University of Leicester

Department of Inflammation, Infection and Immunity

STUDIES INTO THE EFFICACY OF USING NON-PURIFIED ISLETS FOR CLINICAL ISLET TRANSPLANTATION

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by

M'Balu Alena Webb B.Sc (Leicester) M Med Sci (Sheffield)

University of Leicester

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The work recorded in this thesis is my own original research unless otherwise acknowledged in the text or by references.

M'Balu Alena Webb

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Abstract

Studies into the efficacy of using non-purified islets for clinical islet transplantation
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Islet purification prior to allo transplantation currently forms the clinical gold standard, despite the fact that purification protocols result in significant islet losses and remove potential islet precursors and tissue that may provide islets with bio-trophic support Conversely, non-purified islet preparations retain endocrine mass and within the sphere of islet auto transplantation have been associated with excellent long-term graft function.

This study comprises of an analysis of peri-operative factors and the long-term graft function of patients' auto transplanted with either purified (n=14) or non-purified islets (n=23). Complementary *in-vitro* studies were carried out to assess the effect of non-islet tissue on islet viability, integrity and function (n=8), whilst a histology-based study (n=23) assessed whether transplantation of non-islet cells, particularly islet precursors, had a long-term effect on graft function in the clinical setting.

Clinically, non-purified islets did not significantly increase peri-transplant venous pressures and perioperative factors including ITU stay, blood loss and liver function were comparable in both groups. Analysis of the 5 year post-transplant period demonstrated that although insulin release in response to glucose was initially superior following transplant of purified islets, non-purified islets were associated with stable long-term function. *In-vitro* studies reiterated these findings, revealing that islet viability and function were comparable in both groups, however, retention of intracellular insulin was found to be superior within non-purified preparations with some evidence that ductal tissue provided islets with bio-trophic support. Histology-based analysis of patient pancreata suggested a positive role for islet precursors demonstrating significantly superior blood glucose, HbA1c and C-peptide values associated with the transplantation of ductal cells and non-islet PDX-1 and glucagon positive cells.

The results of this study indicate that transplantation of non-purified islets can be performed safely and with comparable long-term graft function as purified islets. Additionally, these studies potentially suggest that ductal tissue may help to preserve islet integrity, whilst certain precursors cells found within the acinar parenchyma and ductal epithelium may improve long-term islet graft function.

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Firstly I would like to thank Mr Ashley Dennison for providing me with the opportunity of carrying out a PhD, your support in truly appreciated. I would equally like to thank Dr Roger James for his unwavering support and assistance over the past 10 years of research.

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Abbreviations

AIDS	Artificial insulin delivery systems
AIC	Akaike information criterion
ADP	Adenosine diphosphate
Alp	Alkaline phosphatase
Alt	Aspartate aminotransaminase
ATP	Adenosine-5'-triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
BRDU	5-bromo-2'-deoxyuridine
CA19-9	carbohydrate antigen 19-9
CAC	Centroacinar cell
CAII	Carbonic anhydrase II
CITR	Collaborative Islet Transplant Registry
CK7	Cytokeratin 7
CK19	Cytokeratin 19
CRLR	Calcitonin receptor-like receptor
CMRL	Connaught Medical Research Laboratories (cell culture medium)
CVP	Central venous pressure
CP	Chronic pancreatitis
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DMC	Measure of enzyme activity. 1 U catalyzes the cleavage of 1µmole peptide bond from dimethyl casein per minute at 25 °C, pH 7.0
DR3 / 4	Human immune-response D-related antigen encoded by the D locus on chromosome 6
dUTP	2´-Deoxyuridine, 5´-Triphosphate

ECM	Extra cellular matrix
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FDA	Fluorescein diacetate
GLUT-2	Glucose transporter 2
GTT	Glucose tolerance test
HARV	High aspect rotating vessel
HbA1c	Glycosylated haemoglobin
HBSS	Hank's buffered salt solution
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPP	Hepatic portal pressure
kATP	Adenosine-5'-triphosphate sensitive potassium channel
IDDM	Insulin-dependent diabetes mellitus
IDX-1	Pancreatic and duodenal homeobox 1
IEQ	Islet equivalent
IL-8	Interleukin 8
IPF	Pancreatic and duodenal homeobox 1
IRP	Islet recovery percentage
ITU	Intensive care unit
LFT	Liver function test
LS Means	Least Squares Means
MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
NBT/BCIP	Nitroblue tetrazolium / 5-Bromo-4-chloro-3-indolyl phosphate
NIECs	Non-islet endocrine cells
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline

PCV	Packed cell volume
PDX-1	Pancreatic and duodenal homeobox 1
PP	Pancreatic polypeptide
PPA	Percentage positive area
PZ units	Measure of enzyme activity Unit definition: 1 U catalyzes the hydrolysis of 1 µmol of 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine per minute at 25 ℃, pH 7.1
Reg 1	Pancreatic regenerating gene I.
RESC	Rat embryonic stem cells
RPMI	Roswell Park Memorial Institute (medium)
STAT3	Signal transducer and activator of transcription 3
STF-1	Pancreatic and duodenal homeobox 1
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBS	Tris buffered saline
Tdt	Terminal deoxynucleotidyl transferase
TGF-β1	Transforming growth factor beta 1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UHL	University Hospitals of Leicester
UW	University of Wisconsin
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

CONTENTS

Figures

Page

Tables

Chapter 1: Pancreas Biology, Diabetes and Islet Transplantation

1.1 Introduction

The prospect of successful islet transplantation is exciting and potentially offers a cure for diabetes, a condition that affects approximately five per cent of the world's population (1) and has a prevalence that is predicted to double between 2005 and 2030 (2). In the western world, diabetes mellitus is generally not considered a fatal condition as exogenous insulin injections (and increasingly insulin pumps), allow patients to control their blood glucose levels and in the majority of cases lead an active and productive life. However, when compared with the response of islets in the native pancreas, it is clear that exogenous insulin offers a relatively crude mode of glycaemic control and commonly leaves patients open to episodes of hyperglycaemia and less frequently hypoglycaemia linked with excessive insulin administration.

Hyperglycaemia, is a chronic problem associated with type 1 diabetes, which can lead to micro and macro vessel damage, and premature death. Approximately 1.1 million people are estimated to have died from diabetes in 2005 and this figure is likely to be a gross under-estimation as many deaths are simply attributed to heart disease or kidney failure (2). At the other end of the spectrum, hypoglycaemia is a potentially devastating acute complication of the exogenous insulin regimens and is a particular hindrance for sufferers of iatrogenic hypoglycaemia (hypoglycaemia unawareness or brittle diabetes), as they cannot sense and therefore act upon signs of hypoglycaemia, rendering everyday tasks (driving, operating machinery) hazardous.

The Collaborative Islet Transplant Registry (CITR) has recently reported that between January 1999 and December 2008, 828 islet transplants had taken place worldwide with the majority of centres targeting patients with severe hypoglycaemic unawareness,

as they have the most to gain from the procedure (3). Lack of transplantable tissue aside, islet transplantation could potentially be of benefit to all type 1 diabetic patients and possibly even type 2 patients with early onset of the disease. Potentially, lives could be saved and the lifespan increased for a significant proportion of the world population. This review chapter will primarily look at pancreas and islet biology and will provide a detailed rational for islet transplantation in comparison with traditional treatments. It will also explore areas where current islet transplant practices can be improved in order to enhance transplant outcomes and maximise the number of patients that could be treated.

1.1.1 The human pancreas

The adult pancreas is a lobulated, soft, flattened and elongated organ of 12-20cm weighing between 70-110g. It lies behind the peritoneum of the posterior abdominal wall and is totally invested in fine connective tissue. The pancreas is intimately associated with more anatomical entities that any other organ in body including the liver, duodenum, spleen, left kidney, aorta, vena cava and superior mesenteric vessels.

The endocrine portion of the pancreas consists of approximately 1 million islets of Langerhans, which are distributed throughout the gland but are relatively concentrated in the tail of the organ. In order to sense nutrient levels in the blood, each islet is associated with a prominent capillary plexus, which has an efferent blood supply leading to the pancreatic acinar tissue. The major islet cell types are α, β, δ and PP cells which secrete glucagon, insulin, somatostatin and pancreatic polypeptide respectively, however, β-cells make up the majority of the endocrine pancreas contributing to approximately 70% of the cells. Research in the last two decades has also revealed a substantial number of non-classical islet hormones and these are outlined in Table 1.1.2.

1.1.2 Insulin

Insulin, secreted by the β-cells, is the dominant hormone in the neurological and humoral pathways that control fuel economy. It is most markedly released in response to raised blood glucose, amino acids, ketone bodies and fatty acids, with glucose as its most potent physiological stimulus. In general terms, insulin is an anabolic hormone, accelerating glucose uptake into a number of tissues while simultaneously suppressing gluconeogenesis and lipolysis (Figure 1.1.1). Insulin release (and indeed release of counter-regulatory hormones) is a precise phenomenon, which, in non-pathological conditions, accurately maintains blood glucose levels between 5-7mmol/l.

Approximately half of all insulin is released during fasting conditions, limiting endogenous glucose production. However in response to increased blood glucose following feeding (>5mmol/l), insulin is released in a bi-phasic manner. Firstly there is a quick release of high concentration insulin (spike) closely followed by a prolonged second phase at a lower concentration, which continues until the stimulus has been cleared. Figures 1.1.1 and 1.1.2 clearly illustrate that insulin secretion is a meticulous and finely tuned response, which would prove incredibly difficult to mimic exogenously. Moreover, studies carried out in the 1970's (4,5) have shown that the mere sight and smell of food and physical satiety stimulate insulin secretion before food absorption has occurred, confirming that there are a number of intrinsic mechanisms that protect against the inevitable hyperglycaemia that follows feeding.

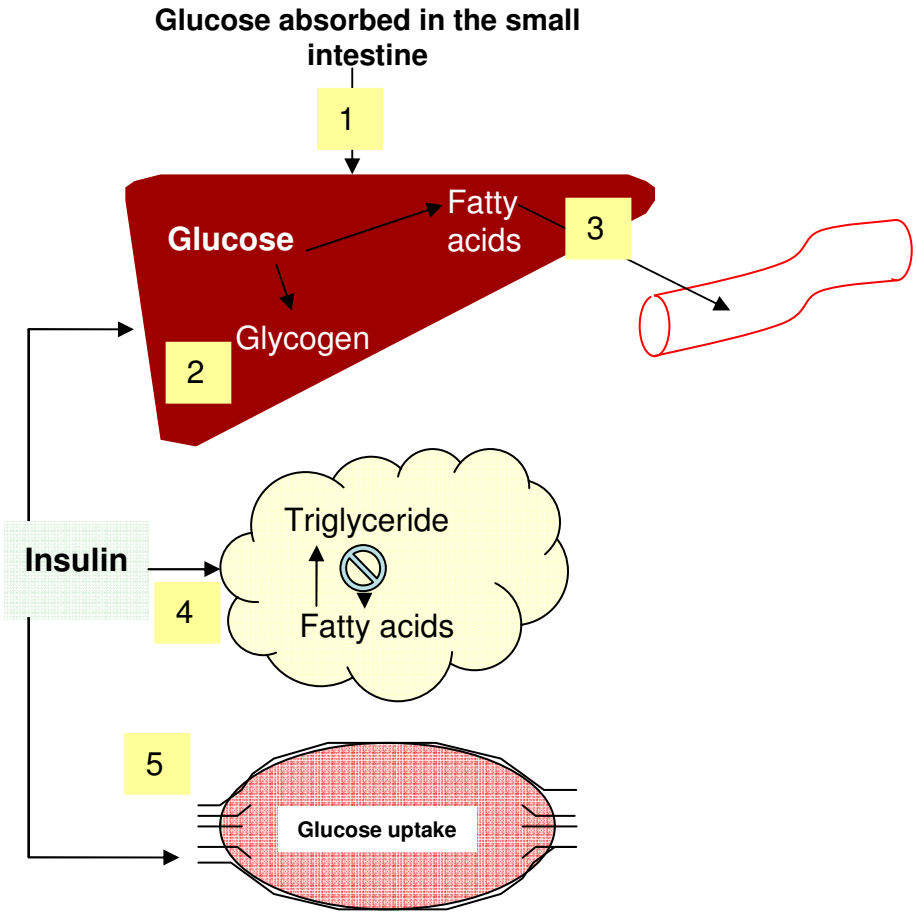


Figure 1.1.1. Insulin action. ***1****. A large fraction of glucose absorbed from the small intestine is immediately taken up by hepatocytes.* ***2.*** *Insulin has several effects on the liver which stimulate glycogen synthesis. Firstly, it activates the enzyme hexokinase, which phosphorylates glucose, trapping it within hepatocytes. Coincidently, insulin acts to inhibit the activity of glucose-6-phosphatase. Insulin also activates several of the enzymes that are directly involved in glycogen synthesis, including phosphofructokinase and glycogen synthase.* ***3****. When the liver is saturated with glycogen, any additional glucose taken up by hepatocytes is shunted into pathways leading to synthesis of fatty acids, which are exported from the liver as lipoproteins. The lipoproteins are broken down in the circulation, providing free fatty acids for use in other tissues, including adipocytes, which use them to synthesize triglyceride* ***4****. Insulin inhibits breakdown of fat in adipose tissue by inhibiting the intracellular lipase that hydrolyzes triglycerides to release fatty acids.* ***5****. Insulin also stimulates the uptake of glucose and amino acids into muscle and other organs of the body.*

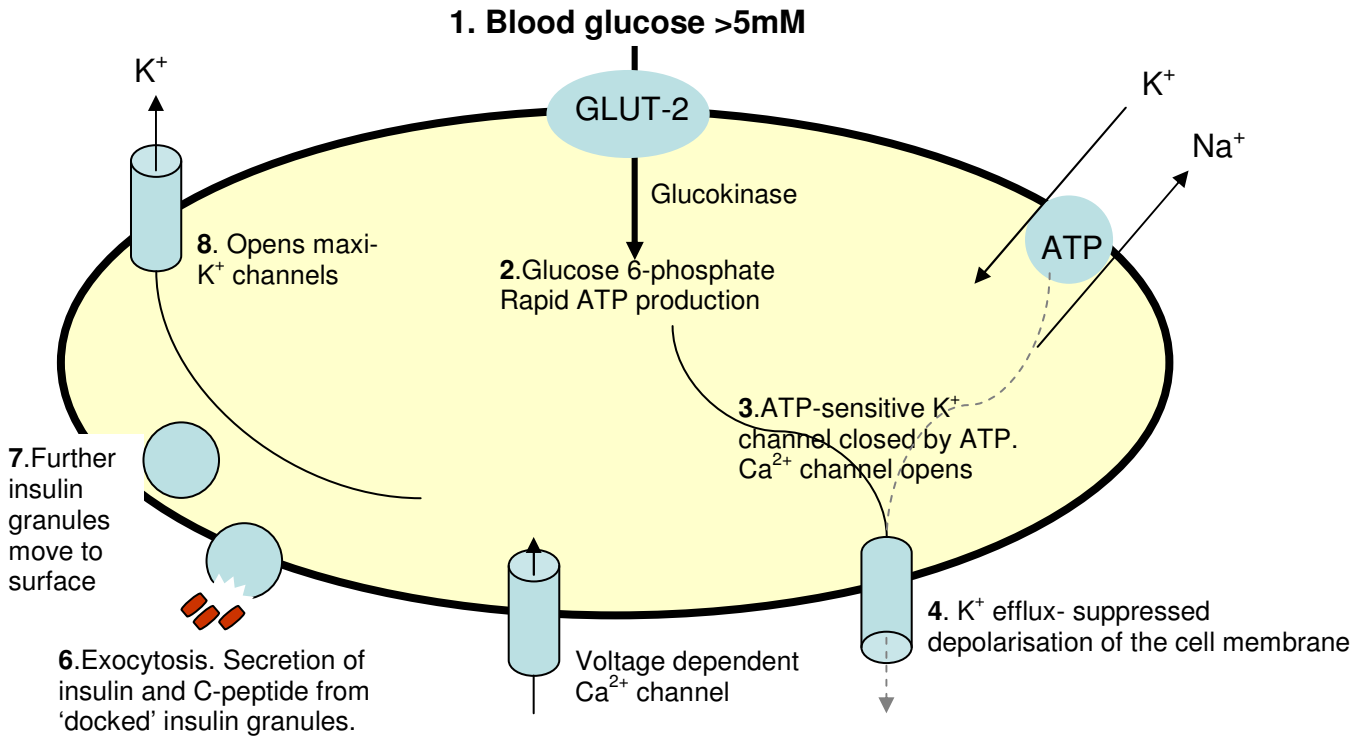


Figure 1.1.2. Insulin release in response to glucose. *In the resting state the kATP channels of the β cell are open and the ATP/ADP ratio is low. The constant outflow of positively charged K+ through the open kATP channels accounts for the negative resting membrane potential of approximately (–70mV). Elevation of extracellular glucose results in glucose transportation into the β cell via GLUT 2 transporters (1). Glucose metabolism is subsequently initiated and this in turn leads to an increase in the cytosolic ATP/ADP ratio (2) which then leads to the closure of the kATP channels (3). Suppression of K+ efflux acts to depolarise the membrane, opening voltage dependent Ca2+ channels and increasing the cytosolic Ca2+ concentration (4&5). The sudden Ca2+ influx initiates exocytosis of insulin granules (6). This first phase response uses insulin granules already docked at the membrane surface and makes up 5% of the insulin released after a meal. The second phase secretion can only be elicited through metabolizable stimuli suggesting that unlike the 1st phase secretion, it is an energy dependent process, and requires ATP to move insulin from a reserve pool to the membrane surface (7). The process continues until the glucose stimulus has cleared.*

1.1.3 Type I Diabetes mellitus

With the onset of diabetes mellitus glucose homeostasis is profoundly disturbed. Diabetes mellitus (DM) consists of a group of diseases characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action or somatic response to insulin. Although the symptoms can be managed by relatively simple means, DM is cited to be amongst the 5 leading causes of death in most countries (1). It exists in two major forms.

