**Figure 5-5b**). Amyloid fibrils form adjacent to the beta-cells and disrupt the cell membrane (**Figure 5-5c**). This results in apoptosis of beta-cells and decreased insulin secretion. None of the islets from the control group stained positive for amyloid.

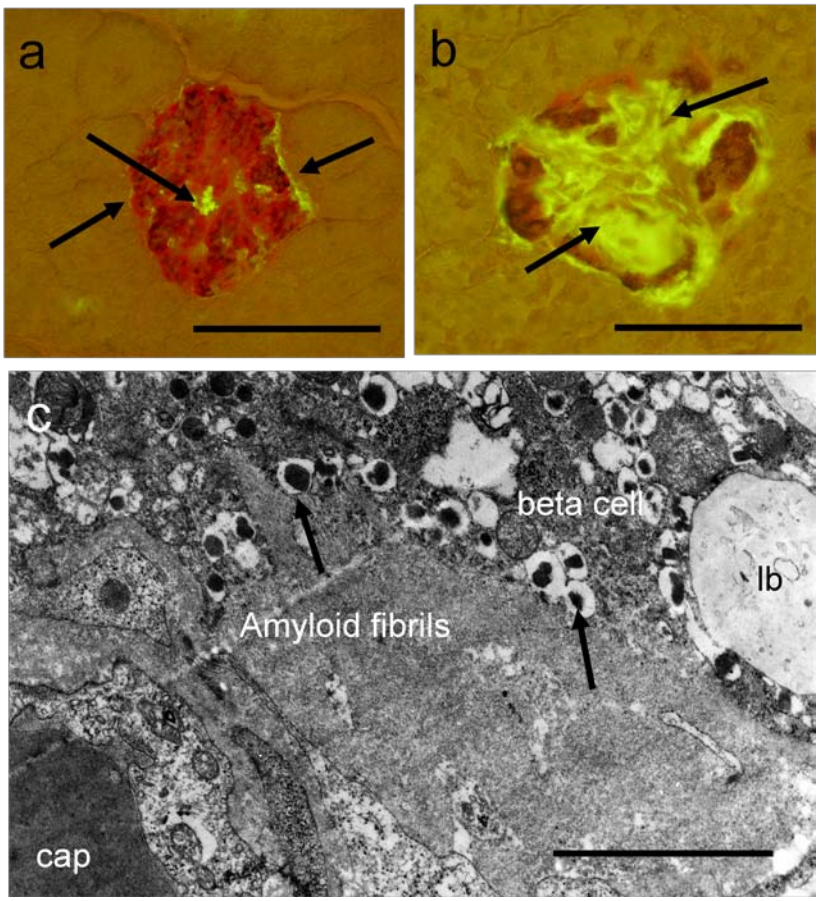


Figure 5-5: Islet amyloid in T2DM

(a) amyloid labelled with the fluorescent marker thioflavin S (green), beta-cells brown, alpha-cells red. Initial small deposits (arrows) are perivascular adjacent to islet capillaries. (b) Extensive islet amyloidosis (arrows) occupying more than 50% of the islet mass in a patient with duration of diabetes <3years. Islet amyloid fibrils are cytotoxic and destroy islet cells. (c) Perivascular amyloid deposits examined by electron microscopy. Amyloid fibrils lie between the islet capillary (cap) and the beta-cells. Typical insulin granules with halos (arrows) and lipofuscin bodies (lb) are present. Scale bars (a),(b),50 microns (c) scale bar 2.5 microns

5.4 DISCUSSION

The lower insulin Stimulation Index observed in the islets from the T2DM donors compared to ND donors is in good agreement with that previously reported by others [179]. The mean duration of diabetes in this donor group was 4.1±0.7years and no patients required insulin therapy. This suggests that insulin secretion was already impaired at a relatively early stage in the disease. The exocytoic capacity of T2DM beta-cells (monitored by measurements of cell capacitance) were comparable to that seen in the ND islets indicating that the T2DM does not result from defects of exocytosis per se but rather more proximal events (including glucose metabolism and initiation of electrical activity). The insulin content of size-matched islets was only slightly lower in T2DM islets than in ND islets but this difference did not attain statistical significance. The threshold for glucose-stimulated-insulin-release has previously been shown to be significantly higher for islets from T2DM donors compared to ND donors [179]. A glucose ramp technique was used by the group [179], whereby the glucose concentration in the perifusate was increased at 1 mM/min from 0 to 50 mM over a 50-min period of time. Using area under the curve measurements it was noted that there was a reduced total insulin secretion in this period with diabetic donor islets producing only 28% of the insulin secreted by islets from control subjects. Maximal insulin secretion was also reduced by 25%. The insulin content of the islets in their study was also not lower in T2DM islets than in ND islets nor was the maximal secretion after application of potassium chloride. The latter stimulation protocol results in membrane depolarization and thus bypasses any proximal events. Our finding that depolarization-evoked exocytosis was not affected is therefore, in agreement with

previous studies suggesting that the intracellular machinery and granule content within the beta-cell required for insulin secretion are not significantly impaired. Thus, it seems justified to conclude that more proximal events of the glucose-sensing of the beta-cell (like glucose metabolism) are affected in T2DM.

A pathogenic role for glucagon secretion in T2DM has long been assumed [234], and there is significant evidence that hyperglucagonaemia plays a key role in the development of hyperglycaemia in T2DM [190, 306]. In non-diabetic subjects, an elevation of plasma glucose (whether induced by an oral or intravenous glucose load), suppresses glucagon secretion [190]. A number of abnormalities in alpha-cell function have however, been recorded *in vivo* in T2DM patients. These include a raised fasting plasma level of glucagon [187, 307] and a blunted or absent alpha-cell suppressive response to hyperglycaemia [188]. In certain cases, glucose has also been shown to elicit a paradoxical stimulation of glucagon secretion in diabetic patients [189, 190, 303]. Although there are conflicting opinions as to whether the absolute fasting plasma levels of glucagon are raised in T2DM patients, in the context of an increased fasting glucose level and hyperinsulinaemia (both of which normally suppress glucagon secretion) it is agreed that a relative hyperglucagonaemia is present [306]. As a result, the elevated glucagon contributes to an increased rate of hepatic glucose efflux which is known to occur in these patients [187].

Although this impaired glucose sensing by the alpha-cell, leading to a loss of hyperglycaemia-induced suppression of glucagon secretion, has been demonstrated *in vivo*, no information on glucagon secretion in isolated T2DM human islets *in vitro* is

available. It is therefore not clear whether the reported abnormalities of glucagon secretion involve defects within the islets or reflect systemic factors. In the current study, the non-diabetic group showed a mean suppression in glucagon secretion of 34±8% between high and low glucose. However, in 5 out of 6 of the islet preparations from the diabetic donors a paradoxical rise was observed. Thus, the glucagon secretion defects we observe in the alpha-cells of islets from donors with T2DM, echo those previously documented *in vivo,* i.e. stimulation rather than inhibition in response to elevated glucose. The capacitance measurements from the alpha-cells of the T2DM donors showed the exocytotic cellular apparatus to be intact, similar to the finding in the beta-cells from T2DM donors. Our data suggests that like the diabetic beta-cell, the alpha-cell in T2DM also shows insensitivity to elevated blood glucose levels resulting in an inappropriate degree of glucagon suppression.

The physiological mechanism whereby hyperglycaemia suppresses glucagon release is not fully understood. Experimental evidence has implicated intra-islet insulin acting in a paracrine mode, or other beta-cell granule components (e.g. zinc) [308], and suggested that the beta-cell dysfunction which occurs in diabetes directly contributes to the alpha-cell secretory defect. The finding that exogenous insulin therapy goes some way to correcting the hypersecretion of glucagon has also been used to support this theory. However, in human [309] and mouse islets [310], increasing glucose from 1mM to 3mM significantly inhibits glucagon secretion, a concentration associated with minimal insulin secretion; it is therefore very likely that the situation is more complex than solely paracrine regulation via beta-cells. Other regulatory factors such as neural signals, nutrients and potentially a direct glucose effect on the alpha-cell are also likely

to be involved. Elucidation of the underlying mechanism causing the impaired glucose-induced suppression of glucagon in T2DM will provide clues of how to prevent hyperglucagonaemia in diabetes.

The glucagon content was very variable in equivalent-sized islets from the T2DM donors. The two preparations where the glucagon content/ islet were considerably higher came from patients with similar duration of diabetes and glycaemic control as the other patients. These two patients did however, have the highest glucagon secretion in the presence of 20mM glucose. The significantly lower ratio of insulin to glucagon content per islet in the T2DM and ND subjects is likely to reflect a reduction in insulin content and an increase in glucagon content. Previously it has been suggested that the alpha-cell population in islets from T2DM patients increases [311]. A recent publication which assessed a large number of pancreatic post-mortem specimens from T2DM donors and controls has refuted this and states that there is no absolute increase in alpha-cell mass but rather, a relative increase as result of the decrease in the beta-cell mass [312]. It would be interesting to correlate islet glucagon content to histological evidence of alpha-cell hypertrophy. However, an increased glucagon content could occur even in the absence of increased alpha-cell area, if the number of glucagon granules and/or glucagon content per granule were increased in T2DM.