Type 1 diabetes or juvenile onset diabetes is characterised by absolute insulin deficiency, abrupt onset of symptoms, proneness to ketosis and a dependency upon exogenous insulin to sustain life. The islets of Langerhans are destroyed, probably as a consequence of a genetic susceptibility (6) followed by the onset of autoimmune destruction triggered by a combination of environmental factors including viral infections (7) (Figure 1.1.3). The number and size of islets is eventually reduced, leading to decreased insulin production and glucose intolerance, which leads to hyperglycaemia.

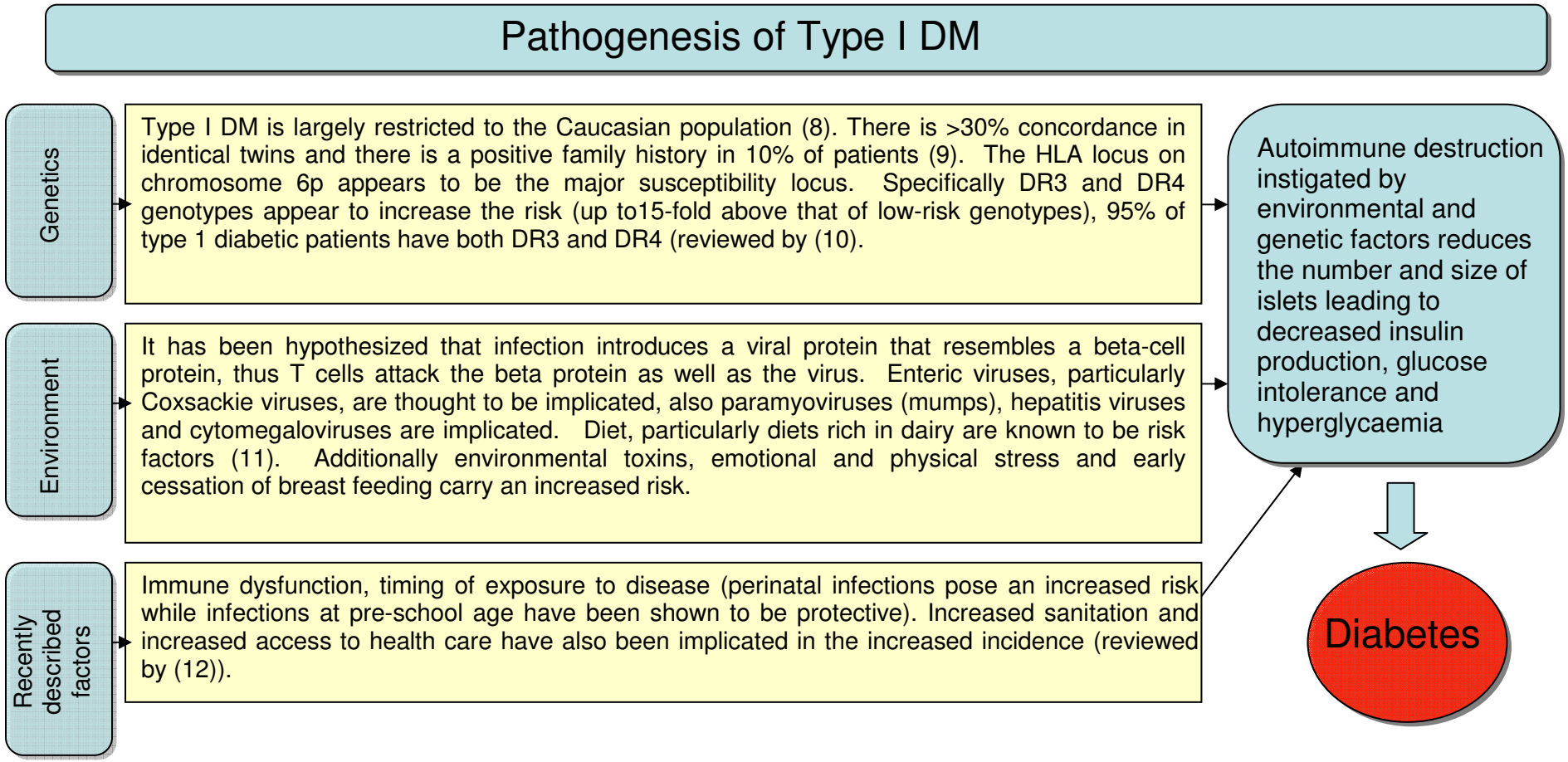


Figure 1.1.3. Factors contributing to diabetes. *The traditional view that a single environmental insult instigates type 1 DM in genetically predisposed individuals has changed with current studies showing a more complex pathogenesis where environmental insults have been shown to both promote and attenuate the disease depending on an the stage of development. The proposal of a more complex model may explain why subtle factors unrelated to the well-recognised environmental insults (e.g. improved healthcare and sanitation) coincide with increased incidence of disease (reviewed in* (10)*).*

1.1.4 Type 2 Diabetes Mellitus

Currently type 2 DM individuals are not considered for islet transplantation, however the progressively early onset of the disease and its growing prevalence makes type 2 diabetes a potential healthcare epidemic within this lifetime. Type 2 DM accounts for approximately 90% to 95% of all cases of DM mainly affecting the over-weight, sedentary and mature (13), although genetics and previous medical history (e.g. hypertension) are risk factors (refer to Figure 1.1.4). Unlike Type 1 diabetes which is largely limited to Caucasian children and young adults, particularly of north-west European origin (8), type 2 DM is not limited to any ethnic grouping, although it's prevalence is particularly progressive in developing nations and amongst ethnic minorities in developed nations. This phenomenon has been expressed as the accumulation of 'thrifty' genotypes in a seminal paper by Neel 1962 (14), predisposing individuals to abdominal fat, energy preservation and insulin resistance which may be beneficial traits during times of famine but may actually shorten lifespan in a 'developed' society.

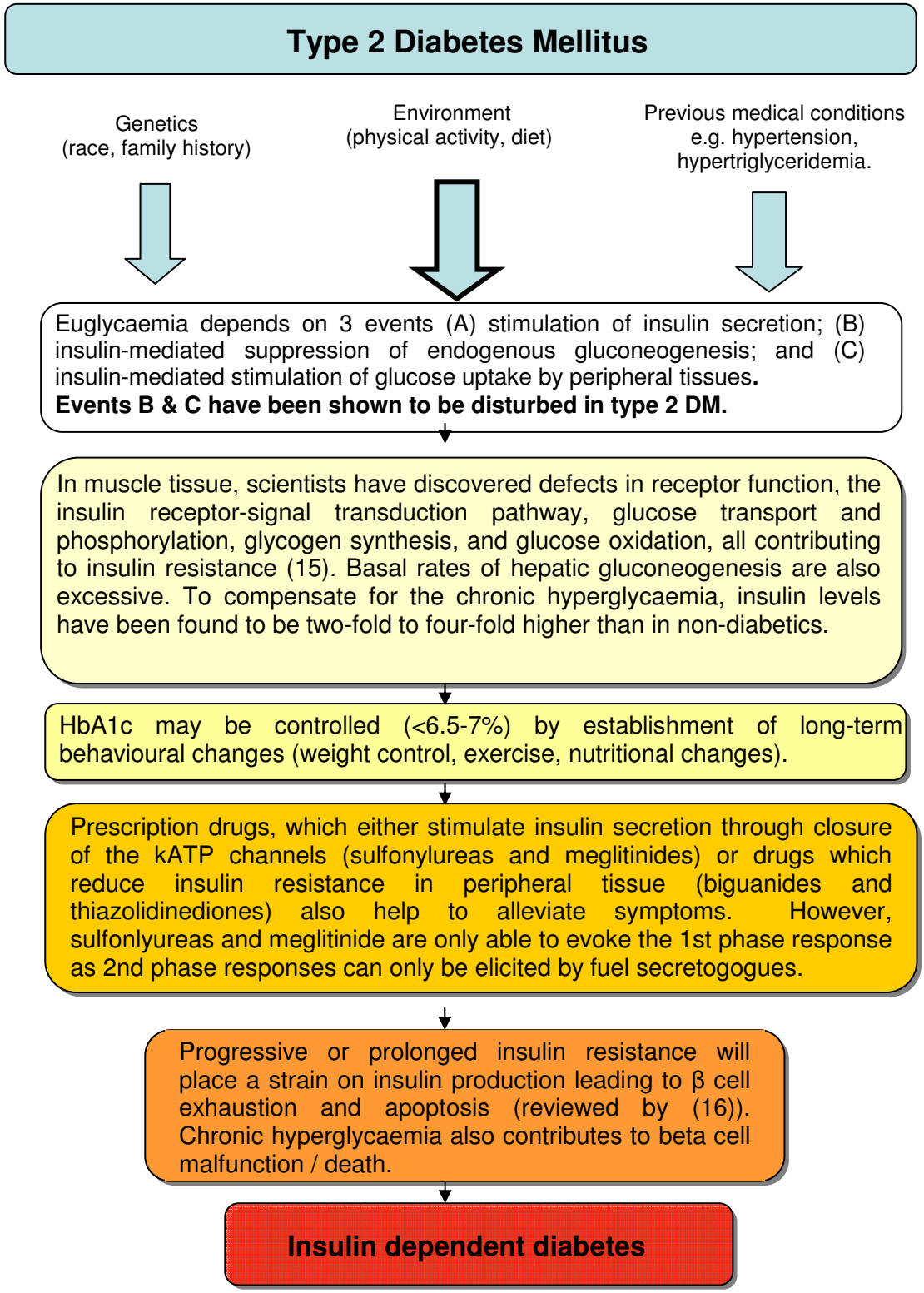


Figure 1.1.4. The progression of type 2 diabetes into insulin dependent disease

Patients that have no residual islet capacity are of primary concern. For these patients, secondary diabetic complications (discussed in Section 1.1.5) dramatically increase morbidity and decrease lifespan. Additionally, within this group of patients exists a group of individuals designated 'brittle diabetics' for whom mortality becomes a daily issue, primarily due to hypoglycaemia unawareness, these factors are discussed in detail below.

1.1.5 Secondary complications of IDDM

The use of daily insulin injections is currently the main treatment of insulin dependent DM and patients can take a combination of basal (long-acting) and bolus injections to maintain euglycaemia during rest and at mealtimes, reducing doses in response to exercise. However, standard exogenous insulin injections are often not precise enough to prevent secondary diabetic complications, caused by chronic hyperglycaemia and although tight glycaemic control can mitigate the onset of complications this can be at the expense of severe hypoglycaemia (Section 1.1.6) Recurring episodes of hyperglycaemia cause a non-enzymatic attachment of glucose to body proteins by the process of glycolation (Figure 1.1.5) which, over time leads to the secondary complications of diabetes which include; neurological and renal damage, retinopathy, peri-natal problems and an increased risk of stroke and heart disease (Table 1.1.1) (17,18). In fact, the global mortality attributable to diabetes has been estimated at between 1.1million (estimated in 2005) (2) and 2.9 million (estimated in 2000), representing up to 5% of all deaths (1).

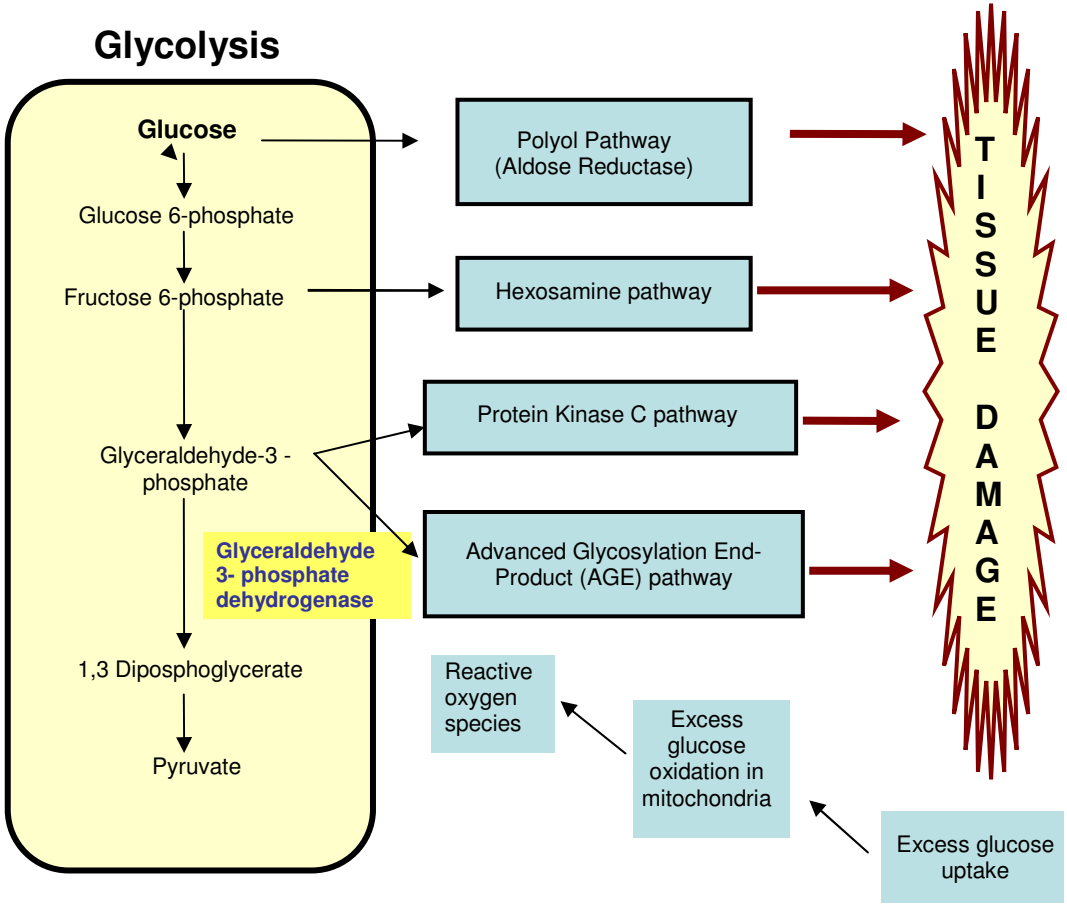


Figure 1.1.5. The mechanisms by which hyperglycemia causes tissue damage. *Vascular endothelial cells are an important target of hyperglycaemic damage, but the mechanisms underlying this damage are not fully understood. Three seemingly independent biochemical pathways are involved in the pathogenesis: glucose-induced activation of protein kinase C isoforms (19), increased formation of glucose-derived advanced glycation end-products reveiwed by (20) and increased glucose flux through the aldose reductase pathway 4 (21).*

Complication	Implication
Neuropathy	Second commonest cause of lower limb amputation
Renal damage	Leading cause of end stage renal failure
Retinopathy	The leading cause of blindness of people of working age
Stroke	4 fold increased risk
Heart Disease	3-4 fold increased risk
Pregnancy	Increased risk of miscarriage and congenital malformation (22,23) and increased death in infancy (17)
Life expectancy	Reduced by approximately 15 years
Erectile dysfunction	3 fold increased risk
Depression	3 fold increased risk

Table 1.1.1. The health implications of secondary diabetic complications. *Information provided by the National diabetes support team unless otherwise stated* (24).

1.1.6 Insulin administration and associated complications

1.1.6.1 Insulin Injections

Subcutaneous injections of insulin are frequently imprecise. A fraction of the dose is delivered intramuscularly and other factors such as temperature of the skin and muscle contractions have an effect on delivery. Perhaps most importantly subcutaneously injected insulin is absorbed into the peripheral venous circulation instead of the hepatic portal system, thus liver related actions (e.g. suppression of gluconeogenesis and glycogen production) are delayed. Furthermore a significant proportion of the insulin is degraded in the bloodstream due to the fact that insulin has a half life of 15 min.

1.1.6.2 Intensive insulin regimes

The major problem associated with diabetes is that the majority of diabetic patients fail to achieve glycaemic targets using standard insulin regimes and when intensive regimes are implemented, this is often leads of increased episodes of severe hypoglycaemia. This was eloquently demonstrated by the Diabetes Control and Complications Trial of 1993 (DCCT) and reiterated in the follow-up study, the Epidemiology of Diabetes Interventions and Complications study (EDIC) of 2005

(25,26). For these studies the effects of intensive insulin management was assessed with intensive management being classed as 1) testing blood glucose levels four or more times a day 2) injecting insulin at least three times daily or using an insulin pump 3) adjusting insulin doses according to food intake and exercise 4) following a diet and exercise plan and 5) monthly follow-up by a physician, nurse educator, dietician, and behavioural therapist. The DCCT study revealed that tight glyceamic control reduced the risk of eye, kidney and nerve disease by 76%, 50% and 60% respectively, while the EDIC showed a 42% reduced risk of cardiovascular disease events and a 57% reduced risk of non-fatal heart attack, stroke, or death from cardiovascular causes. Although both trials undoubtedly demonstrated that tight glycaemic control, including the use of insulin pumps, was important in preventing secondary diabetic complications, the intensive therapy used within both trials was also associated with a 2-3 fold increase severe hypoglycaemia.

Current insulin pumps offer a relatively effective and safe mode of insulin delivery, using glucose values input by the user to calculate and release amounts of insulin that will counteract hyperglycaemia. Although they have been well received, problems associated with the delivery of insulin remain (kinks within tubing are common) and the sometimes cumbersome nature of these devices make them less than ideal. Additionally the increased risk of hypoglycaemia remains an area of concern (25). In the field of insulin delivery, the ultimate goal is the development of a constant glucose monitoring device coupled with insulin pump technology, creating a closed circuit system or ‘artificial pancreas’ which should closely mimic physiological insulin release, radically reducing the incidence of secondary diabetic complications and hypoglycaemic events. Although the development of the artificial pancreas will potentially revolutionise the treatment diabetes and has been

the focus of research for more than 30 years, to date it remains in the research and development stage, as such, glycaemic control remains a problem

Hypoglycaemia from insulin excess results from pharmacokinetic imperfections of all insulin preparations and insulin secretagogues and environmental factors such as food intake, exercise, drug (including alcohol) interactions, altered sensitivity to insulin, and insulin clearance. It represents a particularly dangerous acute complication of the pharmacological treatment of diabetes, principally because low blood sugar can impair both brain and cardiac function and may cause sudden death. Population studies have suggested that hypoglycaemia-related mortality accounts for between 2-10% of deaths in type 1 diabetic patients (27) and is a particular problem for patients with hypoglycaemia unawareness, a condition that results from the interplay of therapeutic insulin excess and compromised physiological (defective glucose counter regulation) and behavioural defences (hypoglycaemia unawareness) against falling plasma glucose concentrations. It has been shown that episodes of severe hypoglycaemia, which is defined as an event requiring assistance of another person to actively administer carbohydrate, glucagon, or other resuscitative actions (28), further impairs the ability to sense hypoglycaemia as such patients remain in a vicious circle of hypoglycaemia and experience great anxiety associated with the condition. Although episodes of hypoglycaemia can be reversed by relaxing glycaemic control in the majority of patients this risks the complications of high glucose. As such the pharmacological treatment of diabetes does not, at present, provide the perfect treatment.

1.1.7 Burden of diabetes on society

DM places a huge burden upon the health care resources throughout the world. In the UK, 9% of all hospital in-patient resources and 5% of the total NHS budget is

used to care for diabetic patients (24). Additionally, The World Health Organisation (WHO) forecast that prevalence will double over a 25 year span (between 2000 and 2025) and consequently the diabetes epidemic will continue to stretch the health-care resources throughout the world**.** Although the growing tide of diabetes sufferers fall into the type 2 category, it is predicted that due to changes in diet and inactive lifestyle, type 2 diabetes (T2DM) will no longer be restricted to the middle aged / elderly but will become a disease of the young obese (29), leaving these individuals open to the severe secondary complications usually found in type 1 diabetic patients.

Complications associated with diabetes mellitus and diabetic hyper/hypoglycaemia create a clear patient-based and financial incentive to seek other methods of providing patients with an improved mode of glycaemia control.

1.1.8 Transplantation, a curative treatment for diabetes?

Allotransplantation of human insulin secreting tissue potentially offers DM patients finely tuned and physiologically accurate glycaemic control, with avoidance of complications related to hyperglycaemia and hypoglycaemia. Additionally, it forms a particularly useful treatment for type 1 diabetic patients who have previously undergone renal transplantation and are already immuno-suppressed.

Criteria other than euglycaemia should also be considered when assessing treatments for diabetes. Although it had been thought that C-peptide was merely a waste product of insulin biosynthesis there is growing evidence that this molecule has a physiological role in its own right. Beneficial effects of C-peptide in diabetic patients have been found to include improved nerve function (30), augmented blood flow in skeletal muscle and skin, attenuation of glomerular hyperfiltration, reduced urinary albumin excretion (reviewed by (31)) and restoration of Na^+-K^+-

ATPase activity which has been shown to diminish in diabetic patients (32). Furthermore, research in the last two decades has revealed a substantial number of non-classical islet hormones, the roles of which are outlined in Table 1.1.2. The majority of these hormones have a profound effect on acinar and ductal cell secretions however, their functions have not been fully characterised and they may have additional unrecognised effects.

Hormone peptides	Localisation	Effects
Amylin, also called IAPP (islet amyloid polypeptide)	Alpha and beta cells	↑release of amylase from the cell-line AR42J but not from isolated acini
Adrenomedullin	PP cells	CRLR not detected Amylase secretion from AR42J cells
Calcitonin gene-related peptide	Subpopulation of somatostatin cells	CRLR not detected. ↓HCO_3^- duct secretion[a] ↓caerulein-evoked trypsin and amylase output in human[b]
C-peptide	Beta cells	Full affects refer to above text. ND of C-peptide receptors on duct cells.
Pancreastatin	Alpha and beta cells	Probably null effect on duct cells. ↓acinar protein output[c]
Secretoneurin	Alpha and beta cells	ND
Ghrelin	Epsilon cells	Inhibits total protein and amylase secretion[c,d]
Cortistatin	Single islet cells	ND
Orexin A	Alpha and beta cells	↑amylase release from AR42J cells
Resistin	Insulin cells	ND
Urocortin III	Beta cells	↑ the volume of pancreatic juice, protein secretion[c,d]
Corticotropin-releasing factor	Alpha cells	↑the volume of pancreatic juice, protein secretion[c,d]
5-hydroxytryptamine	Enterchromaffin Cells	ND
Peptide YY	Alpha cells and some PP cells	Receptors found on duct. ↓ protein content of pancreatic ↓HCO_3^- content of pancreatic juice in unstimulated as well as nutrient-, hormone-, and vagally stimulated animals.

Table 1.1.2 Non-classical hormones of the pancreas*. Adaption from table produced by Bertelli et al 2005 describing the effects of non-classical hormones on primarily ductal and acinar cell function. (33) [a] Action probably mediated via intra-pancreatic neural terminals and induction of somatostatin release. [b] Caerulein-stimulated. [c] Action probably mediated via intra-pancreatic neurons. [d] CCK-stimulated. ND, not determined; CRLR, calcitonin receptor-like receptor—a receptor subunit of either adrenomedullin or calcitonin gene-related peptide receptors. The AR42J cell-line (*) is derived from a chemically induced rat pancreatic acinar carcinoma and secretes amylase and other digestive enzymes.*

1.1.9 Islet transplantation

Islet transplantation is being promoted as the cure for the wider diabetic population. In contrast to the transplantation of solid organs, it is a minimally invasive procedure that utilises a relatively beta-cell sparing immunosuppressive regimen (34). Although, islet isolation is a meticulous procedure, islet transplantation can be performed radiologically and repeated several times without any major discomfort to the patient. Following transplantation into the liver, islets have been shown to engraft within 2 weeks (35) with the most successful groups in the world restoring euglycaemia in 80% of patients for more than 1 year (34).

1.1.10 The islet transplantation protocol

The basis of the islet isolation procedure is explained in Chapter 2 Figure 2.1.1. Recovering the islets uses a modification of a method originally described by Ricordi and colleagues in 1988 (36). In brief, the pancreas is injected with a collagenase enzyme, which digests the pancreas producing a slurry largely containing islets, exocrine tissue and ductal structures. Based on their different densities, islets can be separated from the exocrine and duct tissue on a continuous density gradient; however for islet auto transplantation this step is often omitted to prevent islet loss (discussed in Section 1.1.15). Finally the islets are transplanted into the liver either directly into the portal vein, or via an omental or recannulated umbilical vein which drains into the portal system.

1.1.11 Islet versus other transplant therapies

1.1.11. 1. Pancreas Transplantation

At present, the two types of clinical transplants available for the treatment of diabetes are whole pancreas transplantation and islet transplantation**.** Transplantation of whole vascularised pancreata has been successfully used to produce insulin independence in over 23,000 patients (as of December 2004 (37)) and is a well-established procedure practiced worldwide. However, the operation is a long and complex and the glucocorticoid based immunosuppressive regime required has been shown to be both deleterious to islet viability and to exacerbate insulin resistance (38) and reviewed by (39) sometimes negates the positive effects of surgery. Indeed, a 2003 study by Venstrom and colleagues (40) showed that solitary pancreas transplantation decreased overall survival for those patients with normal kidney function. Therefore, pancreas transplantation is commonly only offered to those patients who are already immune-suppressed for a previous or concomitant kidney transplant.

1.1.11.2. Human 'pseudo-islets'

One of the most anticipated successes of islet transplantation is that surgical protocols developed for transplantation of isolated islets of Langerhans could be transferred to the transplantation of pseudo-islets that have been developed from stem cells and / or pluripotent cells. As embryonic stem cells display the ability to differentiate into a variety of cell lineages *in-vitro*, several groups have successfully created insulin secreting islet-like clusters (41). Similarly, clusters have also been created from transdifferentatied acinar tissue (42), the ductal cells of the pancreas (43,44) and from hepatic cells, which share a similar lineage to pancreatic cells (45-47). Currently pseudo islets are not clinically effective largely due to low levels of insulin secretion (typically 50-fold less than intact primary beta cells) and those propagated from embryonic stem cells carry the added risk of tumour initiation. In

2005 Fujikawa and colleagues described the formation of teratoma after the transplantation of embryonic stem cell-derived pseudo-islets were transplanted into a diabetic mouse model (48). However, such research continues to offer tremendous potential for the future especially when one considers that an auto transplantation of stem cells would omit the need for immune-suppression.

1.1.12.3. Xenotransplantation

The final area of research is xenotransplantation although the transplant of animal islets is not commonly practised due to the marked immunological barriers between species (49). In addition, xenografts potentially pose a risk to public health due to the real possibility of cross species infection due to porcine or other endogenous retroviruses adapting to human hosts (50).

Thus, to date *in-vitro* manipulations have failed to produce cells with a true beta cell phenotype and xenotransplantation is currently deemed unsafe for widespread patient application. Although whole pancreas transplantation undoubtedly provides excellent glycaemic control the substantial immune-suppressive regime required is potentially life-limiting for the treatment of type 1 diabetic patients with minimal renal disease. With these factors in mind, islet transplantation forms the only feasible transplant therapy for the treatment of diabetes, although much research and development is still required to optimise islet isolation / transplantation protocols.

1.1.12 Drawbacks to widespread implementation of islet transplantation

Although islet transplantation is becoming increasingly integrated into clinical practice in America, Europe and more recently Australia (3), many hurdles still exist. Islet recovery remains a significant obstacle and poor islet survival post-transplantation has contributed to disappointing 5 year outcomes reported by the

Edmonton group (51) arguably the most established team in the field. Refinements of the islet isolation and transplantation process (reviewed in Table 1.1.3) will undoubtedly improve current islet transplant outcomes. However, arguably the most pressing issue is the lack of insulin secreting tissue and there is a pressing need to recover the maximum number of islets from each donated pancreas. This issue has never been appropriately addressed particularly in light of the fact that of the 1 million islets within the intact pancreas only approximately one third to one half are recovered routinely for clinical transplantation purposes (3).

It is widely accepted that within the islet isolation process, islet purification is associated with the most significant losses, with in many cases, the majority of islets being contained within the impure fractions following COBE purification (refer to section 2.1.1). Considering the fact that omitting the islet purification step will permit a significantly higher recovery of islets per organ the poignant question is:

Is islet purification necessary?

Stage of Protocol	Target of reform	Current areas of research /improvement
Organ procurement	The loss and damage of islets can occur as early on in the process as organ procurement and each step of the procedure can result in further insult to the islet tissue (52).	The two-layer method using traditional UW preservation solution and oxygenated perflurocarbon to some extent mitigates organ hypoxia.
Enzymatic digestion	Islet isolation, exposes the islet to various forms of cellular stress, including disruption of the cell-matrix relationships, an event that is associated with apoptosis. Studies have shown that the Ricordi system can cause over-digestion of some islets (53)	On-going research into enzyme efficiency (54) may further improve islet viability.
Islet processing	**The majority of islet isolations fail to reach adequate quantity for transplantation** (55) **set for transplantation** (56)**. Islet purification is particularly associated with substantial islet losses** (3)**.**	**Rescue purification gradients recover a percentage of the islets lost during COBE purification** (57)**. The use of non-purified islets** (58,59)
Islet engraftment	Delayed vascular connection resulting in prolonged hypoxia, tissue factor production resulting in activation of coagulation, and inflammation (60,61) have been shown to contribute to islet loss. Graft vessel density remains significantly lower than native islets impairing function (62,63)..	Experimental models show that elevated VEGF improved local angiogenesis (64)
Immune suppression	The side effects of immune-suppression, which include opportunistic infection and reduced tumour surveillance are well documented (65). Moreover the immunosuppressive regimens frequently used have been shown to be diabetogenic (66,67).	Antibody-led technologies may provide safe targeted immune-protection of transplanted islet (68). Islet micro-encapsulation is also under investigation. Studies co-transplanting islets with haemopoetic stem cells show induction of recipient chimerism (69).
Recurrence of beta cell autoimmunity	Recurrent autoimmune beta-cell destruction may contribute to the poor results of clinical islet transplantation (70).	Whole pancreas transplants or inclusion of peri-pancreatic lymph nodes abrogates autoimmunity by passive transfer to the host of an auto-regulatory T-cell subset

Table 1.1.3 The major targets for the improvement of the current methods for islet isolation and transplantation. *Arguably one of the most pressing and easily remedied problems is the failure to recovery the majority of islets from each pancreas.*

1.1.14 Purification gradients

Purification effectively reduces the volume of tissue that will be implanted into the patient, therefore minimizing the risks associated with intra-portal islet infusion (i.e. increased portal pressure and thrombosis) (71). Additionally, it still remains controversial whether purification of the islet preparations may also lower the immunogenicity of the graft (72). On the other hand, current data has also shown that frequently, human islet preparations with adequate quality and potency pre-purification cannot be used for clinical transplantation because the final (post-purification) islet yield falls below the required minimum (55,57). Indeed publications from experienced islet groups in the field show that only between 30% and 50% of clinical islet isolations are actually used for transplantation (55,57,73).

Additionally, studies carried out by the highly reputable Giessen group in Germany have demonstrated that the purification process is associated with an abrupt loss of intracellular insulin, which it is postulated may lead to beta cell exhaustion thus affecting long-term islet function (52).

	N	Mean ($X10^3$)	SD ($X10^3$)	Median ($X10^3$)	Min ($X10^3$)	Max ($X10^3$)
Pre-purification	9	617	307	580	260	1122
Post Purification	403	384	156	355	85	973

Table 1.1.4 Total islet equivalents (IEQs) measured pre and post purification. *Data published by the CITR and represents data collected between 1999 and 2007 in North America* (74) *. Despite the different sample sizes and the fact that non-paired data is available, the pre-purification IEQs far exceed post purification counts.*

It is therefore important to consider whether islet purification is a necessary part of the islet transplantation protocol. This is particularly so in the light of the

outstanding long-term graft survival of patients who have undergone pancreas resection with concomitant islet auto transplantation for the treatment of chronic pancreatitis (75,76). Therefore to complete the present chapter the following section will provide a synopsis of chronic pancreatitis, islet auto transplantation and islet autograft function.

1.1.15 Islet auto transplantation

Islet auto transplantation is offered to patients that have received pancreatectomy for the treatment of intractable pain associated severe chronic pancreatitis. Compared with diabetes, the prevalence of candidates suitable for pancreatectomy with combined islet transplantation is estimated at 150 patients per year in the UK (based on local unpublished data). However, due to the fact that islet auto transplantation is a successful and proven clinical procedure (76), evaluation of islet auto transplantation results have proved invaluable in the field of islet transplantation (77).

1.1.15.1 Background of chronic pancreatitis

Chronic pancreatitis (CP) is an inflammatory disease that causes progressive fibrosis of the pancreatic parenchyma and endocrine component of the pancreas in the later stages. Rates of disease are not available for the UK, however, an exhaustive study of the French population estimated incidence rates of 7.7 per 100,000 whilst prevalence was estimated at 26.4 per 100,000 (78). Severe pain is reported by 80-90% of CP patients (79-82) and has been shown to lead to a poor quality of life, narcotic addiction and increased economic costs (including medical and social) (83). Within the Leicester series, alcohol has been shown to be the cause in approximately one third of cases, although the aetiology may also be idiopathic, genetic, autoimmune, caused by recurrent acute pancreatitis or pancreatic duct obstruction (75). An example of a chronic pancreatic gland compared with a normal pancreas is shown in Figure 1.1.6.

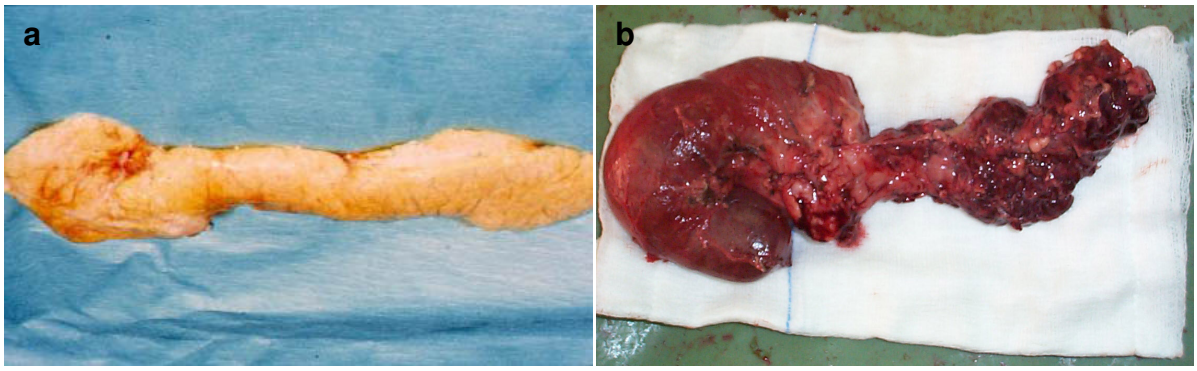


Figure 1.1.6 A normal pancreas versus the chronic pancreatitis gland. *A typical normal pancreas (a) compared with a chronic pancreatitic pancreas (b).*

The exact process by which fibrosis progresses over the long-term is poorly understood however the initiation has been elucidated and is well described by Kloppel et al 2006 (84). It has been shown that one of the compartments (ductal, acinar or mesochymal) of the pancreas is damaged, which itself initially causes local cell death (by necrosis or apoptosis). This event triggers the release of cytokines (e.g. TGFB1) and chemokines from inflammatory cells (e.g. macrophages, local mesenchymal cells or epithelial cells). Next the dead cells are phagocysed by macrophages and the cytokines released cause proliferation of fibroblasts in the site of injury and induce them to transform into myofibroblasts (activated pancreatic stellate cells). Finally the fibroblasts and myofibroblasts deposit ECM, which replaces the inflammatory infiltrate and has an affect on the arrangement of the surviving tissue. At the end of the injury the pancreas shows various patterns of fibrosis.