Islet amyloid was found to be present in 66% of the T2DM subjects. In some subjects the extent of amyloidosis was severe both in terms of the number of islets affected (53% in one case) and the amount of amyloid deposition per islet; there is a known positive correlation of prevalence (number of islets affected) and severity

(amount of amyloid/islet) [313]. Human islets transplanted into nude mice have been shown to develop amyloid deposits [200]; widespread islet amyloidosis has also been identified in transplanted human islets in the liver of a T1DM recipient at post-mortem [314]. This has led to the suggestion that the accumulation of amyloid should be considered as one potential candidate for the long-term failure seen in human islet transplants [315].

This study demonstrates that extensive amyloidosis can occur early in T2DM. As the development of islet amyloidosis is now being hypothesised as a cause of islet graft dysfunction, transplanting islets from T2DM donors, in which the process is already established in the majority of cases, would seem precarious.

Given the *in vitro* data from this current study and other data published previously it would therefore seem that islets from T2DM donors show both beta and alpha-cell defects. Although the donor pool for islet transplantation is severely limited the use of T2DM donors for this therapy, would seem contentious. However, the finding that T2DM beta-cells remain capable of sensing glucose and secrete insulin, albeit not as efficiently as non-diabetic donors, may well be sufficient to stabilise diabetes control in the recipient. Koh and colleague [173] have unintentionally transplanted islets from two donors with an elevated HbA1c. The function of the islets from one of the donors with a marginally elevated HbA1c (6.3%) provided good *in vitro* and *in vivo* graft function. The second donor had a higher HbA1c (7.9%) and although the *in vitro* studies showed the islets to be less functional they were still able to provide a reduction in the recipient's exogenous insulin requirements and an improvement in the recipient's

HbA1c. Transplanting islets from T2DM donors into T1DM recipients however has to be balanced with the potential risks of committing a patient to immunosuppressive medication for what could well be regarded as a sub-optimal islet graft and also to the possibility of a more rapid deterioration of graft function if the defects in islet function are progressive, as seen in many T2DM patients.

5.4.1 Conclusion

The data set out above is the first to correlate the known *in vivo* observations in T2DM patients to the situation *in vitro*. Both beta and alpha-cell glucose sensitivity seem to be impaired in T2DM and both will ultimately contribute to the hyperglycaemia that ensues. More *in vivo* data is required to determine if T2DM islets could potentially be used for clinical islet transplantation but at present, given the *in vitro* results available, extreme caution is required.

As discussed in Chapter 1.3.3, there is a limited ability to increase the donor pool further, by expanding the donor acceptance criteria. The next chapter will focus on the use of medical therapies to increase the transplanted beta-cell function, aiming to potentially reduce the need for sequential transplants and thus increasing the number of organs available in the donor pool.

CHAPTER 6 GLP-1 RECEPTOR AGONIST THERAPY: AN OPPORTUNITY TO IMPROVE PANCREATIC ISLET FUNCTION IN GRAFT AND RECIPIENT ISLETS

6.1 INTRODUCTION

Improving transplanted graft function could potentially reduce the need for sequential islet transplants, therefore increasing the number of organs available in the donor pool. A number of medical therapies are currently used for T2DM to increase insulin secretion and potentially could be adopted to improve insulin secretion from the transplanted beta-cells. Established therapies, such as sulphonylureas, however, are of limited benefit, as they are known to cause hypoglycaemia and weight gain; two factors that should be avoided post transplantation.

Newer therapies in T2DM, target the incretin axis. These include exenatide, a synthetic version of exendin-4, which stimulates the GLP-1 receptor. These drugs offer a number of properties, which would be beneficial to islet transplant recipients. The insulin-releasing effect of GLP-1 is glucose-dependent, thus blood glucose levels are normalized, without causing hypoglycaemia. GLP-1 receptor agonists also promote satiety, slow gastric empting and decrease postprandial glucagon secretion. In rodent models increased beta-cell proliferation [219] and a reduction in apoptosis [221] has also been shown.

Clinically transplanted human islets have been shown to retain the ability to respond to an infusion of GLP-1, resulting in an increase in insulin secretion [230]. This has prompted further studies to assess if the chronic effects of exenatide, most notably an increase in beta-cell mass, could also be demonstrated in transplanted islets. The effects of exenatide on insulin secretion however, were not maintained after the peptide

was stopped, implying that exenatide had no trophic effect on beta-cells [224, 316, 317]. It may however be argued that the exenatide was given in too low a dose or for too short a time to show a trophic effect.

Recent studies have suggested that the use of these agents in patients with T1DM (who have not undergone transplantation) may help improve glycaemic control potentially through stimulation of residual beta-cells spared from the autoimmune process [227] and also by suppressing glucagon secretion in C-peptide negative individuals [228, 229]. GLP-1 analogues and DPP-4 inhibitors could therefore be potential therapeutic agents which may be beneficial for beta-cell replacement therapy (both islet and whole pancreas), not only in terms of the transplanted islets insulin secretion, but also in stimulation of any residual beta-cell function in the recipient's pancreas, as well as the suppression of alpha-cell glucagon secretion [306].

Aim of this chapter

1. To determine the changes in glycaemic control and transplanted graft function that can be attained through the regular use of a GLP-1 receptor analogue.

6.2 METHODS

6.2.1 Exenatide Therapy

6.2.1.1 Patient selection

Four islet transplant recipients from our Oxford cohort were selected for this study. Unfortunately, all patients received a subsequent islet transplant during the 20-week study period. This meant it was not possible to collect any meaningful data at the end of the study period, to determine if the benefits had been attained. It was for this reason that patients with T1DM who had undergone whole organ pancreas transplantation were enrolled in this pilot study.

Five subjects with T1DM who had undergone simultaneous pancreas/ kidney (SPK) transplantation and received the same immunosuppression regime (induction with Alemtuzumab and maintenance therapy with Tacrolimus and Mycophenolate) were recruited (**Table 6-1**). All patients had stable renal function and no history of gastroparesis. Following SPK transplantation, four patients had initially become normoglycaemic, but subsequently developed pancreas allograft dysfunction (median 34.5months, range= 5-40). One patient never achieved insulin independence. Graft failure was defined as a fasting capillary blood glucose >7.0mM, or 2hr postprandial glucose >10.0 mM on three or more occasions in a single week and with an HbA1c value >6.5%. Following the failure of the graft to maintain normoglycaemia all patients had been restarted on exogenous insulin therapy

Table 6-1: Patient Characteristics for the Exenatide study

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**
Age (years)	**45**	**39**	**49**	**42**	**42**
Gender (M/F)	**M**	**M**	**M**	**M**	**M**
BMI (kg/m^2)	**30.5**	**29.4**	**27.4**	**31.2**	**26**
Duration of TIDM (years)	**33**	**31**	**22**	**32**	**30**
Duration of graft function before failure (months)	**40**	**5**	**39**	**0**	**30**

6.2.1.2 Exenatide administration

Subjects were educated in the administration of exenatide and initially started on 5µg once daily and if tolerated, the dose was increased to a maximal dose of 10µg twice daily over a 2-week period. Each was asked to monitor their blood glucose readings 4 times a day using capillary blood glucose sampling and they were contacted by phone on a 2 weekly basis to monitor progress; insulin requirements were adjusted as appropriate. They were seen again in clinic after 8-weeks and again on completion of the study at 20-weeks.

6.2.1.3 Metabolic testing

At baseline insulin requirements were recorded and samples were taken for HbA1c and measurement of renal function. Each patient underwent a mixed-meal tolerance test (MMTT) (see *Appendix 8.4*) during which fasting and stimulated glucose, c-peptide, insulin, and glucagon were recorded. Glucose, C-peptide and insulin samples were analysed by the Oxford Radcliffe Trust Biochemistry Department, Oxford and glucagon samples were sent to the Hammersmith Hospital, London. These indices were repeated again following 20 weeks of exenatide therapy. Prior to the final meal tolerance test, exenatide was administered 30 min before the meal was given. Body weight was recorded before and after the treatment period.

6.2.2 Islet function and morphology in Type-1 Diabetes

During my research, I had the unique opportunity to isolate islets from a T1DM donor pancreas. This allowed me to determine if; 1) any residual beta-cells remained functional in established T1DM and; 2) whether alpha-cell function was preserved in T1DM. This will help to improve our understanding of the hormonal environment that the donor islets are going to be transplanted into. This may also help to better understand the findings that T1DM patients benefit from GLP-1 therapy.