The disease is characterised by disabling pain, steatorhea (malabsorption) and fear of eating due to the associated pain. The actual cause of the pain associated with CP is poorly understood, although possible mechanisms include elevated pressures within the pancreatic duct, abnormal pancreatic blood flow and changes of the pancreatic nerves (reviewed by (85)). Conservative medical management

(e.g. analgesia and digestive supplements) and minimally invasive surgical procedures (e.g. pancreatic drainage) often alleviate the severe pain associated with CP, however the disease tends to have progressive nature and as such patients may ultimately require either partial or total pancreatectomy (86-89).

1.1.15.2 Surgical intervention

Pancreatectomy is considered as the treatment of the last resort as it leads to post-surgical diabetes and exocrine insufficiency. However, a prospective study carried out by Ammann and colleagues demonstrated that 75% of patients with CP developed diabetes over the 10-year time course, at a mean of 5.72 years after onset. (90) Additionally, due to the progressive loss of glucagon and pancreatic polypeptide secretion caused by CP, the type of diabetes which develops (pancreatogenic) resembles the endocrine insufficiency that follows pancreatic resection (91), in that there is an increased risk of hypoglycaemia due to impaired glucagon secretion and patients are liable to complications associated with poor glycaemic control (although there is a paucity of data in this area).

1.1.15.3 Rationale of islet auto transplantation

To abrogate the onset of surgically induced diabetes, several centres around the world, including Leicester, have the expertise and facilities to auto transplant pancreatic tissue from CP pancreata into the liver of the patient. Auto transplantation offers patients the possibility of retaining insulin independence post pancreas resection. Although the majority of patients do require some exogenous insulin post pancreatectomy (75), they still have the benefits of long-term endogenous insulin secretion. Indeed, due to the inevitable loss of beta cell mass in CP patients with time (92), early resection of the pancreas has been shown to be associated with superior islet recovery, improving the chance of insulin independence following islet autotransplantation (93,94).

1.1.15.4 Results of islet autografting

In contrast to islet allo-transplantation, islets have often been auto transplanted in their non-purified state to retain maximal levels of insulin-secreting tissue This factor is particularly pertinent within the Leicester series where assessment of pancreatic digest has shown that over 40% (+/-4.11) of islets are 'mantled' islets (75) and would therefore be largely lost within the acinar rich fractions during islet purification (95).

Islet auto transplantation of non-purified islets goes against the substantial body of experimental evidence which suggests that islet-acinar co-localisation may be deleterious to islet integrity (96,97). Although, this experimental data and in-depth analysis cannot be dismissed, ***clinical*** follow-up data has clearly demonstrated that transplantation of non-purified islets can provide prolonged insulin independence following total pancreatectomy (76). In fact, to date, auto transplantation of non-purified islets seems to provide the most successful insulin independence rates within the entire field of islet transplantation (Table 1.1.5) although the superior success of auto transplantation is clearly multi-factorial. As the problems related to rejection and the recurrence of autoimmunity, are avoided, the role of non-purified islets both in the auto and allo transplantation warrants further attention.

	Percentage of insulin independent patients				
Institution reporting results	**1yr**	**2yr**	**3yr**	**5yr**	**10yr**
Collaborative Islet Transplant Registry n=112. Purified islets.	65	45	26	10	nd
University of Minneapolis n=55. >90% Non-purified islets.	83	74	68	46	28

Table 1.1.5 Table showing the stability of islet graft function in those patients that have become insulin independent through islet allo and auto transplantation. *Patients auto transplanted with non-purified islets show a more sustained islet function. All data is correct as at December 2006* (74,98).

1.1.16 Summary

Islet transplantation potentially offers patients a cure for diabetes mellitus which is a life-limiting disease associated with a wide range of co-morbidities. Although long-term outcomes of islet transplantation will undoubtedly be improve with continued research and development aimed at refining the original Edmonton Protocol, one of the most pressing issues remains the lack of insulin secreting tissue. In fact the success of the Edmonton protocol has been largely attributed to the use of consecutive islet transplants and insulin independence has been directly correlated with the islet numbers transplanted (34,51). Omitting the purification process therefore has considerable logic, in that it will increase numbers of islets available for transplantation and avoid the potentially damaging purification process. Additionally there is growing evidence that non-islet pancreatic tissues both support islet viability and have the ability to propagate/transdifferentiate into the beta cell phenotype. These phenomena will be discussed in the following 2 sections in

order to question the current assumption that islet purification is a necessary step for successful islet transplantation.

1.2. Is Islet Purification Necessary?

Since the advent of islet transplantation, there has been a significant emphasis on the importance of islet purity, with many of these assumptions being based on small animal and cell culture models. This assumption warrants further assessment as the initial success reported by the Edmonton group in 2000 (34) followed islet transplantation averaging 24% beta cell purity and not exceeding 52%. Furthermore, to date auto transplantation of non-purified islets provides the most successful insulin independence rates within the entire field of islet transplantation (Table 1.1.3). This data suggests that in the clinical setting, significant acinar and ductal contamination is well tolerated. However, one could go further and hypothesize that extra-insular tissue including acinar tissue, ductal tissue, peri-pancreatic lymph nodes and vascular tissue actually confer an advantage to islet survival and function. It is well recognised that the extracellular matrix improves islet survival (90,99) however this chapter will assess the influence of non-islet pancreatic tissue based on published evidence.

1.2.1 Transplantation of non-purified islets

The Minnesota transplant group headed by Prof. D Sutherland currently holds the largest series of (non-purified) islet auto transplant procedures in the world (n= 193 adults as at the 1st December 2007 (77)) and undoubtedly have reported the most important long-term islet transplantation results in the field (76). Bucking the trend of the majority of islet allo transplantation scientists and physicians, the Minnesota group have long recognised the benefit of transplanting non-purified islets in order to conserve endocrine mass (98). To date, they have published a substantial body of work on their highly successful program (76,98,100) and have consolidated the auto transplant clinical findings with experiments in several canine models (101,102).

Interestingly, in the 1990's when islet allo transplantation results largely remained poor (12.4% insulin independence at one week, 8.2% after more than 1yr) (103) the Minnesota group were able to show that insulin independence could also be achieved with non-purified islets in the allo transplantation setting. Following the transplantation of tissue containing in excess of 600,000 IEQ (an IEQ count rarely procured following purification (3)) a 32 year old female T1D patient (47kg, insulin dose 0.7U/kg, HbA1c 10.8%) became insulin independent for an initial period of 6 months. Further studies carried out by the group compared the ability of non-purified and purified islets to engraft and function within the peritoneal cavity of dogs. Purified islets were shown to fail early, with only 46% functioning at 3 weeks, whereas at 6 months post-transplant 67% of non-purified islet transplants functioned (104). These early studies have undoubtedly lead to the growing theory that islet purification removes beneficial cells and exploration of the potential benefit of these cells is essential if current graft survival rates are to be improved.

1.2.2 Improved transplant outcomes associated ductal epithelial cells

Until recently ductal tissue had largely been considered as an unwanted contaminant of the islet isolation procedure (105). This is despite the fact that ductal epithelia has been shown to give rise to islets *in-vivo* (106-108) and *in-vitro* (43,44,109). However, a growing number of studies have demonstrated the trophic role of ductal tissue on islet *in-vitro*. Ilieva *et al* 1999 demonstrated that co-culture with ductal tissue abrogated islet central necrosis and that this was associated with their ability to secrete IGF-II among other factors (110). Furthermore recent studies carried out by Murray *et al* in 2009 provided evidence that ductal co-culture enhanced both islet function and viability rates (111).

Despite these positive attributes, little attention was paid to the fate/function of ductal tissue post transplantation until 2004 (112). The clinical study by Street and

colleagues reported that co-transplantation of high levels of ductal epithelial cells within islet allo transplant preparations correlated with superior metabolic outcomes, measured as the response to an intravenous glucose tolerance test post transplant. Additionally, a difference nearing statistical significance was found between graft success and failure with regard to the total number of ductal cells infused. Due to the collagenase resistant nature of the larger duct (Figure 2.1.4) it is the cells of the most peripheral ducts (centroacinar cells and intercalated ducts) that are transplanted, which is encouraging as there is substantial evidence that it is these cells that give rise to islet cells during cell regeneration (113-115).

In addition to their potential for islet regeneration and bio-trophic support, ductal epithelial cells have also been shown to have high angiogenic potency. A recent study by Movahedi and colleagues was able to demonstrate that cultured pancreatic ductal cells expressed 3-fold and 10-fold more VEGF and IL-8, respectively, than cultured human islet endocrine cells. In addition both proteins were expressed at angiogenic levels suggesting that duct-islet co-transplantation may lead to enhanced islet vascularisation (116).

1.2.3 Induction of a chimeric state by non-islet pancreatic cells

Induction of mixed chimerism (i.e. the coexistence of donor and recipient cells) through the transplantation of allogeneic donor bone marrow under appropriate host conditioning, is one of the most reliable strategies to induce transplantation tolerance (117). In the case of bone marrow, once donor stem cells have engrafted they can sometimes co-exist and differentiate in concert with recipient stem cells giving rise to all haemopoietic cells types. Consequently, thymically derived T-cells directed at either self or donor antigens are deleted by negative selection, leading to a robust state of tolerance.

Interestingly, an assessment carried out by Sever and colleagues in 1989 demonstrated that within a preparation of purified islets, populations of lymphoid cells, macrophages and dendritic cells were present and it can be assumed that higher numbers of such cells are present in non-purified islet preparations. In addition to haemopoetic cells, other cell types have also been shown to be present in non-purified islet preparations (96,118) which also have the ability to induce host chimerism these include: lymph nodes (119), vascular endothelial cells (120) and, more recently, stem cells (121).

Potentially the most potent method of inducing mixed chimerism involves the use of stem cells, as evidenced by Fandrich and colleagues. They demonstrated that stable engraftment of rat embryonic stem cells (RESC) could be achieved without any form of host pre-conditioning, such as irradiation, cytotoxic drug regimens or T cell depletion. Secondly, long-term partial mixed chimerism by use of RESC could be achieved without triggering graft-versus-host reactions, whereas severe graft-versus-host disease is often observed in the clinical setting of allogeneic hematopoietic stem cell associated transplantation (69). Although definitive characterisation of the pancreatic stem cells remains inconclusive, pancreas regeneration in the response to injury is well documented (122,123) with the highly plastic ductal epithelium thought to be the primary source of cells with stem cell-like characteristics (113).

To date, there is no evidence that the transplantation of non-purified islet preparations induces a state of patient chimerism, as most non-purified islet preparations are auto-transplanted. However, the work of Fandrich, Barlett and Johansson (119-121) certainly suggest that inclusion of haempoetic cells, soft tissues and stem cells may confer an immunological advantage to the recipient.

1.2.4 Inclusion of mantled islets

Pancreas digestion using collagenase and subsequent islet purification has the effect of destroying the islet microenvironment. The extracellular matrix (ECM) that surrounds each islet prevents cellular stress and supports ß-cell function, survival, proliferation, differentiation and migration through integrin mediated signals. However, upon islet isolation ECM support is lost and islet cells, particularly peripheral cells (alpha, delta and PP cells), are liable to undergo anoikis which is defined as integrin-mediated cell death. Indeed a number of studies have associated standard islet isolation protocols with malfunction, apoptosis, necrosis and loss of both peripheral islet cells (124) and beta cells (53,125,126).

However under normal islet isolation conditions, a significant percentage of islets (coined 'mantled islets') remain intact, embedded within a rim of acinar parenchyma. As the purification gradient process is governed by the density of each tissue group, 'mantled islets' are not routinely transplanted as they largely remain within the dense acinar fraction of the density gradient. This represents a loss of structurally and functionally intact tissue which in theory should provide superior function to conventional purified islets (127). Furthermore, paediatric donors have been deemed poor for islet isolation/transplantation purposes due to the fact that islets are difficult to cleave from the surrounding acinar tissue using commercially available collagenase blends and, as such, give rise to high ratios of mantled islets (128). This factor is disappointing as paediatric pancreata show superior function following transplantation of whole vascularised pancreas (129). Moreover, common sense and indeed published data dictates that younger islets are associated with longevity and superior function (130-133). Presently older obese donors are preferentially chosen due to the relative ease of islet dissociation from surrounding acinar tissue (128,134), however it is likely this has contributed to the poor graft survival reported with the allo transplantation field (51)

One negative aspect of utilising embedded islets is that central necrosis following transplantation will naturally be exacerbated as the acinar tissue creates a further barrier for the diffusion of oxygen and nutrients. However logical this conclusion may appear, local islet auto transplant results have shown that mantled islets are associated with transplant longevity (75). One could conclude that on balance maintenance of islet structure, function and viability outweighs the increased potential of islet central necrosis.

1.2.5 Inclusion of acinar tissue

Bluth and colleagues have suggested that the depletion of Reg I production, an acinar-derived factor, is associated with the pathogenesis of impaired glucose tolerance of pancreatitis (135). There is a paucity of information in this area and additional research may reveal further reveal the importance of acinar derived factors in glucose homeostasis.

1.2.6 Negative factors associated with the transplantation of non-purified islets

Key studies demonstrating the negative outcomes associated with non-purified islet versus purified islets are outlined in Table 1.2.1. Reasons for reduced islet function have been related to enhanced rejection and increased islet damage. In the majority of cases these assumptions are based on rodent models using transplant sites, largely the kidney, that are not routinely used clinically (97,118,136-138). In addition to this, further models (*in-vitro*) have suggested that ductal cells can be deleterious to beta cell viability (139,140) despite the fact that this data contradicts more recent clinical observations (112). Indeed, one key publication that is often cited (96) has indicated an increase in cell death when islets were co-cultured with high levels of contaminating non-islet tissue, in a serum free medium. However, in the absence of serum which contains a number of enzyme inhibitors, it is possible

that the positive signals derived from the ductal and possibly acinar cells were affectively outweighed by the negative effects of deleterious proteolytic enzymes released.

Rodent	Both purified and non-purified islets restored normoglycemia in rodent recipients, however the insulin response to glucose challenge 12 weeks after transplantation was negatively correlated with the amylase content of the transplant (136).
Rodent	In a DBA/2 (H-2d) pancreatic islet-to-B6AF1 (H-2b/k.d) recipient combination (no immunosupression) all non-purified islet grafts were rejected by 18 days (10.2 +/- 2.5 days). In contrast, 33% of the purified islet allografts survived more than 100 days. (118)
Rodent	Following transplantation under the kidney capsule, insulin content of grafts contaminated by either 50 or 90% exocrine tissue was significantly reduced when compared to pure islet grafts (97).
Rodent	Highly purified neonatal rat islet tissue, derived after 10 days in vitro, was allografted and was found to be non-immunogenic or weakly immunogenic. In contrast, non-islet pancreatic components, derived from the same culture system, transplanted with highly purified islet tissue, resulted in rejection in 88% of cases (137).
Rodent	Either purified or non-purified islets were transplanted syngeneically into the striated muscle of dorsal skin fold chambers. non-purified islets were associated with low engraftment and increased immune response rates, inflammation and graft loss. (138)
In vitro	In vitro studies have suggested that duct cells contribute to early beta-cell damage after islet transplantation through their involvement in tissue factor-mediated thrombotic and inflammatory events (105).
In vitro	Duct cells are suggested to directly contribute to ß-cell damage through their production of nitric oxide, indeed, 50% of nitric oxide production has been attributed to ductal cells (140).
In vitro	Significant levels of non-islet contamination (60-70%) was shown to be deleterious to islet survival *in-vitro*. (96,141)

Table 1.2.1 Key publications advocating islet purification prior to transplantation. *The majority of publications in the field are based on small animal studies and in-vitro work. To date no large mammal or human studies have been carried out that support the above data.*

1.2.7 Thrombosis related morbidity

In real terms the largest drawback to transplanting non-purified islets in the allo and auto transplant setting is the threat of thrombosis at the transplantation site due to the high volumes of tissue transplanted.

The potential lethality of transplanting large volumes of non-purified islets was underlined by Froberg et al (1997) (142) who published a case report of severe portal hypertension (49cm of H2O) and fatal disseminated intravascular coagulation immediately after islet auto transplantation of 20ml of tissue into the spleen. In the sphere of auto transplantation reports of thrombosis are almost exclusively associated with transplantation into the spleen (142,143) and as a consequence of this, the spleen has since been viewed as an unsafe transplantation site. Although the previous mortality and morbidity data can be explained, transplanting large amounts of tissue must be treated with caution. Data reported by the CITR reveal that of the 717 allo transplants carried out in North America between 1999 and 2008, seven have resulted in (non-fatal) portal vein thrombosis following the transplant of purified islets (74) and one would expect that transplantation of non-purified islets would increase this rate of morbidity. However, local islet auto transplantation results (75) underline the fact that in the clinical setting, transplant volumes of up to 32ml are well tolerated and safe, although physicians must stringently base infusion volumes on transient rises in portal and central venous pressure. Additionally, strategies such as transplanting sub-therapeutic amounts of tissue over a prolonged period (14 days), have been associated with superior graft outcomes in animals (144) and may further increase the safety of the procedure.

1.2.8 Summary

Transplantation of non-purified pancreatic digest preserves endocrine mass and may lead to the ideal scenario of single donor islet transplantation. Fortuitously,

transplantation of non-purified islets also allows co-transplantation of not only ductal tissue, which has been shown to be beneficial but also other cells which may provide islets with bio-trophic support and possibly promote immune tolerance. A further facet of non-purified islet transplantation is the possibility that non-islet cells may have the potential to convert into the beta cell phenotype post transplantation and this is discussed in section 1.3.

1.3 Insulin secreting potential of non-beta cells

The primary objective of islet transplantation is to implant tissue that will provide long-term glucose-responsive insulin secretion. The previous chapter reviewed evidence that extra-insular cells may provide bio-trophic support to islet tissue or induce graft tolerance in the recipient, however there is another important factor to consider, do non-islet pancreatic cells have the ability to differentiate and contribute to the insulin secretion of a graft post transplant? This concept is relatively novel, however there is a growing body of evidence that the cells of the pancreas have an extensive capacity to adapt and proliferate both *in-vitro* and *in-vivo*.

1.3.1 Pancreatic compartments

Traditional histological descriptions of the pancreas distinguish between the exocrine and the endocrine pancreas as if they were two functionally distinct organs. An endocrine compartment consisting of hormone-secreting islets of Langerhans and a relatively expansive population of acinar cells that secrete pro-enzymes via the ductal tree into the intestine. However an alternative view could be that the pancreas is a mixed organ with a clear acinar-ductal-endocrine axis Figure 1.3.1 clearly illustrates that due to physical intimacy there is an obvious continuity between acinar parenchyma, centroacinar cells (CAC) and ductal cells of the pancreas. Moreover, it is generally considered that it is the ductal epithelium that gives rise to the islets of Langerhans during both embryogenesis and natural β-cell turnover (145,146) and this in essence, completes the continuity between the pancreatic compartments. Of all pancreatic cells, it is perhaps the CAC that best highlights the blurred boundaries between the compartments of the pancreas. As its name suggests this cell type can be considered part of the acinar compartment due its location within the lumen of each acini and its influence on zymogen secretion (147). However, the CAC has been shown to have a close phenotypical

similarity with the most peripheral cells of the ductal system and indeed most authors characterize the CAC as a ductal cell type (145). In addition to its relationship with the acinar and ductal cell, there is also evidence that within the normal pancreas (rodent), 0.21 & 0.30 % of centroacinar cells express glucagon and insulin respectively (148). From this description, it is clear that the pancreas is not comprised of discreet independent cellular compartments but is an amalgamation of cell populations with indistinct boundaries. This chapter will review published articles that outline the potential for non-islet pancreatic cells to transdifferentiate into insulin secreting cell types, *in-vitro* and *in-vivo*.

1.3.2 Ductal cell plasticity *in-vitro* and *in-vivo*

Traditionally, associations between islet tissue and ducts were considered by most researchers as a transient epiphenomenon of embryonic endocrine development, however numerous *in-vitro* studies and animal models have shown that beta cell propagation from the ductal epithelium. (44,108,149,150). It is therefore surprising that little attention has been paid to the fate of dispersed ductal tissue post transplantation.

As previously discussed, observational studies by Street *et al* (112) showed superior metabolic outcomes when patients were transplanted with high volumes of ductal epithelial cells. In the same year, Bogdani and colleagues looked into the fate of human ductal tissue transplanted into the nude mouse model. In this study, human pancreatic ducts with less than 1% contaminating insulin positive cells were cultured and subsequently transplanted under the kidney capsule of normoglycaemic nude mice. Between weeks 0.5 and 10 the ductal cell volume had decreased by 90% whilst the change in insulin positive cells was variable and dependentant on donor age. Donors over 10 years old showed a 3 fold decrease

whilst donors under 10 years exhibited a 2.5 fold increase in insulin positive cells. Moreover, 3% of the insulin positive cells were shown to co-express cytokeratin19 and up to 1% co-expressed for BRDU. This study provides good evidence that, post transplant, *in-vivo* conditions induce the transplanted ductal tissue of a young donor to transdifferentiate into an endocrine phenotype and furthermore these cells have proliferative qualities. Due to the lack of lineage based data in this study, it is possible that the existing beta cells were able to proliferate via a ductal phenotype (Cytokeratin 19 positive). However if this is the case, this study suggests a bio-trophic role of the ductal epithelium to islet tissue which has already been discussed (section 1.2.2).

The findings of Bogdani and Street concur with the regeneration studies of Hayashi *et al* 2003, De Haro-Hernandez *et al* 2003, Bouwens *et al* 1993 and Lui *et al* 2007 (107,113-115). '*En masse'* these studies suggest that the source of islet cell neogenesis are centroacinar cells, intercalated ducts and intralobular ducts or latent precursor cells that lie within these cell populations. Moreover, to quash any uncertainty, later cell lineage-based experiments carried out by Bonner-Weir and colleagues in 2008 demonstrated a clear lineage between duct cells and islet neogenesis (150). They took the direct approach of genetically marking ductal cells using CAII (carbonic anhydrase II) as a duct-cell-specific promoter to drive Cre recombinase in lineage-tracing experiments using the Cre-Lox system. This study showed that the CAII-expressing pancreatic ductal cells acted as progenitors that gave rise to new islets and acini both after birth and after injury (ductal ligation) and adds credence to the hypothesis that beta cell genesis derives from the ductal epithelium.

1.3.3 The centroacinar cell

Areas where epithelia of divergent differentiation coincide have been proposed as niches for organ specific stem cells within the intestine (151) and liver (152,153). The centroacinar cells (connecting the exocrine and ductal compartments) may represent the stem cells of the pancreas. Like true ductal epithelial cells, the CAC have been shown to display significant proliferative abilities and, pertinently, have the ability to propagate into beta cells. Both of these phenomena are efficiently shown in a study by Hayashi and colleagues in the 2003 (113). This study revealed that following partial pancreatectomy a population of BRDU-positive cells emerged amongst the intercalated duct epithelium and centroacinar cells. These cells were shown to rapidly proliferate, migrate, and form endocrine clusters within the acinar texture after a few days. Such studies bridge the gap between the exocrine, ductal and endocrine compartments of the pancreas and show that transdifferentiation events are not dependentant on artificial tissue culture conditions and non-physiologically high levels of growth factors.

1.3.4 Acinar cell plasticity *in-vitro* and *in-vivo*

Numerous studies report that acinar to duct transdifferentiation (*in-vitro*) is a spontaneous event based on the apparent loss of acinar cells and a reciprocal increase in the ductal phenotype in culture (154-156). Rigorous assessment of these studies would suggest that acinar preparations, by their very nature, cannot be pure due to the fact that every acini of the pancreas is associated with terminal ductal elements (section 4.2.2.7). Additionally, due to the well documented fragility of acinar tissue coupled with the proliferative capabilities of ductal cells, it is likely that contamination of acinar tissue with even a small percentage of ductal cells would lead to marked ductal cell overgrowth (43,157).

The most convincing evidence of true acinar to ductal transdifferentiation was provided by Means and colleagues in 2005 (158). This study used lineage tracing techniques to prove that acinar-to-ductal metaplasia represents a true transdifferentiation event, mediated by initial dedifferentiation of mature exocrine cells into a population of nestin-positive precursors, similar to those observed during early pancreatic development. In the same year, time-lapse videomicrography studies carried out by Spyhris and colleagues provided rigorous evidence that acinar to duct conversion was a direct process with no intermediate steps (156).

The studies of Means and Sphyris substantiate the acinar-ductal transdifferentiation theory and again add credibility to other less rigorous studies. Such proof of acinar to ductal transdifferentiation is invaluable as it opens up the possibility of acinar to islet transdifferentiation. Although this proposition may appear unlikely, it has already been shown that the acinar tumour cell line AR42J can convert into a β-cell phenotype under the influence of activin A, betacellulin, hepatocyte growth factor and glucagon-like peptide-1 (159,160). The prospect of successful acinar to islet transdifferentiation offers great potential as it suggests that all major non-islet tissues of the pancreas (acinar and ductal tissue) can be converted into the beta cell phenotype.

1.3.4.1 Acinar transdifferentiation in response to injury

Another compelling facet of acinar to islet transdifferentiation is that acinar cells displaying endocrine characteristics have long been recognized within the acinar parenchyma in association with certain patho-physiological conditions. Pancreatic cells displaying endocrine and exocrine phenotypes were first defined as intermediate cells by Melmed and colleagues in 1972 (161). The intermediate cell (IC) is rarely observed in the normal pancreas but can be observed with some

frequency in patients with chronic pancreatitis (162). Whilst in animal models ICs have been shown to emerge in response to pancreatic ductal obstruction (163).

Although the mode by which CP induces the appearance of ICs has not been elucidated, biological analysis of the CP pancreas reveals marked over-expression of growth factors and their receptors, principally; TGFa, EGF(R) (164) and acidic and basic FGF (165); factors that have been shown to induce the beta cell phenotype *in-vitro* (166,167). However, histological investigations have revealed that the intermediate cell frequently displays features of damage, such as irregular nuclear profiles, vacuolization of the rough endoplasmic reticulum and swelling of mitochondria (168). Despite this, the presence of insulin secretory granules and generous levels of rough ER suggest that such cells are metabolically active and may present a means of preserving the insulin secreting capabilities of the pancreas following injury (169).

Although ICs are negligible in normal pancreas, they are frequently observed in chronic pancreatitis (CP), (see above) thus auto transplantation of non-purified islets following pancreatectomy for CP will almost certainly involve transplantation of intermediate cells, although their impact on graft function is currently an unknown quantity.

1.3.5 The pancreatic stem cell

Organ stem cells are slow-cycling cells with capacity of unlimited self renewal, asymmetric cell division and maturation into the mature cell type. There have been numerous attempts to characterise the pancreatic stem cell, however to date it remains an elusive entity (reviewed in (170,171); possible candidates of putative pancreatic stem cells are outlined in Table 1.3.1 In a way, the elusiveness of the pancreatic stem forces one to question their importance in endocrine and even

exocrine regeneration, particularly in light of the well accepted and frequently observed regenerative capacity of the ductal epithelium, in particular the centroacinar cells of the pancreas (113).

1.3.6 Haemopeotic stem cells and pancreatic vasculature

Nestin, an intermediate filament protein expressed by neuroepithelial stem cells, has been proposed as a marker for putative islet stem cells (Table 1.3.1). Although it is widely accepted that ductal cells are the primary foci of nesidioblastosis, numerous research groups have shown that the nestin positive cells of the pancreas have an unusually extended proliferative capacity and the ability to give rise to numerous cell types, including beta cells, *in-vitro* (172) . Subsequent in-depth studies have shown that nestin is largely restricted to cells of the pancreas vasculature including reactive stellate cells, pericytes and endothelial cells during active angiogenesis and as such nestin has now been abandoned as a marker for endocrine progenitor cells (173). However, earlier embryology studies by Bouwens et al 1996 (174) demonstrated that within the rodent pancreas ductal cells co-expressing the mesenchymal cell marker vimentin were shown to form the mantle around which islets developed. Additionally vascular endothelial cells may be involved in nesidioblastosis within the adult pancreas particularly as there is known to be paracrine action between blood vessels and neighbouring islets during embryogenesis (175). However, due to the paucity of research in this area, the influence of haemopoetic cells and pancreatic vascular tissue on islet generation is in doubt.

Stem Cell Description	Evidence
Pancreatic and duodenal homeobox 1 positive cells *(pseudonyms: IPF-1, IDX-1 or STF-1)*	PDX-1 is transiently expressed in all foetal pancreatic epithelial cells before its expression becomes restricted to islet cells. As such, hormone negative-PDX-1 positive cells can be presumed to be latent endocrine progenitor cells. This presumption is strengthened by the fact that PDX-1 expression is associated with the conversion of ductal cells into a beta cell phenotype (176)
Nestin positive cells	Pancreatic cells expressing nestin (type VI intermediate filament) have been shown to differentiate into insulin expressing cells *in-vitro* (172,177,178). However, later studies have shown that nestin is not a specific stem cell marker and is expressed in a variety of proliferating and metabolically active endothelial cells.
Peripheral cells of the ductal epithelium	The ductal epithelium propagates beta cell neogenesis in-vivo and *in-vitro* (discussed above) whether duct cells represent a population of pluripotent cells or harbour as of yet uncharacterised facultative stem cells, is unclear.
Stellate Cells	There is histological evidence that stellate cells contribute to regeneration following acute pancreatitis (179).

Table 1.3.1 Potential stem cells of the non-endocrine adult pancreas.

1.4 Introduction Summary

Clinical islet transplantation may provide a viable treatment of diabetes mellitus. However its widespread success has been hindered by a number of factors including the availability of donor pancreata and loss of function of purified islets post transplant. In its broadest sense, the aim of this study was to investigate whether islet purification is necessary for optimal islet function post transplantation and is underlined by the data obtained from the Leicester islet auto transplant program which has been published by Webb *et al* 2008 (75) and further elaborated in Appendix I.

1.5 Description of work

Firstly a retrospective analysis of the long-term outcomes of patients receiving purified versus non-purified islets auto transplants following pancreatectomy for chronic pancreatits was carried out. In order to elucidate the factors involved in the long-term function of transplanted islets, an *in-vitro* system was developed in order to assess the impact of non-islet tissue on islet viability, structure and function. Finally a histology-based study was used to assess the presence of cells with the capacity to convert into a beta cell phenotype in chronic pancreatitis patients that have undergone total pancreatectomy and islet auto transplantation. From the work outlined above it is proposed that the following null hypothesis will be proven:

'The transplantation of purified islets provides no advantage over non-purified islets based on in-vitro, histological and clinical data'

1.6 Aims of study

The study presented in Chapter 3 comprises of a retrospective analysis of patients either transplanted with non-purified islets or purified islets. The main aim was to

directly compare islet graft outcomes within these two groups. Secondly, peri-operative factors that have cited to be adversely affected by transplanting non-purified islets / large islet graft volumes were also assessed. Specifically the study aimed to:

- Compare clinical markers of islet graft function in the two patient groups (including C-peptide, serum glucose and HbA1c) at 3 and 6 months post transplant and then annually thereafter up to 5 years post transplant.
- Compare peri-operative data within the two groups including intra-operative venous pressures and blood loss, operation length and hospital stay.
- Compare the liver function of patients within the two groups (alkaline phosphatase, aspartate aminotransaminase, bilirubin and albumin synthesis) within the 1st year of transplantation.

For the *in-vitro* study presented in Chapter 4 the initial aim was develop a tissue culture system that would allow the survival of primary non-purified islets over the short-term (8 days). Once this had been developed the primary objective was to:

- Compare the viability, function and hormone status of islets either cultured as non-purified islets or purified islets *in-vitro*.
- Assess the composition of islets within the purified and non-purified islet cultures and specifically assess relationships between islet and non-islet cells within the non-purified islet culture.

The aim of the histology-based study presented in Chapter 5 was to assess whether levels of islet-cell precursors and non-islet hormone positive cells within the pancreas of chronic pancreatitis patients undergoing pancreatectomy and

concomitant islet auto transplantation positively correlated with islet autograft outcomes. Specifically the aim was to:

- Correlate levels of hormone positive non-islet cells, ductal cells and pancreatic and duodenal homeobox 1 positive cells with clinical markers of islet graft function at 3 and 6 months post transplant and then annually thereafter up to 5 years post transplant.

Chapter 2: Materials and Methods

2.1 Islet Procurement

Islets were prepared from human, porcine and rodent pancreata using the following protocols.

2.1.1 Clinical preparation of islets for auto and allo transplantation

Figures 2.1.1 to 2.1.6 outline islet isolation, transplantation and peri-transplant assessments based on the on-going clinical program run by the University Hospitals of Leicester. The data used in the present study relates to islets prepared for islet auto transplantation, however for the purpose of comparison, processes relating to islet preparation for allo transplantation (particularly islet purification) have also been described.