6.2.2.1 Donor History

A pancreas from a donor with T1DM was offered to the islet research programme. The donor was a white female aged 32 years old who weighed 73.8 kg (BMI 28 kg/m^2) with no family history of diabetes [318]. At diagnosis (at the age of 19) she was treated with gliclazide and metformin initially. However seven weeks after diagnosis, despite maximal doses of oral therapy, she developed ketonuria and had a fasting blood glucose of 24 mmol/l. She was therefore started on insulin treatment (0.27 U/kg insulin twice daily). This was increased to 0.6 U/kg using a basal bolus regimen over a 6 month period. She underwent four admissions for severe diabetic ketoacidosis. The cause of death was brain-stem death as a result of hypoxic brain injury. Unfortunately no serum samples were taken for determination of c-peptide or antibody status at any time. Samples taken at the time of death showed HLA-DR3 and -4 positivity. The donor's pancreas was retrieved for research with the informed consent of the family and with the approval of the local ethics committee.

6.2.2.2 Islet isolation

Islets were isolated using established protocols (see *Chapter 2)*. An islet count was omitted as poor dithizone uptake was predicted. Islets that appeared structurally intact were hand-picked, using a 10µl pipette, from the purified digest for the secretion experiments and the residual islets were retained for morphological analysis. The islets used for the functional experiments were therefore not a true representation of the whole isolated population. Islets isolated from a cohort of non-diabetic donors of similar age (n=5), were used as controls for the *in vitro* studies.

6.2.2.3 Islet functional studies

Insulin and glucagon secretion/ content was measured from batches of 15 islets (in quadruplicates) using techniques described in *Chapter 2*.

Membrane capacitance, as a measure of granule exocytosis, was recorded from isolated alpha and beta-cells using the patch-clamp technique described in *Chapter 5*.

6.2.2.4 Immunohistochemistry

Pancreatic tissue samples taken from the body and tail regions of this donor pancreas before collagenase treatment and from the pancreases of three additional patients with T1DM (diabetes duration >10 years, n=2; diabetes duration <48 h, n=1; tissue retrieval at post mortem was approved by the local ethics committee) were prepared for light and electron microscopy as described in *Chapter 3*. The samples were immune-labelled with anti-insulin (in-house antibody), anti-glucagon (Sigma, UK), anti-somatostatin (DAKO, Denmark) and markers for macrophages (anti-human CD68; DAKO) and lymphocytes (anti-human CD45; DAKO). The cellular composition of islets isolated from this donor with T1DM was compared with islets of a non-diabetic donor using immune-labelling for insulin, glucagon and somatostatin. Fluorescent markers for the different hormones were used and the labelling examined by confocal microscopy. Successive Z-sections were reconstructed to provide a three dimensional image of the islet structure.

Electron microscopy analysis was undertaken as described in (*Appendix* 8.6).

6.3 RESULTS

6.3.1 Exenatide Therapy

6.3.1.1 Tolerance for exenatide treatment

Four of the five patients continued exenatide for the 20-week period. Patient 5 dropped out of the study shortly after starting due to side-effects of the treatment and was excluded from further analysis. Three patients achieved the maximal dose of exenatide 10µg twice daily but one could only tolerate 5µg twice daily. Renal function in all cases remained stable during the study period.

6.3.1.2 Glycaemic control and Graft function

After the 20-week period of treatment, there was a significant reduction in exogenous insulin requirements (0.42± 0.09 vs 0.13± 0.04 units/kg/day; mean± SEM p=0.01) (see **Figure 6-1** for changes in individual insulin requirements). Stimulated c-peptide concentrations taken at 90mins following a MMTT were significantly higher after treatment (0.7±0.2 vs 1.2 ±0.1 nmol/l $p<0.05$) and increased in all patients (**Figure 6-2**). In one patient, the stimulated c-peptide was ~430% higher than at baseline (**Figure 6-3**).

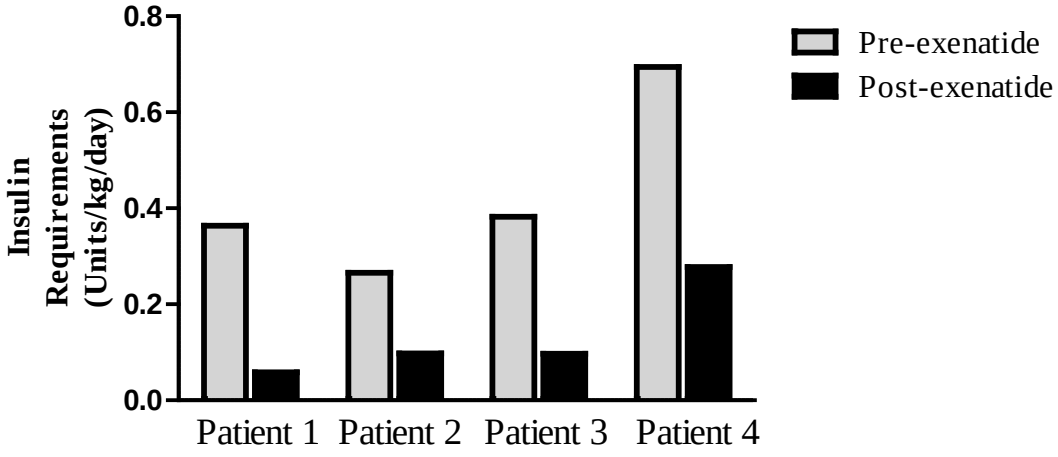


Figure 6-1: Exogenous insulin requirements before and after 20 weeks exenatide therapy for each of the four patients.
Grey bars represent exogenous insulin requirements before exenatide therapy and black bars after 20 weeks of therapy.

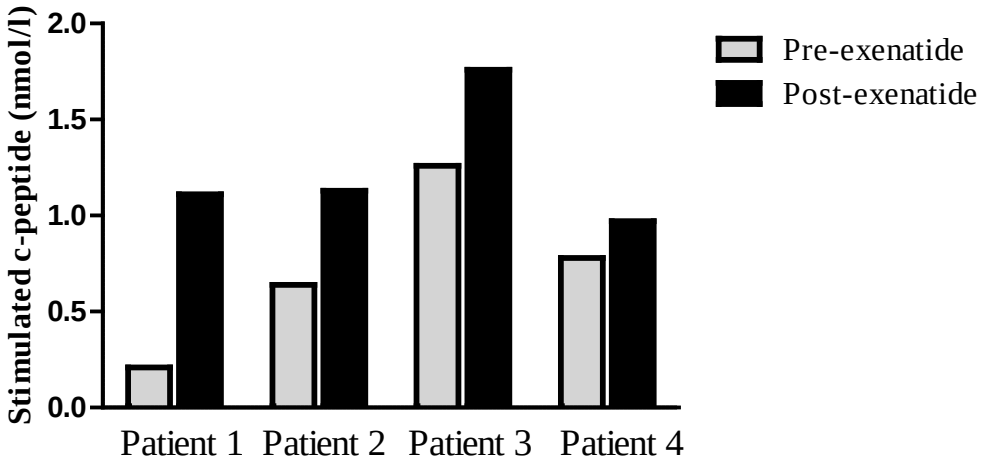


Figure 6-2: Stimulated C-peptide 90min after a mixed meal tolerance test for each of the 4 patients. Grey bars represent stimulated c-peptide before exenatide therapy began. Black bars represent the stimulated c-peptide after 20 weeks of therapy. Exenatide was given 30min before the 20 week MMTT.

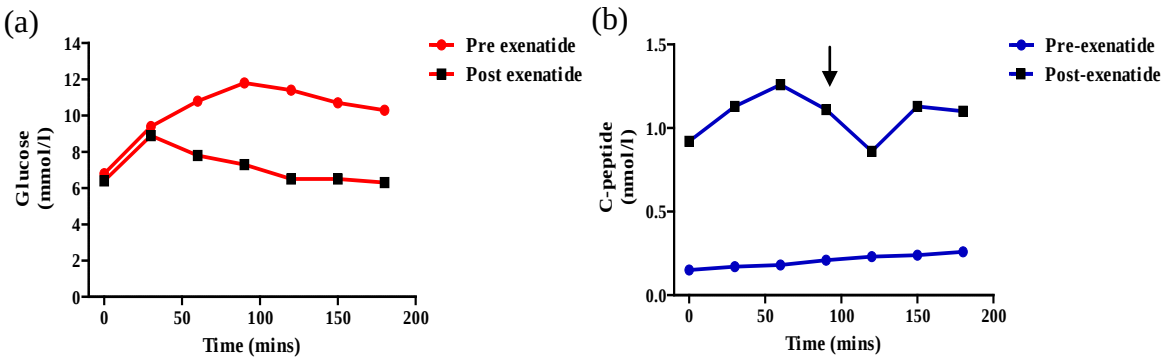


Figure 6-3: Glucose (a) and c-peptide profiles (b) before and after exenatide treatment for patient 1. Red lines indicate glucose (mM) and blue represents c-peptide (nmol/l) concentrations. Black square represent post-exenatide. Arrow indicates the increase in C-peptide at the 90mins point (~430% increase following treatment)

The mean HbA1c for the group was 8.6 ± 1.7% before exenatide and 7.1 ± 0.07% at the end of therapy, showing that glycaemic control was markedly improved, despite the decrease in insulin dose. No episodes of severe hypoglycaemia were observed and no patients recorded home blood glucose readings less than 3.9 mM. Two patients had lost substantial weight during the study period (9.4% and 7.1% of body weight) and the other two had remained weight neutral.