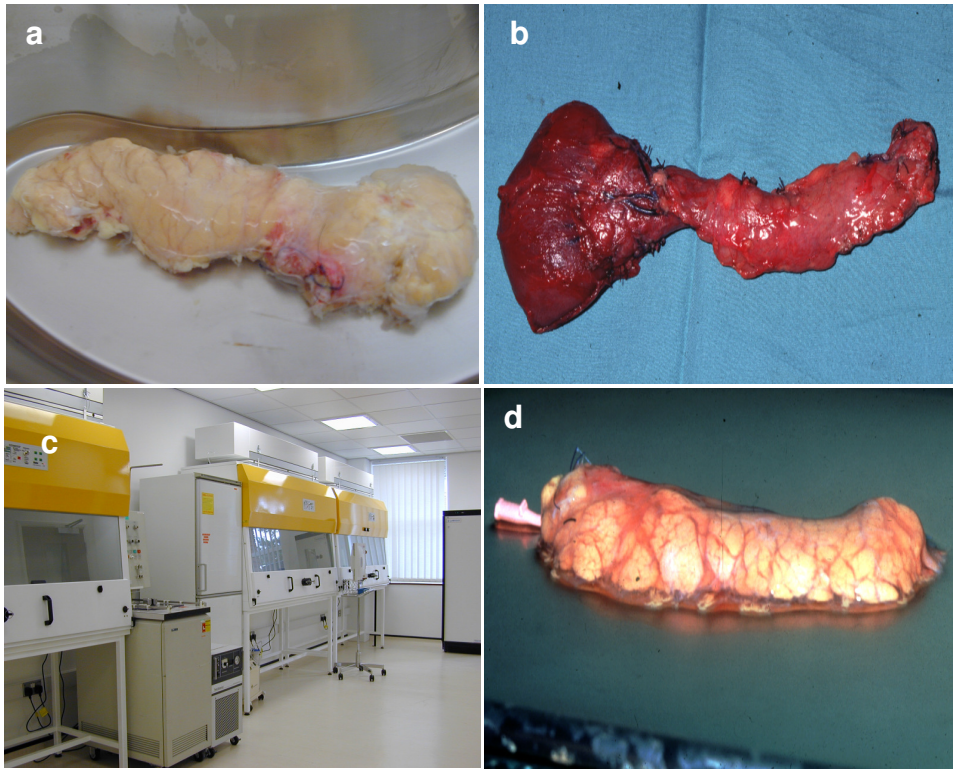


Figure 2.1.1 Islet isolation. *Islet isolations were carried out on cadaveric pancreata (a) or from pancreata resected from patients with severe chronic pancreatitis for the purpose of islet auto transplantation (b). Once in the clean room facility, all manipulations were carried out under the sterile laminar flow hoods (c). At the outset of the procedure the duodenum was carefully removed and the pancreas cleaned of extraneous fat and tissue. Next a cannulae was inserted into the pancreatic duct and the organ was perfused with double it's weight of collagenase enzyme. Formerly NB1 collagenase (3-4mg/ml, Serva, UK) was used followed by Liberase enzyme (3.33mg/ml Roche, Mannheim, Germany) and latterly GMP grade NB1 collagenase (20 PZ units per gram of pancreas, Serva, Denmark) coupled neutral protease (1.5 DMC units / gram of pancreas, Serva, Denmark (d).*

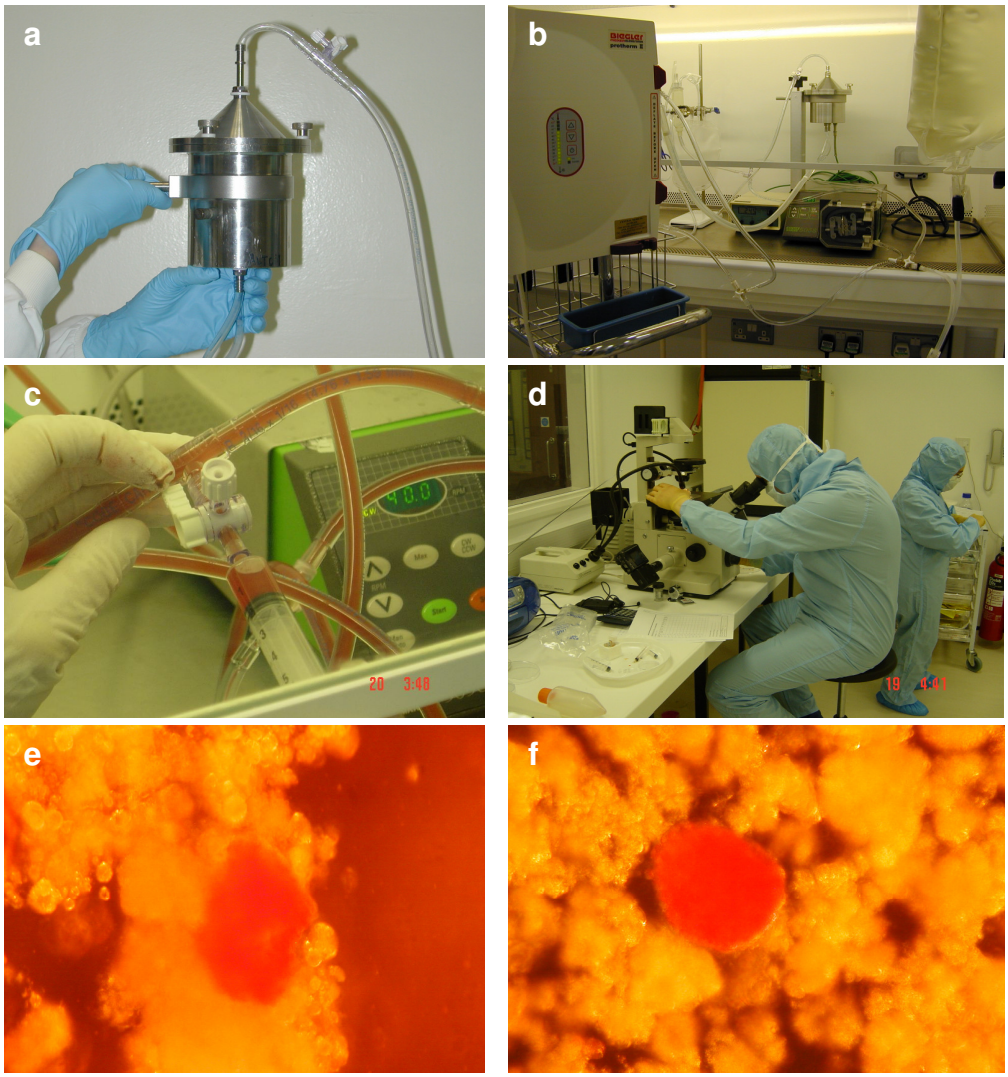
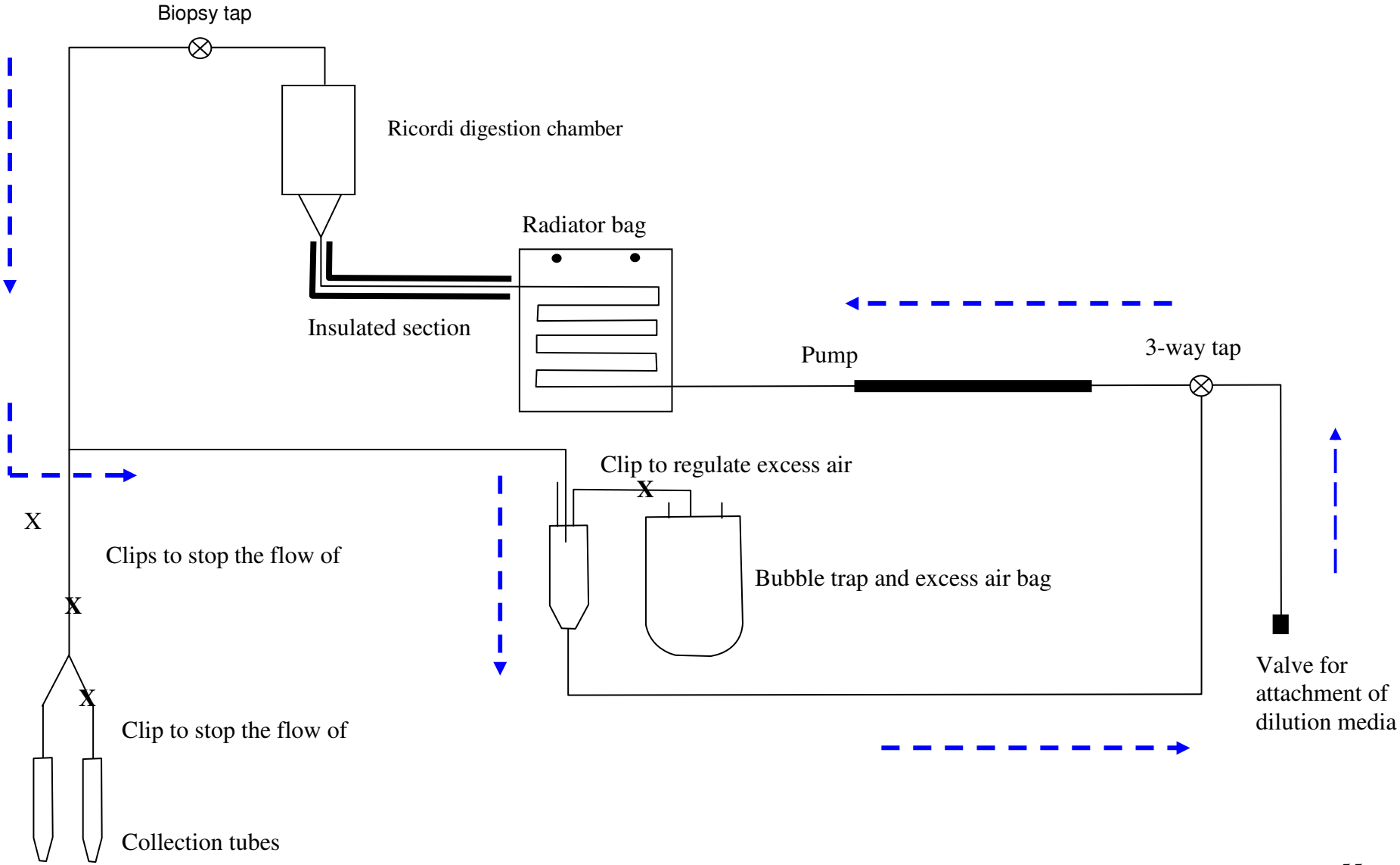


Figure 2.1.2 Pancreas digestion. *Following distension, the pancreas plus any residual enzyme, was transferred to a specialised 'Ricordi' chamber (a). Through a system of tubing, media was warmed to 37° C and re-circulated over that pancreas (b). The chamber was manually shaken to aid digestion and the design of the Ricordi chamber is such that only digested tissue of sufficiently small size (500µm) is able to flow out of the chamber into the tubing circuit. Regular samples were taken from a biopsy port located directly above the chamber and were viewed under an inverted microscope to assess whether the pancreas had adequately digested (c&d). At the stage when 50% of islets were cleaved of acinar tissue (e & f represent embedded and cleaved islet respectively) tissue collection was initiated by opening the tubing circuit (refer to Figure 2.1.3).* Figures e & f photographed at a magnification of x 200.

Figure 2.1.3. Schema of the pancreas digestion circuit. *Dashed lines represent the flow of the closed (re-circulating) circuit. When digestion is evident the circuit is 'opened' and flows from the dilution media, through the chamber in the direction of the collection tubes.*



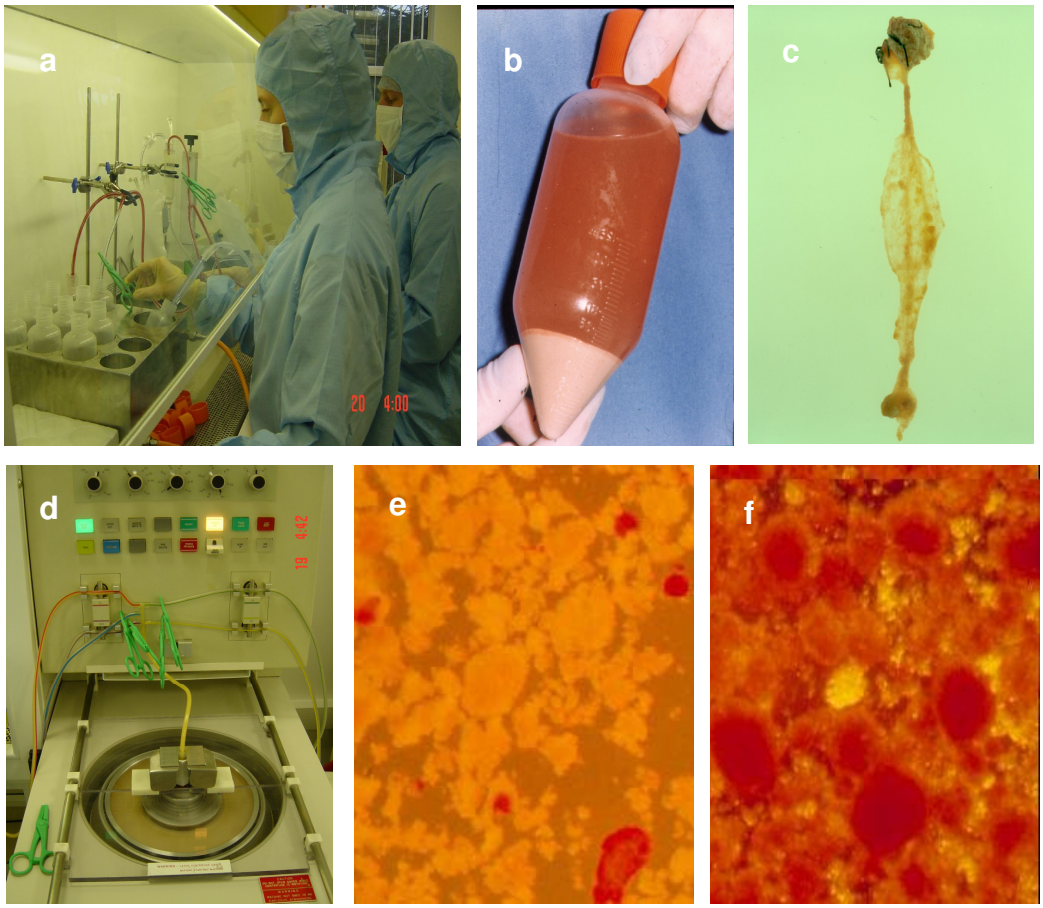


Figure 2.1.4. Collection of purified / non-purified islets. *By opening the circuit the pancreas was continually flooded with fresh media (Dilution media, Lonza, Basel, Switzerland), diluting the enzyme and preventing over-digestion (a). The digested tissue slurry was collected into pre-chilled collection tubes that contain 20% human serum albumin (Zenalb 20, Bioproducts Laboratories, Elstree, UK, Figure a). In combination, the serum and low temperature prevent the enzymes found within exocrine tissue, or any residual collagenase, damaging the islets. The digested tissue slurry was collected and the packed cell volume measured in graduated centrifuge tubes (a&b). Following digestion the main pancreatic the duct and associated larger ducts remains undigested (c). Pancreatic digest consisted of around 2% islets (red), the remaining tissue was largely exocrine/ ductal tissue (e). At this stage islets could be purified on a density gradient in a COBE 2991 processor (CA, USA, Figure d), producing highly purified fractions of islets (f vs. e) although, it is of note that a substantial proportion of islets were often discarded in less pure fractions (see Figure 2.1.5). In the sphere of islet auto transplantation, whole pancreas digest is often transplanted to conserve islet numbers.* Figures e & f photographed at a magnification of x 100.

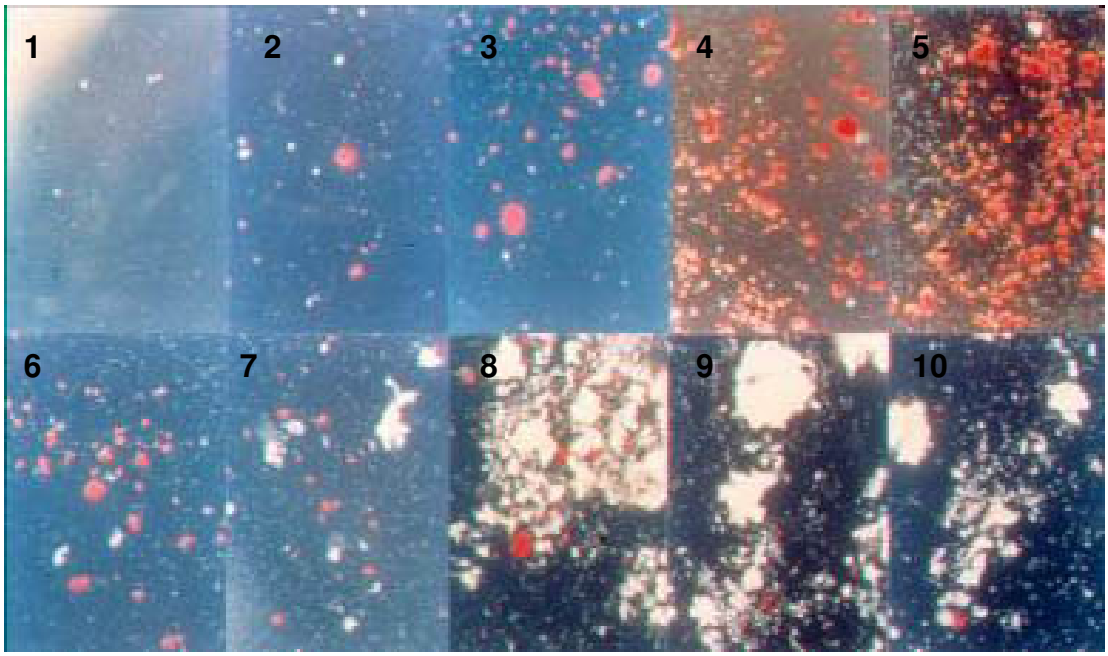


Figure 2.1.5. Fractions of a purification gradient used in the preparation of islets intended for islet allo transplantation. *The fractions are initially duct rich containing increasing levels of islets (Figure 1-3). Fractions 4 and 5 represent particularly islet rich fractions, whilst fractions 6-10 contain increasing levels of acinar contamination. For transplantation purposes it is likely fractions 3-7 would be pooled together. However, fractions 8-10 contain by far the largest volume of tissue, and as such, absolute numbers of islets within these fractions would substantial.* All figures photographed at a magnification of X40. *Photographs courtesy of Dr Sue Swift, formerly of the University Hospitals of Leicester.*

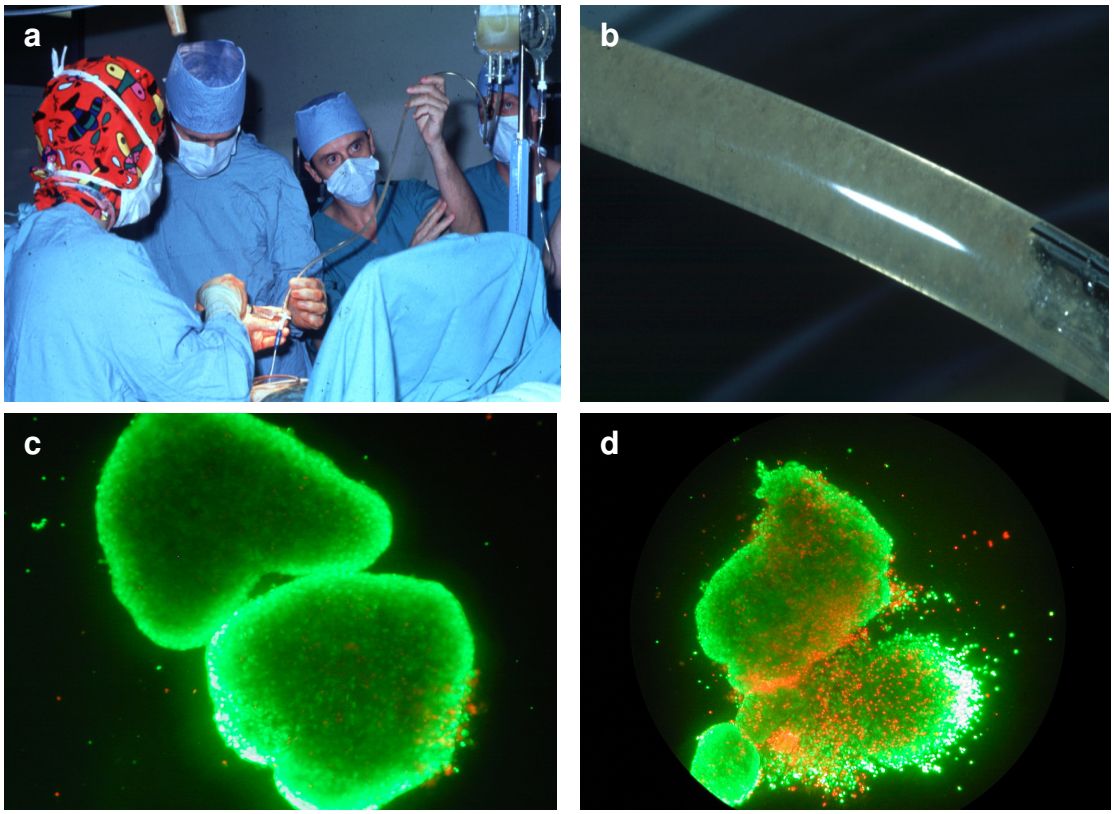


Figure 2.1.6 Islet transplantation and islet viability. *Prior to transplantation the digested pancreatic tissue/islets were pooled together and suspended in transplant media (Lonza, Basel, Switzerland) again containing 20% human serum albumin (not shown). Immediately prior to transplantation, patients received 5000 IU of intravenous heparin to prevent thrombosis. Subsequently, islets were infused into the liver and less frequently the spleen, usually via the portal vein or more recently the re-cannulated umbilical vein (a&b). Vital staining using fluorescein diacetate (0.67µm) and propidium iodide (4µm), Sigma, UK, was used determine the viability of the transplanted islets (c & d) with highly viable islets showing little propidium iodide (red) staining (c).* Figures c & d photographed at a magnification of x 400.