Unfortunately the laboratory which the samples were sent to for glucagon analysis (Hammersmith Hospital, London) did not have a sensitive enough assay to detect changes in the plasma glucagon, as values were below the standard curve. As the samples had been thawed for a prolonged period they were unsuitable for further analysis in an alternative laboratory.

6.3.2 Islet function and morphology in Type-1 Diabetes

6.3.2.1 Function of islets isolated from a donor with T1DM

Islet insulin content from the T1DM donor was lower than the median of size-matched control islets but still within the same range (21.1 vs median 37.7 ng/islet [range 16.1–47.2], n=5) (**Figure 6-4a**). Glucose-stimulated insulin secretion was comparable to that of controls (2.9-fold vs median 3.7-fold increase between 1 and 20 mM glucose [range 2–6.1]) (**Figure 6-4b**). (Stimulated insulin secretion at 20 mM was 0.24 vs median 0.31 ng/islet/h [range 0.18–0.49].) Beta-cell exocytosis in response to voltage-clamp depolarisation steps was also comparable to controls.

Glucagon content per islet was higher than that found in controls; 2,107 pg/islet vs median 827 pg/islet [range 761–1,054], n=5) (see **Figure 6-5a**). Glucagon secretion was not suppressed by elevation of glucose concentration (see **Figure 6-5b**) (controls: median 32% suppression [range 20–55%]). However, exocytosis in response to voltage-clamp depolarisations was similar to that of controls. Thus, the defect of glucagon secretion is more pronounced than that of insulin, suggesting that T1DM somehow directly influences the function of the alpha-cells.

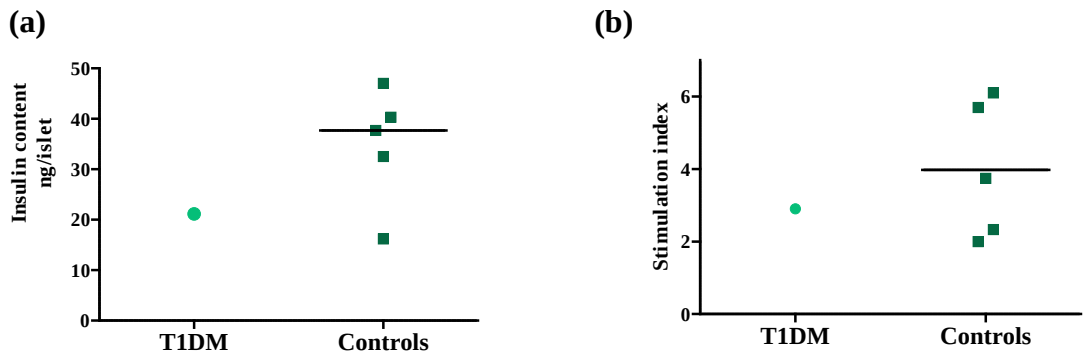


Figure 6-4: (a) Insulin content per islet for the T1DM donor and 5 similar aged controls. (b) Insulin stimulation index for islets isolated from the T1DM donor and controls.
Bar represents the median for controls

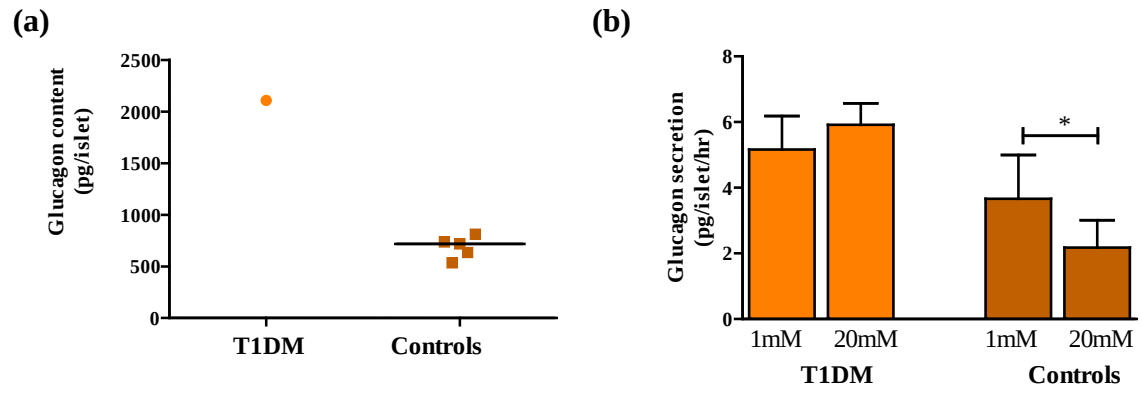


Figure 6-5: (a) Glucagon content per islet for the T1DM donor and 5 similar aged controls. Bar represent the median glucagon content for controls. (b) Glucagon secretion for islets isolated from the T1DM donor and controls.
Bars represent the mean glucagon secretion per islet during 1hr incubation in 1 mM or 20 mM glucose. *p <0.05

6.3.2.2 Histological analysis

Analysis was made of 182 islets in histological specimens taken from the body and tail regions. This revealed that whilst the majority (85%) of the islets from the T1DM donor

contained no insulin-positive cells; 15% of islets did contain insulin-positive cells. All of the insulin-positive islets (**Figure 6-6 a, b**) were found in a localized region of the specimen from the tail region. A subgroup of these insulin-positive islets (40%) contained a normal proportion of beta-cells (>50% of insulin-positive area per islet area [192]). There were varying degrees of cellular inflammatory infiltrate in the insulin-positive islets; extensive infiltration (**Figure 6-6 c)** was associated with islets with reduced insulin positivity. Infiltrates contained CD68 (**Figure 6-6 d**) and CD45 positive inflammatory cells and were similar in distribution to those seen surrounding islets in the new-onset T1DM patient and in other reported literature [319] . The insulin-negative islets contained no infiltrate. Islets containing insulin were not dysmorphic in structure (Figure 6-6**e**) but the majority of islets with no insulin positive cells were no longer ovoid with well-defined margins (**Figure 6-6 f**). It is unlikely that these dysmorphic islets with well defined margins would have survived the collagenase isolation process. Electron microscopy demonstrated lymphocytic infiltrates in islets (**Figure 6-7a**) and well granulated alpha-cells. Lipofuscin bodies were noted within the alpha-cells, suggesting that the islet cells were not neogenic [146]. Confocal Z-stack reconstruction of an islet isolated from a non-diabetic donor (**Figure 6-7b**) and from the T1DM donor (**Figure 6-7c**) illustrates an islet with low levels of insulin-positive beta-cells relative to glucagon-positive alpha-cells in the T1DM donor. Islets from the other patients with long-standing disease (n=2) examined by histology contained no insulin positive cells or inflammatory infiltration, were glucagon positive and had a dysmorphic structure.