2.1.1.1 Characterisation of the graft

Directly before transplant, samples of the islet preparation were assessed for identity, viability and following transplant, beta cell function. Islets were counted (refer to section 2.1.4.4 and Table 2.1.1) and sized after dithizone staining and results were normalised to islet equivalents (IEQs). One IEQ equated to one islet of 150um diameter. At this time assessments were also made to calculate the proportion of cleaved /un-cleaved islets. Vital staining using fluorescein diacetate and propidium iodide (Sigma, UK) determined the viability of the transplanted islets. The functional assays carried out were targeted at the insulin producing beta cells as these are the main therapeutic cells of the islet transplant. Under cell culture conditions, the islets were exposed to glucose of different concentrations and the resulting insulin secretion is quantified (Section 2.3.3). Other assessments included microbial screening (carried out by the UHL Microbiology Laboratories and endotoxin content assays.

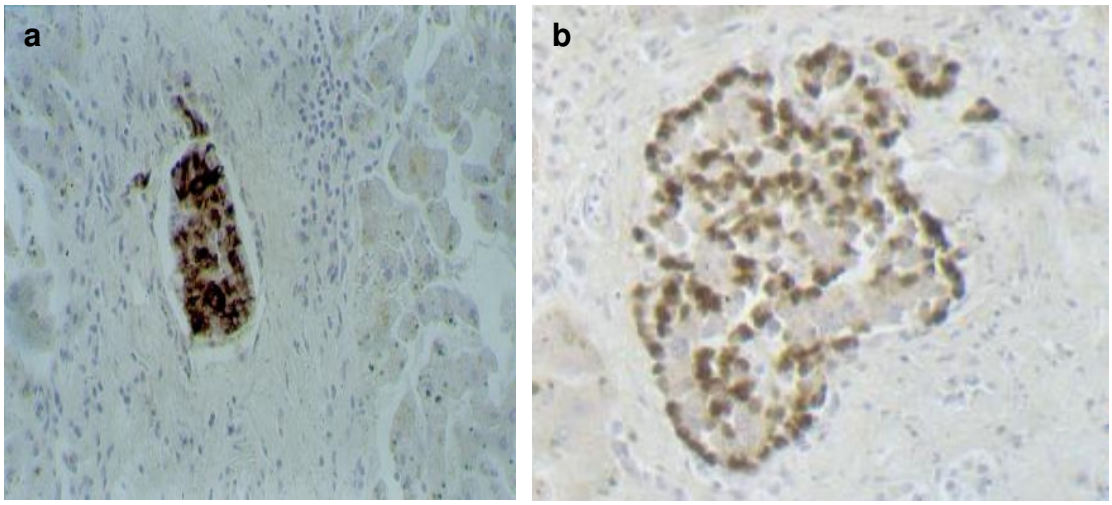


Figure 2.1.7 Examples of engrafted islets 1 month post-transplantation. *Following transplant, the islets pass from the hepatic portal vein into the liver where they typically become vascularised within 2 weeks. Local post-mortem samples of one islet transplant patient, who unfortunately died 30 days after transplant of a peri-operative stroke, revealed that the islets were tightly embedded in the left lobe of the liver. Moreover immuno-histochemistry staining showed the presence of alpha (a), beta (b) and delta cells (not shown) within the transplanted islets.* (180). *Photograph courtesy of Dr Sue Swift, formerly of the University Hospitals of Leicester.* Figures a & b photographed at Magnifications of x 200 and 400 respectively.

2.1.2 Clinical assessment of islet function pre and post-transplant

Follow-up assessments were undertaken at 1, 3, 6 and 12 months post-transplant and annually thereafter, using the 75g OGTT. Prior to the assessment, patients were fasted overnight and had their insulin stopped for at least 12 hours. Blood samples were taken for glucose and C-peptide levels at 0 (fasting), 30 and 120 minutes and additional fasting samples were taken for assessment of glycosylated haemoglobin (HbA1c), Na^+, K^+, urea and creatinine. Since many patients were recruited from centres at some distance from Leicester, many patients failed to attend an assessment every year.

2.1.2.1 Preparation of blood samples

Serum preparation for the assessment of C-peptide was carried out by centrifuging whole blood (that had been previously clotted for 1 hour and 30 min at 4℃) for 10min at 1500g. Serum samples were divided into aliquots and stored at -20℃ until use. Plasma samples for HbA1c Na^+, K^+, urea and creatinine assessment were collected in EDTA plasma tubes and were immediately processed by the UHL Pathology Service, Leicester, UK.

2.1.2.2 C-peptide and HbA1c assay

C-peptide assay was carried out using the C-peptide ELISA kit supplied by DRG Diagnostics, Nottingham, UK. The lowest limit of sensitivity was 0.04ng/ml and the intra and inter assay variability was 6.70 and 9.92 CV% respectively. Controls were provided by running the Lyphochek® Immunoassay Plus Control system with each Elisa plate, which consisted of 3 quality control serums containing levels of C-peptide consistent with values expected within human serum. Serum samples were assayed for HbA1c by the UHL Pathology Service, Leicester, UK using the Tosoh HLC-723

GHb G7 high performance liquid chromatography analyser (Tosoh Europe, Tessenderlo, Belgium)

2.1.3 Liver function tests

Post transplant liver function tests were undertaken at 6 and 12 months post-transplant and annually thereafter. Four main tests were undertaken by the bio-chemistry services within the University Hospitals of Leicester using patient serum, these were: alanine aminotransferase (3-40 iu/L), alkaline phosphatase (30-300 iu/L), bilirubin (3-17 µol/L) and albumin (local reference ranges are in parenthesis). Again, as some patients failed to attend follow-up assessments every year the data was incomplete.

2.1.4 Human pancreas digestion and islet isolation for research

2.1.4.1 Digestion of Human Pancreata

Standard human islet isolation procedures (Section 2.1.1) requires a minimum of 2 people, as such a single person technique based on the protocol developed by O'Neil and colleagues was employed for this project. Human pancreata, not suitable for transplant, were obtained from the UK Human Tissue Bank, Leicester. Upon arrival the pancreata were washed with a Videne solution (Ecolabs, Leeds, UK,) a solution of cefuroxime (at 5mg/ml) diluted in HBSS and finally the pancreata were rinsed with HBSS. Next, all extraneous tissue, including the pancreatic capsule, was removed and the pancreatic duct was cannulated in the head of the pancreas. A syringe was connected to the cannula and the pancreata were perfused with Liberase HI enzyme (Roche, UK) at a volume double that of the weight of the pancreas and at a concentration of 3mg/ml. The pancreas was then placed (intact) within a 1 litre closed tub with any remaining collagenase solution. The organs were digested by static incubation in a water bath set at 37° C for between 25 to 65 min. At full digestion the pancreata became loose and tissue was easily sloughed off using

tweezers. The glands were then manually disrupted, passed through a 500µm mesh and collected into a cold receiver dish containing HBSS with 10% FCS. The tissue effluent was then decanted into 250ml conical tubes and centrifuged for 2 min at 100g at 4˚C to produce a tissue pellet tissue was washed X 2 in HBSS prior to culture.

2.1.4.2 Human Islet Isolation for research

Islets were isolated using a modification of a technique described by Huang *et al* 2004 (181). Islets were isolated on a discontinuous gradient of Biocoll and UW. The post-digestion cellular pellet was suspended in 80ml of Biocoll UW at (1090 g/cm^3) and placed in a 250ml conical tube. Lighter gradients were sequentially added: 50ml of 1080 g/cm^3 50ml of 1070 g/cm^3 and 20 ml of 1060 g/cm^3. After centrifugation at 856*g* for 17 min, islets were collected from the 1.080/1.070 and 1.070/1.060 interfaces. Islets, from each interface, were suspended in 2 tubes containing MEM with 10% FCS. The tubes were centrifuged at 280*g* for 3 minutes. The supernatant was removed, and the cells were washed with 150 ml HBSS. This procedure was repeated 3 times and islets were subsequently cultured.

2.1.4.3 Formulation of islet isolation gradients

To calculate volume of gradient concentrate (e.g. Biocoll solution) versus the volume of diluent required, the following formula was applied per 100ml:

$$\frac{100dd - 100d2}{d1 - d2}$$

Where:
DD = Desired density
D1 = Density of gradient concentrate solution
D2 = Density of diluent

Once made, the densities of all solutions were checked for accuracy on the AM255-5M Densitometer (Anton Paar K.G, Austria).

2.1.4.4 Islet counting and IEQ calculation

A sample of the islets were then stained with dithizone immediately after purification and counted under a phase contrast microscope. The purity of each preparation was determined through dithizone staining of the sample. Islet equivalents (IEQs) equated to one islet of 150um diameter. As such, to calculate IEQs per sample, individual islets within a sample were sized, categorised multiplied by an equivalence factor (refer to table 2.1.1).

50 100 (75)	**100 150** (125)	**150 200** (175)	**200 250** (225)	**250 300** (275)	**300 350** (325)	**350 400** (375)	**400 plus**
0.167 ×	0.648 ×	1.685 ×	3.500 ×	6.315 ×	10.352 ×	15.833 ×	22.750 ×
I.E:							
Total I.E.Q within sample			×		=		

Table 2.1.1 Method used for the calculation of islet IEQ. *Using an inverted microscope (at x 100 magnification) and an eyepiece graticule, all islets measuring ≥ 50 μm were counted, allocated to a size (50-99 μm, 100-149 μm, 150-199 μm, 200-249 μm, 250-299 μm, 300-349 μm, 350-399 μm, 400+ μm) and marked in the appropriate column on the islet quantification sheet. An indication how many islets were mantled was also noted. After counting, the number of counts per column was multiplied by the equivalence factor at the base of the column. The total from each column gave the total IEQ per sample, the actually volume of the entire islet preparation was used to calculate the total number of IEQs.*

2.1.5 Rodent pancreas procurement digestion and islet isolation for research

2.1.5.1 Tissue Procurement and digestion

Adult Wistar rats weighing 180-300g were used as organ donors, although the median weight was 250g. Rat were stunned and culled by dislocation of the vertebrae.

Rat pancreata were digested using a modification of the method described by Lacy and Kostianovsky (1967) (182). In brief, the pancreatic ducts were clamped at the distal end, next to the duodenum. The pancreata were distended in-situ by an intraductal injection of ice-cold collagenase solution (NB8 Serva, 1mg/ml in HBSS). The pancreata were resected and next transported to the laboratory on ice, with cold ischemia times ranging from 35-90mins. Each resected pancreas was put into MEM (Invitrogen, UK) pre-warmed to 37°C and then incubated at 37°C in a water bath for exactly 18 minutes. Subsequently the warm media was replaced with ice cold MEM (Invitrogen, UK) and each pancreas was disrupted with vigorous shaking for 60 sec. The pancreatic digest was passed through a 500μm mesh where upon the digestion was stopped with ice cold FCS (Invitrogen, UK). The digest was then washed twice with MEM & 10% FCS (centrifugation at 100g, 2 minutes). At this stage non-purified islet were ready for culture

2.1.5.2 Isolation of Rodent islets

Pancreatic digest was centrifuged at 200g for 2minutes, after aspiration of media the tubes were inverted on absorbent towel to ensure total removal of media. Bovine Serum Albumin (stock solution; density of 1.1g/ml and osmolarity of 500mmol/kg, First Link UK Ltd.) was used to create a discontinuous gradient (section 2.1.4.3). The pancreatic digest was re-suspended in a BSA gradient of 1.086 density and gradients of 1.079 and 1.059 were layered on top. The gradients were centrifuged at 800g for 20 minutes with no brake. The resulting gradient routinely revealed 2 separate layers of

islets, one within the 1.079 layer and at the 1.079 and 1.059 interface. Each islet layer was examined for purity under a dissecting microscope.

2.1.5.3 Rescue Purification

Commonly the uppermost islet layer (at the 1.079 and 10.59 interface) was heavily contaminated with duct structures (commonly 50%). In such cases a 'rescue purification' procedure was developed which involved re-suspending the islet/duct suspension into dish at a depth of 5mm, leaving at R/T for 60 seconds and removing the duct structures which generally floated. The islets were then consolidated, washed and counted and purity after this procedure was approximately 85% (70-95%) based on observations following dithizone staining. The islets within this layer were largely smaller than the second islet layer and contained more fragmented islets.

2.1.6 Porcine Pancreas Digestion and Islet Isolation for Research

2.1.6.1 Tissue Procurement and digestion

Porcine pancreata were procured and digested using a modification of the method described O'Neil et al 2001 (183). Market grade pigs (Nottingham Trent University, Soton Bonnington, Nottingham, UK) were culled by electrocution and were then exsanguinated. A midline incision was made and the pancreas was resected. The pancreatic duct at the duodenal end was located and cannulated with a 16 gauge or 18 cannulae. The glands were subsequently infused with UW solution via the pancreatic duct and transported on ice to the islet isolation laboratory, cold ischemia times varied from between 65 and 120 min. Upon arrival pancreata were decontaminated in Videne solution, rinsed with HBSS and trimmed of all extraneous tissue including the pancreas capsule. The pancreata were distended with 2 x volume of Serva NB8 collagenase (1mg/ml) and placed in a sterile 1L tub along with any remaining collagenase solution. The organs were digested by static incubation in a water bath set at 37° C for between 25 to 65 min. At full digestion the pancreata

became loose and tissue was easily sloughed off using tweezers. The glands were then manually disrupted, passed through a 500um mesh and collected into a cold receiver containing HBSS with 10% FCS. The tissue effluent was then decanted into 250ml conical tubes and centrifuged for 2 min at 100g at 4˚C to produce a tissue pellet.

2.1.6.2 Isolation of Porcine Islets

The tissue pellet was suspended in a 1.13g/cm^3 ficoll/Euro-Collins which in turn was overlaid with a 1.096g/cm^3 and then 1.061g/cm^3 ficoll/Euro-Collins gradient. The discontinuous gradients were subsequently spun at 850g for 20mins and the islets were collected from the 1.096-1.061 interface. Samples of the islet layers were stained with dithizone and viewed for purity under a dissecting microscope. The islets were then washed with HBSS & 10% FCS and cultured

2.2 Tissue Culture

2.2.1 Standardisation of Seeding Density of Non-purified Islets

Directly after the pancreatic digest had been washed in MEM + 10% FCS, tissue was suspended in cold UW and centrifuged at 200g for 2 minutes with the resulting packed cell volume (PCV) noted. As small quantities of tissue were handled (between 0.5 and 4ml of digest) tissue pellets were first diluted with a known amount of HBSS and accurate tissue volumes were ascertained using a 2 or 5ml-graduated pipette taking into account the dilution factor.

2.2.2 Supplemented CRML for Human and Rodent Islet Culture

RPMI 1640 was used for the culture of rodent and porcine tissue while CRML media was used for human tissue culture (both Invitrogen, UK). Both media were supplemented with; 10% FCS, 5mM glucose, 2mM L-glutamate, 1mM sodium pyruvate, 71.5μM β-mercaptoethanol and 50pmol hydrocortisol and 10mM sodium

bicarbonate. All cultures were incubated at 37°C with 95% air and 5% CO_2 and fed every 48hours unless stated. All materials were obtained from Sigma Aldrich unless otherwise stated.

2.2.3 Memphis media

CRML1066 (Invitrogen, UK) was supplemented with 10µM water soluble vitamin E, 10mM nicotinamide, 2mM Glutamax, 25µM Hepes, 5mM sodium pyruvate, 16.7µM zinc sulphate. Subsequently for every 1L of media the following supplements were added; 2ml ciprofloxacin IV, 6.25mg insulin, 6.25mg human transferrin, 6.25µg selenium, 5.35µg water soluble linoleic acid, 25ml human serum albumin.

2.2.4 Replenishment of media, centrifugation method

The tissue suspension was placed in a sterile tube. Any tissue attached to the petri dish was immediately submerged in fresh media. The tissue suspension was centrifuged at 200g for 2 min, re-suspended in the petri dish and returned to the incubator. Spent media was stored at –20ºC for subsequent analysis.

2.2.5 Replenishment of media, sedimentation method

This protocol was similar to that outlined in section 2.2.4. The cell suspension was left at room temperature to sediment for 15 min, spent media was removed and the tissue pellet was re-suspended in the petri dish and returned to the incubator.