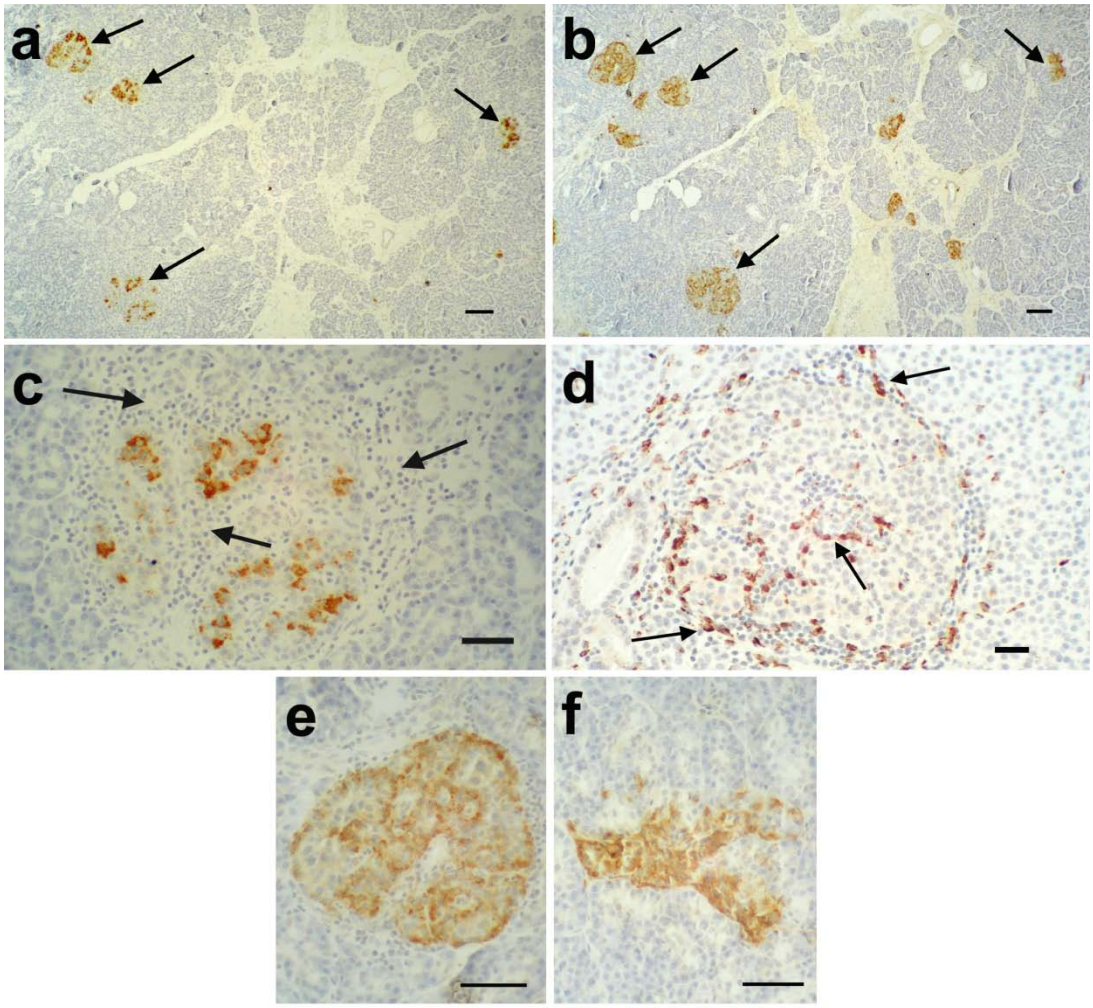


Figure 6-6 Pancreatic islets from the T1DM donor

(a) insulin-positive islets (arrows) were restricted to small numbers of exocrine lobules of the pancreatic tail region; (b) adjacent section labelled for glucagon identified more islets in other lobules. (c) Higher magnification of the islet indicated with a red asterisk in (a) shows an insulin-positive islet surrounded by extensive mononuclear infiltrates (arrows). (d) The inflammatory infiltrate surrounding insulin-positive islets contained cells that were positive for the macrophage marker CD68 (arrows). (e) Higher magnification of an islet in (b) (known to contain insulin-positive cells) stained for glucagon showed normal ovoid morphology with glucagon-containing alpha-cells on the periphery and adjacent to islet capillaries; (f) an islet (containing no insulin-positive cells) that consisted almost entirely of alpha-cells and was dysmorphic in structure. Scale bars (a, b) 150 µm; (c) 50 µm; (d) 25 µm; (e, f) 50 µm;

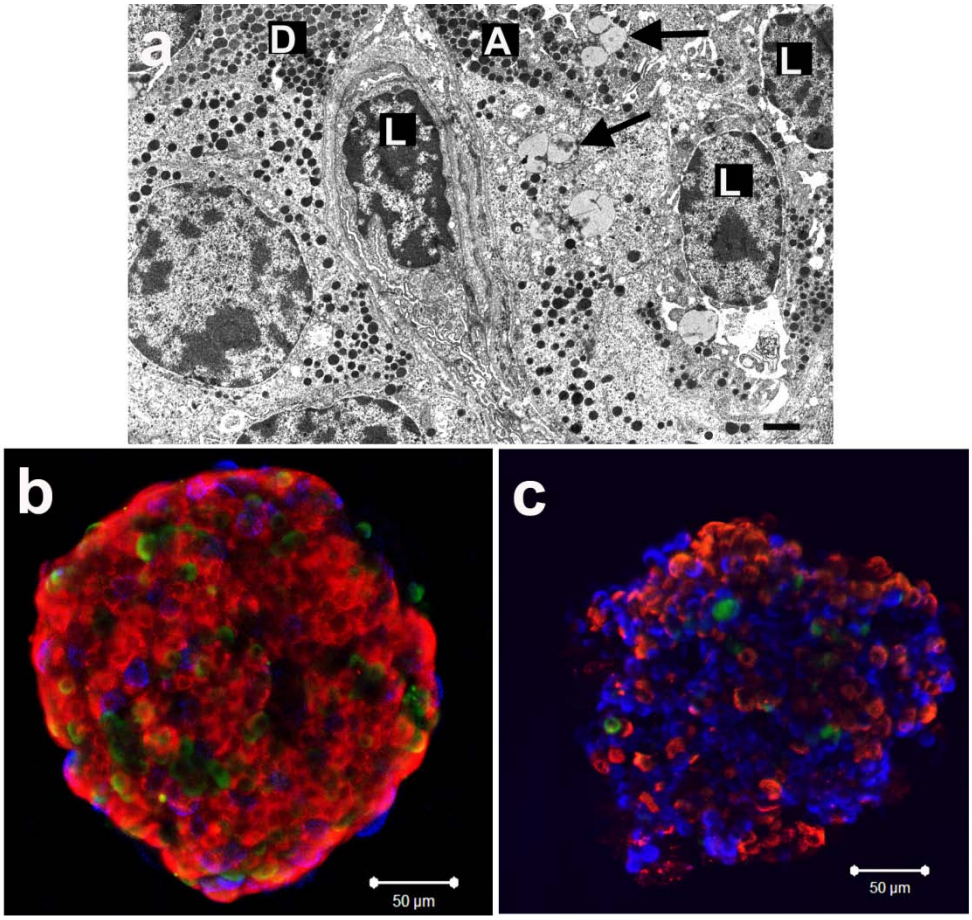


Figure 6-7 Pancreatic islets from the T1DM donor and controls

(a) Electron microscopy demonstrated; L, lymphocytes within an islet. A,alpha-cell; D, delta cell. Lipofuscin bodies are noted in the alpha-cell (arrows) indicating that this was not a neogenic cell. (b) Shows a confocal reconstruction of an isolated islet from a non-diabetic donor and (c) the T1DM donor immunolabelled for insulin (red), glucagon (blue) and somatostatin (green); this illustrates an islet with low levels of insulin positivity. Note the relative increase in alpha-cell number in the islet isolated from the T1D donor that correlates with the increase islet glucagon content. Scale bars: (a) 1.0 µm; (b,c) 50 µm

6.4 DISCUSSION

GLP-1R agonists were initially introduced as a diabetes therapy for use in the treatment of T2DM. Preliminary studies have however, shown that their scope within diabetes may extend far beyond this and they may be of benefit in T1DM patients and in patients undergoing beta-cell transplantation. In this study whole pancreas transplant recipients with chronic allograft dysfunction were used to determine changes in islet function. Following treatment with exenatide for a 20 week period a considerable reduction in exogenous insulin requirements was seen in all cases (mean reduction ~74%). Many factors could have contributed to this improvement in islet function. It is likely that the predominant reason for the insulin reduction stems from the increase in transplanted beta-cell function (as the stimulated c-peptide was significantly increased when exenatide was administered prior to the final MMTT). However, delayed gastric emptying, postprandial glucagon suppression, and weight loss secondary to increased satiety are all likely to have contributed.

All four patients were know to be c-peptide negative before transplantation but none had undergone a stimulation test prior to their pancreas transplant. It is now well recognised that the autoimmune destructive process may not be absolute in T1DM and that a number of T1DM patients may contain insulin positive cells long after diagnosis [320]. Previous studies have shown that ~30% of patients without measurable basal levels showed the presence of C-peptide following an arginine test [321]. Other studies have reported that 23% otherwise c-peptide negative patients had detectable C-peptide levels following a glucagon-challenge [322]. Here I show that some islets isolated from

a T1DM donor contained beta-cells which responded normally to glucose with stimulation of insulin secretion, suggesting that the beta-cells in this subgroup of islets had escaped the autoimmune destructive process. It is therefore likely that endogenous insulin production from the recipients own pancreas could be stimulated by exenatide treatment, although the relative contribution of this to the overall secretory response is likely to be small. The only way to confirm this however would be to undertake intricate selective venous sampling.

A lack of suppression of glucagon in response to a carbohydrate rich meal is typically seen *in vivo* in patients with T1DM [323, 324]. This was demonstrated in the *in vitro* experiments undertaken in the islets isolated from the T1DM donor. Unfortunately, the glucagon levels were not available for the transplanted patients undergoing exenatide treatment; however previous literature would suggest that the ability of GLP-1R agonists to suppress glucagon [325] may also have a contributory effect on the overall reduction in exogenous insulin requirements and benefits for glucose homeostasis.

To assess if there are long-term benefits (e.g. proliferation, improved islet sensitivity to glucose and reduced apoptosis) in addition to the acute benefits from GLP-1R agonist therapy, would require the need to stop treatment for a period of time and recheck the meal tolerance test off therapy. This however was not part of this preliminary study and given previous observations [224, 316, 317] it was unlikely that chronic benefits, if present, would be significant enough to detect after a 20-week period. As all patients were keen to continue with exenatide treatment after the study

period the aim will therefore be to continue treatment for a period of 1 year and reassess all parameters at that stage to allow time for any chronic benefits to be established.

Although GLP-1R agonist therapy thus has a number of beneficial effects, recent reports have highlighted potential areas of caution. The most concerning is the reported increase in thyroid C-cell hyperplasia/ malignancy in rodent models treated with liraglutide. Studies have however indicated that GLP-1R expression in rodent thyroid C-cells is significantly higher than in humans and that GLP-1R agonists did not activate adenylate cyclase or generate calcitonin release in primates [326].

A recent review of the US Food and Drug Administration's database of reported adverse events has however revealed pancreatitis, pancreatic cancer and thyroid cancer were more commonly reported among patients who took sitagliptin or exenatide as compared with other therapies [327]. Whilst these observations highlight potential safety concerns the data from adverse event reporting system databases are hampered by certain biases. These include the major issue of reporting bias, including the greater tendency for adverse events of new drugs to be reported, as opposed to older drugs. Incomplete reports also mean that important information on potential confounding factors can also be overlooked. It is for this reason that the paper [327] was withdrawn by the journal's editorial committee [328].

Reassuringly *in vitro* work using four human cell lines has shown no direct impact of exenatide on cancer cell formation [329]. As the transplanted patients are on

immunosuppressant treatment, which in itself leads to a higher risk of cancer formation, it would seem prudent to monitor all patients on GLP-1R agonist treatments closely.

Whether the effects of exenatide therapy are durable or merely offer a temporary, improvement prior to islet graft deterioration is unclear. At worst, it may act as a transient measure to stabilize control whilst waiting for a further transplant. However, GLP-1R agonists may conceivably offer additional benefits. It is worthy of note that all four patients studied here had markedly reduced insulin requirements and it will be interesting to see whether this effect is maintained and if a further long-term improvement is observed. The appropriate time to introduce GLP-1R agonist therapy is also debatable; if longer-term benefits are shown, it would seem logical to begin treatment in the early stages following islet or whole organ transplantation, possibly as part of the regular post transplantation therapy. If such treatment does prolong graft function, reducing the need for further transplantation, this would significantly increase the availability of pancreases to allow other patients to undergo islet transplantation. In addition, this would reduce the patient risk associated with sequential islet transplants (e.g. increased sensitization and repeated hepatic injections) and also reduce the costs incurred.

6.4.1 Conclusion

This study demonstrates the positive effect of exenatide therapy on transplanted beta-cells, significantly reducing the need for exogenous insulin, and is the first to show this in the whole pancreas transplant model. Although the islet mass is considerably less in

islet transplantation it is likely that these results are transferable to islet transplantation. For the treatment of islet and pancreas allograft dysfunction incretin-based therapies could be used prior to the initiation of insulin therapy, however further studies are needed to determine at what stage intervention would be most beneficial. This is also the first study to report on the glucose-responsiveness of beta-cells in islets isolated from a patient with long-standing T1DM. The findings also support *in vivo* studies undertaken previously, which show that glucagon suppression is likely impaired in T1DM patients.

CHAPTER 7 OVERALL CONCLUSIONS

CONCLUSIONS AND FUTURE WORK

The main focus of this research has been to conduct a number of studies aiming to answer the question of whether obese donors provide a suitable source of islets for clinical islet transplantation. Detailed *in vitro* and *in vivo* studies have been undertaken to assess if a previous obese environment has a detrimental effect on isolated islet function and transplant outcomes. Assessment of islet isolation yields and pancreatic morphology have been compared in obese and non-obese donors to better understand why islet yields are higher from obese donors. In addition, these studies have provided insights into the question of whether donors with T2DM, could be considered as a source of islets for clinical transplantation. This has also provided additional understanding into the pathogenesis of the T2DM disease process. Finally, preliminary data was obtained to identify potential medical therapies that could be used to improve islet graft function.

7.1 FUNCTIONAL STUDIES IN ISLETS FROM OBESE DONORS

Pancreatic tissue lipid concentrations have been shown to be elevated in both obese humans [64] and in animal models of obesity [330]. Intra-islet triglyceride content was also found to be increased in the samples analysed here (*Chapter 2*). This suggests that triglyceride storage in islet cells is elevated in obesity as shown in high fat-fed rodents [330]. This elevation in triglyceride, as well as the increased local NEFA concentrations, as a consequence of increased pancreatic fat, could lead to lipotoxicity effects on the islets, affecting insulin-stimulus secretion coupling and decreasing

responses to glucose. However, the *in vitro* function of islets isolated from obese and non-obese donors remained similar for both insulin and glucagon secretion and intra-islet ATP concentrations. This suggests that if lipotoxicity had been present *in vivo* it was either not severe enough to cause significant islet dysfunction or the effects were reduced when the islets were isolated, possibly as a result of recovery during the post-isolation period.

Whole pancreas transplants from obese donors have been shown to be unsatisfactory, not from an islet function aspect, but from other complications [63]. As a result current pancreas allocation algorithms now offer obese donors preferentially to islet transplantation [17]. Analyses of the Oxford Islet Isolation database indicates that 32% of all pancreases offered for clinical islet transplantation (from donors <65 years old) between 1st Jan 2008- 1st Jan 2011, were from donors with a BMI 30-35kg/m^2. The transplanted function of these islets however, has not been assessed in detail. In Oxford, we showed 5/5 donors achieved good recipient graft function using transplanted islets, isolated from obese donors. The conclusion that islets from obese donors are suitable for islet transplantation is reinforced by a more extensive and detailed analysis of work conducted in Edmonton. Three months following transplantation, both the obese and non-obese groups showed significant metabolic benefits and these benefits were similar between the groups. A longer period of follow-up would have been preferable. However, in the Edmonton cohort the majority of recipients went on to have further transplants, preventing meaningful analysis as variables from more than one donor are involved. As the number of patients requiring only a single transplant increases, it is hoped that longer-term follow-up studies would become more feasible. This work

disproves the original hypothesis which proposed that 'Although islet yields from obese donor pancreases are increased compared with non-obese donors, these islets are suboptimal in terms of islet function and transplanted islet graft outcome'.

A larger number of donors and the inclusion of pancreases from donors with BMIs at the extremes of the spectrum (e.g. <20kg/m^2 and >40kg/m^2) would have been desirable to include in both the *in vitro* and *in vivo* studies, but due to limitations in organ donation and the current pancreas allocation algorithms, this was not possible. Other factors for example, the age of onset and duration of obesity, recent changes in body weight, and body fat distribution would all have been useful to help determine correlations however, these were not available.

As only a small proportion of the obese donors, in both the *in vitro* and *in vivo* components of this study had a BMI >35 kg/m^2, caution must be used in extrapolating the data to donors with a BMI >35 kg/m^2. However, as the upper limit for donor BMI set by the UK Islet Transplant Consortium is 40kg/m^2 and most of the pancreases used for the clinical islet programme are from donors <35kg/m^2 the data in thesis is of considerable clinical use. Further detailed studies are required to determine if donors above this upper limit could provide islets which are functionally similar to non-obese donors and therefore the donor pool could be increased further.

7.2 MORPHOLOGICAL STUDIES ON THE EFFECT OF OBESITY ON ISLET STRUCTURE AND ISOLATION YIELDS

Donor BMI has been clearly shown to have a positive effect on islet yields, expressed as IEQ which takes into account islet volume [122, 123, 150]. The aim of *Chapter 4* was to determine how this occurs. The results show that there was an increase in the proportion of larger islets isolated, rather than an increase in islet number. Morphometric studies of pancreatic specimens from obese and non-obese donors however, showed the proportion of larger islets to be similar in the two groups. The increase in islet yields seen in obese donors is therefore a result of the islet isolation process liberating the larger islets more efficiently from obese donor pancreases rather than the pancreas containing more or larger islets. This work disproves the original hypothesis which proposed that 'The higher islet yields obtained from obese donor pancreases, as measured by islet equivalents, are due to obese donors having larger islets rather than having an increased total islet number'.

It was established in *Chapter 2*, that obese donor pancreases had a larger intra-pancreatic adipocyte content compared to non-obese donor pancreases. Previous studies have shown that fat content is an important determinant of islet isolation success [149, 150] and hence this is likely the reason why obese donors are also associated with increased islet isolation success. The mechanisms by which increased pancreatic fat leads to an improved recovery of larger islet remain unclear. Studies have shown that the digestion time is shorter in obese pancreases [112]. As collagenase has been shown to penetrate inside the islets after distension [293], this reduction in digestion time could

result in larger islets being less susceptible to fragmentation, hence a larger proportion are present in the final digest. An alternative hypothesis would be that the potential of collagenase to penetrate inside the obese donor islets is altered by changes in the pancreas structure; future studies will be aimed at addressing this.

7.3 ISLET FUNCTION AND MORPHOLOGY IN TYPE-2 DIABETES

All avenues are currently being explored to determine if the donor pool for islet transplantation can be increased further [62, 161, 170]. The use of T2DM donors in the early stages of the disease, when islets are still able to secrete significant concentrations of insulin [331] has been suggested [173]. It is recognised that T2DM is multi-factorial in origin [332]; insulin resistance and impaired insulin secretion are usually present but the relative contributions of insulin sensitivity and impaired beta-cell function in T2DM have been controversial. A spectrum likely exists where insulin resistance has a higher contribution in some T2DM subjects, whereas beta-cell dysfunction does in others. If an individual with T2DM, even with severe hyperglycemia, is rendered euglycemic by either changes in lifestyle or pharmacological means [333], beta-cell function, in particular glucose responsiveness, can be restored [334]. Therefore, removing islets from a severely insulin resistant environment and placing them into a lean insulin sensitive recipient, may provide sufficient insulin secretion to attain control of glycaemia and also allow any functional abnormalities to be restored.

Only one small study has assessed the insulin secretory capacity of islets isolated from donors in the early stages of T2DM [177]. This showed the beta-cells to be

responsive to a glucose challenge and although secretion was reduced compared to controls at high glucose levels, it was similar at 6mM glucose. The experiments in *Chapter 5* aimed to determine potential reasons that may preclude the use of T2DM donors. Hormonal secretion studies showed that the beta-cells from T2DM pancreas donors have the ability to respond to a glucose challenge, but were in agreement with the previous study showing a reduction in insulin secretion in response to high glucose, compared to that seen in our age-matched controls. The cohort of patients was however diverse with a range of donor ages and BMI. The alpha-cell glucagon secretory response to glucose was abnormal in the majority of the T2DM donors studied. This inappropriate alpha-cell response has also been shown *in vivo* [189]. An increase in glucagon secretion has the potential to increased hepatic production of glucose [187], which may compromise a transplanted graft comprised of T2DM islets. The prevalence of amyloid deposition within the islets of the T2DM was high. Amyloid deposition is a well characterised progressive destructive process [198] and has recently been found to develop in transplanted islets from non-diabetic donors [314]. It would therefore not be advantageous to transplant islets in which this process has already commenced. Although good islet isolation yields have been demonstrated from T2DM donors [173] the functional and morphological abnormalities described above cast doubt on the use of these islets in clinical transplantation. This work supports the original hypothesis, which proposed that 'Islets from donors in the early stages of T2DM are unsuitable for islet transplantation'.

7.4 MEDICAL STRATEGIES TO IMPROVE GRAFT FUNCTION

As there is a worldwide shortage of pancreatic donors, it is vital that transplanted graft function and longevity is maximised. GLP-1R agonists stimulate beta-cell insulin secretion *in vitro* [335] and transplanted islets have been shown to be responsive to this class of drugs [224, 230]. The results from *Chapter 6* highlight the significant benefits GLP-1R agonist therapy can have on *in vivo* islet function. The initial intention was to perform these studies in islet transplant patients, but this was not feasible as the patients went on to have sequential transplants during the treatment period. The studies were therefore undertaken in a cohort of whole pancreas recipients who had experienced allograft dysfunction. Although the vascular supply and transplant site are different between these two methods of beta-cell replacement, the benefits seen in the whole organ patients are likely to be applicable to the islet transplant patients. This work supports the original hypothesis, which proposed that 'GLP-1 receptor agonists offer a therapeutic strategy for improving islet function post-transplantation'.

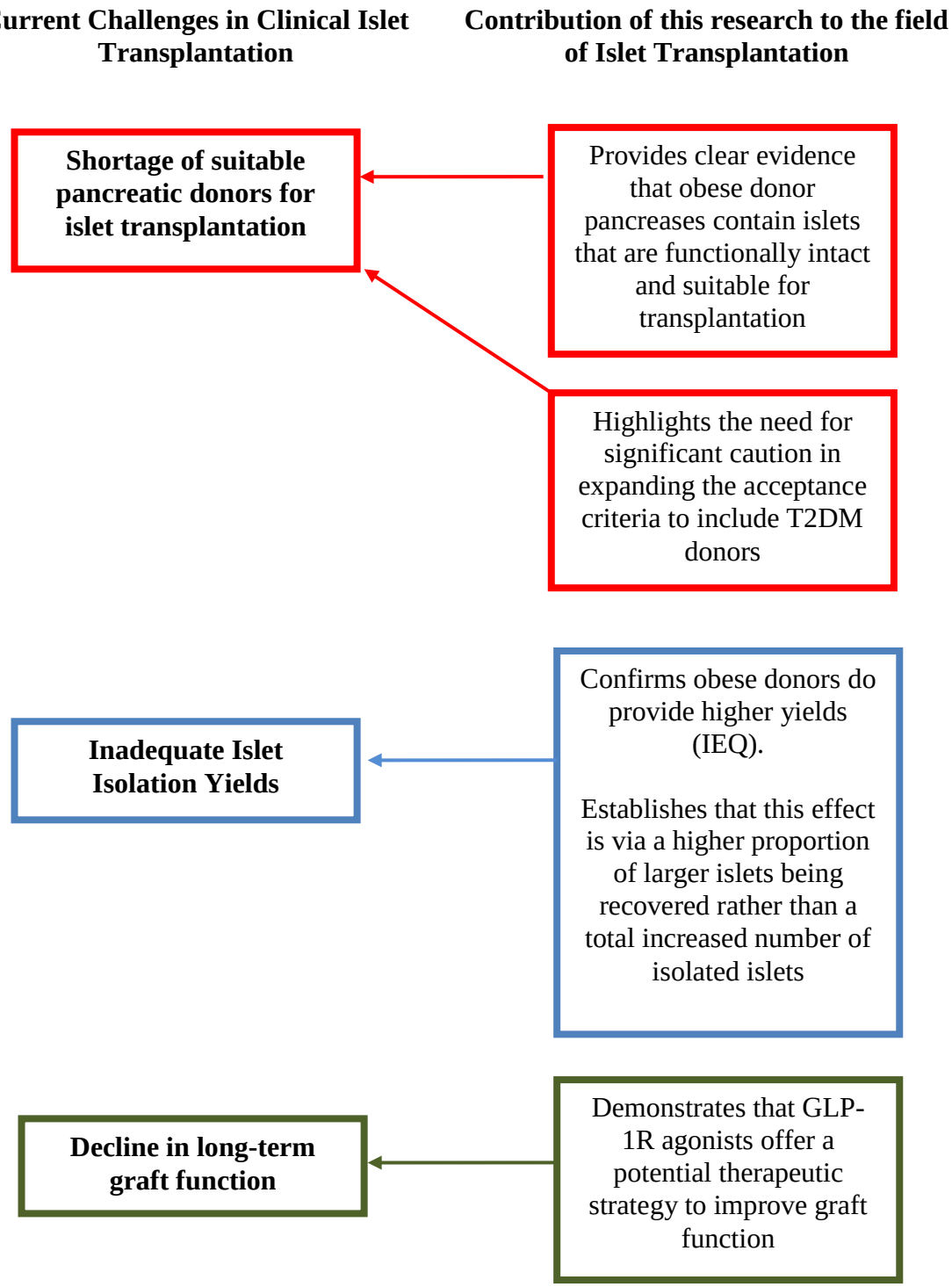
The benefits resulting from GLP-1R therapy are likely to be more than just increased beta-cell stimulation; glucagon suppression [215], improved insulin sensitivity through weight loss [336] and potentially increased islet mass through increased beta-cell replication and neogenesis [219] may also be contributing factors.

The studies undertaken on the T1DM donor pancreas highlight that residual beta-cells can be present and remain functional. It is therefore likely GLP-1R therapy would also benefit the function of any residual beta-cells present.

This class of drug would seem a logical inclusion for treatment for allograft dysfunction and if used early after transplantation may reduce the need for sequential transplants in some instances, thus helping to increase the number of organs available in the donor pool.

The results from the *in vivo* GLP-1 studies documented here and preliminary reports from others highlight the need for a prospective randomized placebo-controlled study. A multi-centered trial is soon to be started, for which our centre will be contributing patients, in order to determine the effects of the GLP-1R analogue Liraglutide on islet transplant outcomes.

7.5 SUMMARY


Current Challenges in Clinical Islet Transplantation
Contribution of this research to the field of Islet Transplantation
Shortage of suitable pancreatic donors for islet transplantation
Provides clear evidence that obese donor pancreases contain islets that are functionally intact and suitable for transplantation
Highlights the need for significant caution in expanding the acceptance criteria to include T2DM donors
Inadequate Islet Isolation Yields
Confirms obese donors do provide higher yields (IEQ).
Establishes that this effect is via a higher proportion of larger islets being recovered rather than a total increased number of isolated islets
Decline in long-term graft function
Demonstrates that GLP-1R agonists offer a potential therapeutic strategy to improve graft function

CHAPTER 8 APPENDICES

8.1 INTRA-ISLET TRIGLYCERIDE AND FATTY ACID ANALYSIS

Islet lipids were extracted according to the method of Folch et al [337]. An internal triglyceride standard was added before extraction so that the concentration of fatty acids could be measured. Total phospholipid and triglycerides were separated by spotting lipid extracts onto silica gel 60 (Merk) thin-layer chromatography plates and running in a solvent system of hexane:dieythyl ether:acetic acid (85:15:1). Lipid bands were visualized under UV light after spraying with 0.1% ANS (8-anilino-1-naphthalene sulphonic acid), then identification verified using commercial standards. Total phospholipids bands were scraped into glass tubes and methylated at 80°C with 6% H2SO4 in methanol for 12 h. Triglyceride bands were scraped into glass tubes and methylated at 80°C with 1.5% H_2SO_4 in methanol for 2 h. Total phospholipid and triglyceride fatty acids were eluted into hexane. Separation and quantification of the fatty acid methyl esters (FAMEs) from adipose tissue and islet total phospholipids and triglyceride was achieved using gas chromatography, on an Agilent 6890 GC (Agilent Technologies, UK) fitted with a 30 m x 0.53 mm (film thickness 1 μm) capillary column (RTX-Wax). Individual fatty acid peaks were identified by reference with known FAMEs. Fatty acid composition (μmol/100 μmol total fatty acid) in these fractions was then determined.

8.2 PRINCIPLES OF RADIOIMMUNOASSAY

Radioimmunoassay (RIA) is based on the antigen-antibody reaction in which tracer amounts of the radio-labelled antigen competes with endogenous antigen for limited binding sites of the specific antibody against the same antigen. The radio-isotope I^{125} was used for both insulin and glucagon measurements.

A fixed concentration of radio-labelled antigen in trace amounts was incubated with a constant amount of antiserum such that the total antigen binding sites on the antibody are limited such that the only 30–50% of the total radio-labelled antigen may be bound in the absence of the antigen. When unlabeled antigen, either as standard or test sample, is added to this system, there is competition between radio-labelled antigen and unlabeled antigen for the limited constant number of binding sites on the antibody. The amount of radio-labelled antigen bound to antibody decreases as the concentration of unlabeled antigen increases. Following optimal incubation condition radio-labelled antigen bound to antibody is separated from unbound radio-labelled antigen. Antibody-bound radio-labelled antigen is separated from free radio-labelled antigen, by an antibody precipitation using polyethylene glycol (PEG). The amount of unlabelled hormone present is inversely proportional to the amount of radioactive tracer measured. The amount of hormone present in the samples is determined using a standard curve. This is known as the double antibody/PEG technique. The bound or free fraction is counted by a gamma counter with appropriate settings for the particular radioisotope.

A standard curve is generated with a set of known concentrations of the unlabeled standards and from this curve the amount of antigen in unknown samples can then be calculated. Thus, the four basic necessities for developing a radioimmunoassay are: a

specific antiserum to the antigen to be measured, the availability of a radioactive labelled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

8.3 CONTINUOUS GLUCOSE MONITORING

Continuous glucose monitoring (CGM) was performed before and at 3 months after islet transplantation using the CGMS iPro (Medtronic). All CGM monitoring was performed in seven complete and consecutive 24-hour periods. The CGMS probe was inserted into the subcutaneous tissue of the abdomen. Patients were asked to keep a diary and record home glucose readings for a minimum of four times per day. The probe was removed after 7 days and information downloaded using Medtronic software.

8.4 MIXED MEAL TOLERANCE TEST

Patients were asked to fast from midnight the night before and withhold insulin treatment on the morning of the test. They can take water, but no other drinks. They are also requested to avoid excessive alcohol and exercise for 48 hrs prior to the MTT. Patients attended for the Mixed meal tolerance test (MMTT) between 0800 and 1000am. Following the application of topical anaesthetic cream, a standard gauge cannula was placed into an antecubital fossa vein for blood sampling. This was kept patent by occasional flushing with sterile Normal saline. At time zero minutes, the patient was asked to drink 240ml Fortisip Liquid drink (CHO 18.4g, protein (caseinates) 5g, fat 6.5g, per 100 ml) over a period of 2-4 mins. Blood was sampled at times -10, 0,

30, 60, 90, 120, 150 and 180 mins. The sample volume was 5mls. The blood at each time point was placed into a fluoride oxalate tube (1ml) for plasma glucose and into an EDTA tube (4ml) for plasma C-peptide and insulin. The samples were then stored at -80^{o}C. Later the samples were transported on frozen ice to Core Biochemical Assay Laboratory, Cambridge University.

8.5 PRINCIPLES OF CAPACITANCE MEASUREMENTS

During exocytosis, the cell membrane surface area is increased by the fusion of granules. Cell membranes behave like electrical capacitors and changes in cell capacitance therefore reflect changes in the cell area. Monitoring capacitance using the patch- clamp technique can thus be used to study dynamic cellular phenomenon involving rapid changes in cell surface, such as exo- and/or endocytosis. Using the equation (C = Cs *A), cell capacitance (C) is thus proportional to membrane area (A), depending otherwise only on a constant, the specific capacitance (Cs) which has been found to be $\approx$10 fF/μm^2. To measure capacitance a voltage signal is imposed onto a cell which is altered continuously by application of a high frequency (600-1000 Hz) sine wave to charge and discharge the membrane. In addition to the capacitive current there is a resistive current through the ion channels within the cell membrane. A lock-in amplifier is used to separate the capacitive from the resistive component of the resulting current. This is known as the Lindau-Neher technique. Voltage-clamp depolarizations can be applied to single alpha and beta-cells to evoke granule exocytosis causing a measureable increase in capacitance.

Freshly isolated islets were dispersed into single cells by trypsin digestion and plated onto plastic Petri dishes. The cells were cultured in RPMI medium containing 10 mmol/l glucose prior to the experiments. Patch pipettes were generated from borosilicate glass capillaries (Harvard Apparatus, UK) using a PIP5 temperature controlled pipette puller (Heka Electronics, Germany). The patch pipettes were coated with Sylgard (Dow Corning) and fire-polished using a Microforge (Narishige, Japan). The tip resistance was 4-7MΩ when filled with the intracellular solutions described below. The pipettes were moved into position using a hydraulic micromanipulator. Cells were visualised using an Axiovert 10 inverted binocular microscope (Zeiss, Germany) mounted on an anti-vibration table (Technical Manufacturing Corporation, USA). A peristaltic pump was used to change extracellular solutions. The bath temperature was maintained at 32°C using temperature controller (Warner Instrument Corporation).

The bath solution was composed of (mM) 128 NaCl, 10 TEACl, 5.6 KCl, 2.6 CaCl2, 1.2 MgCl2, 5 HEPES, and 5 glucose (pH 7.4, adjusted with NaOH). The intracellular solution contained (mM) 125 Cs-glutamate, 10 CsCl, 10 NaCl, 1 MgCl2, 5 HEPES, 0.05 EGTA, 3 MgATP, 0.5 mg/mL biocytin, and (unless indicated otherwise) 0.1 cAMP (pH 7.2, adjusted with CsOH).

To facilitate immunocytochemical identification of cells subjected to capacitance recording, the pipette solution was supplemented with 0.5 mg ml-1 biocytin (Molecular Probes, Eugene, OR). Immunocytochemical identification of the specific cells used for patch-clamp was performed using antibodies against insulin and glucagon following the electrophysiological experiments.

8.6 FIXATION AND EMBEDDING OF SPECIMENS FOR ELECTRON MICROSCOPY

Pancreatic biopsies 1-2mm^3 in size were taken from the body of the pancreas. Each biopsy was immediately placed in freshly prepared 2.5% glutaraldehyde (Sigma, UK) in 0.1M phosphate buffer pH 7.2 and stored at 4^oC for a minimum of 24hrs. Tissue was placed into a glass pot and washed in phosphate buffer (0.1M pH 7.2) three times. 2. 1% Osmium tetroxide (Agar scientific) in phosphate buffer was then added the pot placed on a shaker for 1hr. The specimens were then washed in distilled water three times. The distilled water was discarded and 70% alcohol added for 15 mins. This was followed by a wash in 90% alcohol for a further 15 mins, then three washes in absolute alcohol for 10 mins each. The alcohol was then discarded and replaced with 50/50 Spurr (AGAR scientific) / absolute alcohol and left overnight. The specimens were then placed in pure Spurr and left for a further 24hrs. This was then replaced with fresh pure Spurr once again, then spun and put into oven (50^oC) overnight. Ultrathin sections (70nm thick) were cut onto nickel grids , contrast was enhanced in 2% uranyl acetate and lead citrate and sections were viewed in Joel 1010 electron microscope.

CHAPTER 9 REFERENCES

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