2.3 Islet assay and biochemical assessment

2.3.1 Islet Recovery

Islets were purified by the method specified above and the exocrine rich pellet was saved from the isolation preparation. One hundred islets were seeded alone, co-cultured with exocrine rich tissue (10µl pcv/ml) into 12 well plates. After 8 days, the cultures were again assessed for islet numbers. Islet counts were used to determine

islet recovery percentage (IRP) using the formula [IRP= islet count at day 8 divided by islet count at day 0 multiplied by 100]. Each experiment was carried out in triplicate.

2.3.2 Glucose Responsiveness

To assess insulin release of the islets a static incubation assay was performed. Static incubations were set up in 24 well plates, islet preparations were placed upon Millicell well-plate inserts (12µm pores, Nunclon, UK) and were pre-incubated with 1ml of 1.8mM glucose for 1h to wash the islets. Subsequently each set of islets was exposed to 1ml of 1.8mM, 25mM and 1.8mM glucose solution for 1h each and the resulting supernatants were collected and stored at –20˚C. The glucose solutions were made up in glucose free RPMI media with IV quality 50%w/v glucose solution. The stimulation index was measured as the insulin released in response to high glucose divided by the average of the insulin released in response to low glucose solutions.

2.3.3 Insulin secretion analysis

The insulin concentrations of culture media and tissue lysates from human and porcine experiments were measured using human insulin ELISA kits purchased from Biosource Europe S.A. The inter-assay variability of was between 5-6%. Rodent samples were measured on the standard or ultra sensitive rat insulin ELISA kit (DRG supplied by IDS Tyne & Wear, UK). Tissue lysates were commonly diluted 1:20 before assay whilst media was assayed undiluted.

2.3.4 Insulin Content of Islets

Samples were washed with PBS. After 3 washes the cell pellets were frozen at –80˚C until insulin extraction and DNA measurement. Stored samples were suspended in 200µl of sterile filtered water and sonicated on ice for 2 X 10 sec at 14 microns

(Soniprep, MSE, Worsley, UK). The lysate samples were then split into two 100ul samples for subsequent insulin content and DNA assay. For insulin content assay, 400ml acid/ethanol (98.35% ethanol, 1.65% HCL) was added to the 100ul sonicate and the preparation was extracted overnight by incubating the samples at 4°C, Directly after insulin extraction samples were samples were diluted 1 in 20 with PBS and assayed for insulin using ELISA (following the manufacturers instructions). DNA measurement of sonicated samples is outlined in section 2.3.6 and insulin content of tissue was expressed as ng insulin/µg DNA.

2.3.5 Amylase Assay

Amylase was measured on spent media samples using the Phadebas amylase test kit (Magle AB, Lund Sweden). Prior to assay samples were centrifuged at 1000g for 1 minute to remove cellular debris and the assay was carried out on the supernatants diluted 1:10 and 1:20 with CRML. The kit method was followed and all samples were read in a spectrophotometer at 630nm against a CRML blank. The amylase activity was calculated using a batch-coded standard curve supplied with each kit.

2.3.6 DNA Assay

DNA assay was used as an indication of cell numbers, and also insulin content was expressed in terms of DNA. DNA assay was also used to assess the homogeneity of initial seeding densities. Samples that had previously been sonicated were stored at -20°C until assay. Samples were measured on the Hoefer DyNA Quant 200 flurometer with the flurochrome disbenzimdazole (Hoechst 33258, Sigma UK), using a modification of the protocol provided. In brief, in standard curve was produced using salmon sperm DNA at a concentration of between 20 and 500ng/ml (final cuvette concentration). Samples were assayed by adding 10µl of sample to 2ml of

DNA assay buffer. Final concentrations of DNA in the cuvette were expressed as ng/ml DNA.

2.4 Morphometric studies

2.4.1 Tissue Fixation

Fresh and cultured tissue were washed in cold PBS and subsequently fixed for 3hrs in 4% paraformaldehyde (in PBS), embedded in 2% agar, dehydrated in graduated alcohols (70-100%), cleared of dehydrating agents and finally infiltrated with paraffin wax.

2.4.2 Immuno-histochemistry

Before staining, tissue blocks were cut into 4µm sections and dried at 37°C for a minimum of 24hrs. Sections were subsequently rehydrated by placing the sections in; xylene (x2) 99% IMS, 95% IMS 90% IMS and distilled water for 3 minutes each. At this stage antigen unmasking was carried out if required by microwaving sections in 0.01M sodium citrate buffer for 20mins at 850W. Sections were soaked in 3% H_20_2 for 10mins to block endogenous peroxidases, washed with distilled water and equilibrated in TSB +0.1% BSA

2.4.2.1 Immuno-histochemistry, standard protocol

Detection of the primary antibody was carried out using the ChemMate Envision™ Detection (Rabbit/Mouse) kit. In brief, the primary antibody was applied to each section, incubated at 37˚C for 1 hour and then washed with TBS + 0.1% BSA (3 x 5 min). A secondary antibody attached to horseradish peroxidase, was applied and incubated at room temperature for 30 min. Finally, after 2 thorough washes (x 5 min) with TBS + 0.1% BSA and a final wash with PBS, the diaminobenzadine substrate was used as the agent of antigen detection with haematoxylin used to counter-stain. Sections were then dehydrated through graded alcohols to xylene and coverslips

were mounted using XAM (BDH, UK) all products were purchased from Dako (Glostrup, Denmark) unless otherwise stated. Non-immune serum was used as a negative control.

2.4.2.2 Immuno-histochemistry, sensitive protocol

Detection of the primary antibody was carried out using the Catalyzed Signal Amplification System (Dako, Glostrup, Denmark). For this system TBS+0.1% tween was used for all washing steps. In brief, a protein block was applied to each section and incubated at room temperature for 5 minutes this was then replaced by the primary antibody and sections were incubated for 15 minutes. After this the sections were incubated with: link antibody, ABC complex, amplification reagent and streptavidin-peroxidase for 15 minutes each in a step like manner. Between each step sections were washed 3 times for 5 minutes. After the streptavidin step, diaminobenzadine was used as the agent of antigen detection. Sections were then counterstained with haematoxylin and coversliped.

2.4.3 Assessment of necrotic and apoptotic cells in islets

Central necrosis was characterised on sections stained with haematoxylin & eosin. Central necrosis was characterised by 1) a zone of dark cells that were clearly demarcated from the surrounding live to tissue 2) an a zone of cells showing widespread pyknosis or karyorrhexis a zone of cells devoid of nuclear material. The number of islets with central necrosis was counted and expressed as a percentage of the total.

Assessment of apoptotic cells was carried out using the terminal deoxynucleotide transferase dUTP Nick End Labelling (TUNEL) assay using APO-BRDU assay supplied by Biosource, CA, USA, using the protocol provided. Methyl green was used as a counterstain. When assessing digested pancreatic tissue which displays

marked levels of cell death the TUNEL assay was unable to discriminate whether the predominant mode of cell death was apoptosis, autolytic cell death or necrosis due to the fact that like apoptosis, necrosis and autolysis also result in the DNA strand breaks which represent a target for Tdt-mediated dUTP labelling and this finding has already been discussed in a previously published study (184).

2.4.4 Counting hormone staining of fixed tissue

Calculation of the percentage of insulin positive cells within each tissue sample involved a 2 stage approach. Initially 10 fields were analysed at x10 magnification to assess the mean percentage of insulin positive cell aggregates (insulin positive aggregates largely refers to islets, although following 8 days of culture, non-purified islet preparations contained aggregates of islet and non-islet tissue). Aggregates were classed as being insulin positive as long has they expressed cellular insulin staining in at least one cell.

Secondly at x20 magnification, 10 insulin positive aggregates were chosen at random and the mean percentage of insulin positive cells within the 10 islets was calculated. Therefore the percentage of insulin positive cells per tissue sample was calculated as the: [mean % insulin positive clusters x mean % percentage insulin positive cells].

For these analyses insulin was used as a survey stain for islets as it consistently gave a more intense stain than pan endocrine markers including chromogranin and synaptophysin.

2.4.5 Counting attached versus free islets within non-purified islet cultures

Following 8 days of culture non-purified islet preparations were shown to contain both free islets and islets attached to non-islet tissue. Therefore percentage of attached islets per tissue section was calculated as:

The total number of attached islets per tissue section x 100

The total number of islets per tissue section

2.4.6 Calculation of the proportions alpha, beta and delta cells within islets

To calculate the percentage of alpha, beta and delta cells within islets, 5 islets were chosen at random and the percentage of, for e.g. alpha cells was calculated using the following formulae:

The total number of alpha cells (within 5 islets) x 100

The total number of cells (within 5 islets)

2.4.7 Estimation of the insulin (ng) / µg of beta cell DNA

To calculate insulin (ng) / µg of beta cell DNA, intracellular insulin (Section 2.3.4) was normalised against the percentage of insulin positive cells that had been characterised using immunohistochemistry (Section 2.4.4), thus allowing an estimate of ng of intracellular insulin per µg of beta cell DNA.

Intracellular insulin (ng/µg DNA) X 100

Percentage of beta cells in sample

Antibody	Product code	Supplier	Dilution	Special conditions
Insulin	HB124	Hybridoma Bank Maryland US	Ascitic fluid 1:10,000	N/A
Glucagon	A0565	Dako	1:1000	HER
Somatostatin	A0566	Dako	1:5000	HER
Synaptophysin	M0776	Dako	1:25	HER
Amylase	A8273	Invitrogen	1:800	HER
Laminin [4C7]	M0638	Dako	1:500	N/A
PDX-1	Ab47267	Abcam	1:500	HER
PDX1	AB3243	Chemicon	1:500	HER
PDX1 [267712]	Mab2419	R&D systems	1:800	HER
Connexin 43 [CXN-6]	C8093	Sigma	1:1000	N/A
Cytokeratin 7 [OVTL]	AM255	Biogenix	1:100	HER
Cytokeratin 7 [OV-TL 12/30]	M07018	Dako	1:500	Human specific, HER
Cytokeratin 19 [BA17]	M0772	Dako	1:50	Human specific, HER
Vimentin [Vim 3B4]	M7020	Dako	1:100	HER
Glut-2 [5D1]	Ab85715	Abcam	1:2000	HER, CSA detection, biotin block required
CD133	Ab19898	Abcam	1:1000	HER, CSA detection, biotin block required
CA19-9 [TA888]	18-7265	Invitrogen	1:50	HER
VEGF [VG1]	M7273	Dako	1:1000	CSA detection, biotin block required
Ki-67	M7240	Dako	1:100	Requires Immediate staining after being cut (<48 hrs). HER

Table 2.4.1. **Antibodies used including product information and optimal directions for use**. *Although the majority of antibodies utilised were accompanied with manufacturer recommendations with regards to antibody concentration and antigen retrieval, optimisation experiments were carried out to elucidate the detection method judged to be the most sensitive. HER = Heat epitope retrieval.*

2.5 Molecular biology

2.5.1 *In-situ* hybridisation

Sections were stained for insulin mRNA using a human insulin oligo cocktail synthesised by Sigma biogenesis. The 6 oligo probe sequences used were: Probe1) TCC CTG CAG AAG CGT GGC ATT GTG GAA CAA. Probe 2) TCC CTC TAC CAG CTG GAG AAC TAC TGC AAC. Probe 3) CAC CTG GTG GAA GCT CTC TAC CTA GTG TGC. Probe 4) ATG GCC CTG TGG ATG CGC CTC CTG CCC CTG. Probe 5) GAC CCA GCC GCA GCC TTT GTG AAC CAA CAC. Probe 6) CTT CTT CTA CAC ACC CAA GAC CCG CCG GGA. Before use, the oligo probes were end-labelled using digoxigenin (Roche, UK). Tissue sections were incubated with the probes overnight at 37°C and RNA detected using the anti-digoxin/alkaline phophatase ascites, NBT/BCIP detection system (Roche, UK).

2.6 Statistical analysis

The statistical analyses used for each study is described within the methods section of each results chapter and p values of <0.05 were considered statistically significant. However the following is a description of the model used to compare the clinical islet graft outcomes (e.g. serum glucose, HbA1c and C-peptide) of patients either transplanted with purified or non-purified transplant taking into account 5 years worth of data.

- The model used was, a repeated measures multiple regression analysis and was used to compare the purified and non-purified groups, over the 5 year period of the study after taking into account the effect of year.
- Random intercepts and random slopes models were fitted to the data.
- This model took into account that clinical patient data can widely vary as such instead of fitting a common slope and a common intercept as would be carried out in a normal regression model, the intercept and slope was

allowed to vary across individuals, as such each individual had their own intercept and slope.

- As well as fitting random intercept and random slope models, random intercept only models were also fitted. Subsequently a comparison of the goodness of fit (using AIC criteria) was made and the model was selected (either random intercepts and random slopes or random intercepts only) based upon which had the lowest AIC.
- LS means (least squares means [mean score after adjusting for other effects in the model]).
- All data was analysed using SAS version 9.1 using Proc Mixed and statistical analysis was carried out in association with Dr John Bankart, Senior Lecturer, Statistics, University Hospitals of Leicester. P values of <0.05 were considered statistically significant.

2.7 Ethical approval

Rodent and Porcine pancreata were procured in line with schedule 1 Home Office guidelines.

Ethical approval for the use of cadaveric human pancreata were procured from the UK Human Tissue Bank, consent was obtained at the source and multi-centre ethical approval was obtained (04/MRE04/77). Local ethical committee approval was also obtained for the use of archived human chronic pancreatic samples (07/H0304/65). Work with all human tissues had gained Trust approval from the University Hospitals of Leicester before research commenced.

Chapter 3: Auto transplantation of non-purified islet vs. purified islets; a clinical study

3.1 Introduction

Within the sphere of islet transplantation there has been a common conception that high levels of contaminating non-islet tissue are detrimental to islet survival. This is thought to be due to activated acinar zymogens causing direct damage to both the islets and the cells of the transplant recipient (136,141), the effect of nitric oxide produced by ductal cells (140) and through acinar, ductal, endothelial and lymphoid elements eliciting a supra-immune response (105,118,138). While there is some evidence to support these assertions, islet losses during purification are often substantial and post purification counts are so reduced that they often do not satisfy the minimum numbers of islets required for transplantation (56).

In the field of islet auto-transplantation (following pancreatectomy for the treatment of chronic pancreatitis) it has been established that long-term outcomes, in terms of the need for exogenous insulin, is related to the number of islet equivalents transplanted (185). Consequently the majority of groups performing islet auto transplantation have chosen to transplant non-purified islets in order to preserve islet numbers (75,98). Results published over almost 20 years have demonstrated that patients auto-transplanted with non-purified islets following total pancreatectomy show stable function and extended insulin-independence rates, far superior to those reported following islet allo-transplantation (of purified islets), especially as the established method of sequential islet allo-transplant involves infusing, on average, 4-5 times more IEQs (51,98). In many ways the data for these two patient groups is not directly comparable and most obviously, auto-transplantation avoids the need for potentially diabetogenic and beta cell damaging immunosuppressive drugs (66). In islet auto transplantation, allogenic rejection of

tissue is avoided, as is (and perhaps more importantly) the recurrence of the beta cell targeted immune response (70), which is the signature of type 1 diabetes and is thought to play an important role in the loss of islet mass and function following islet allo transplantation (reviewed by (67)). However, auto transplantation of non-purified islets clearly demonstrates that islets can survive, engraft and function when co-transplanted with significant amounts of contaminating acinar and ductal tissue, even when the islet equivalents transplanted are low (75,186,187).

Within the Leicester series, islet purification prior to transplant was initially regarded as essential, similar to the vast majority of islet transplantation centers throughout the world (3). However, due to the observed losses during purification, this process was restricted to larger weight pancreata (with higher digest volumes) and later phased out completely. Consequently, the series from the University Hospitals of Leicester provides an opportunity to compare the long-term outcomes of patients receiving purified or non-purified islet auto transplants. The main aim of this study was to compare the long-term (5 year) graft function of patients transplanted with either purified or non-purified islets. Secondly, perioperative outcomes and patient liver function (at 6 and 12 months post transplant) were examined.

3.2 Methods

3.2.1 Patient data, islet transplantation and postoperative care

Of the 55 patients within the Leicester series, 24 were excluded due to the criteria discussed below. All assessments carried out were part of standard patient care. A description of the patient pre and post operative assessments can be found in sections 2.1.2 and 2.1.1 respectively and surgical aspects of the pancreatectomy, which are outside the scope of this study, have been previously described (75,188). In addition, routine biochemistry for liver function was assessed, and specifically, tests for albumin, bilirubin, aspartate aminotransaminase and alkaline phosphatase were performed at 6 months and annually thereafter as part the surgical follow up for post-hepaticojejunostomy and islet auto transplantation.

3.2.2 Exclusion Criteria

Thirty one of the 55 patients within the Leicester series were included in this study and 13 of those patients had received purified islets. A number of patients where confounding factors could potentially influence peri-operative and transplant outcomes were excluded. Islets transplanted via the re-cannulated umbilical vein (rather than the middle colic vein into the portal vein) were excluded, as the umbilical approach restricts islets to the left lobe of the liver; (although there is no definitive evidence that this should significantly affect graft function). Patients receiving a transplant following partial resection were also excluded as assessment of islet graft function would be masked by function in the residual pancreas. Finally, all patients included within this study had a minimum of 5 years post operation follow-up to allow assessment of long-term graft function, although since many patients were recruited from centers at some distance from Leicester, a number of patients failed to attend an assessment every year.

3.2.3 Statistics

Median values and ranges or mean values +/- SEM were used as descriptive statistics. The results of purified versus non-purified outcomes were measured using the two tailed-Students t-test and non-parametric analogues of this test on SPSS version 16. Comparisons of purified versus non-purified transplant outcomes over the entire 5 year follow up period were analysed using repeated measures multiple regression (random intercepts and random slopes were initially fitted), using SAS version 9.1 using Proc Mixed. The full description of the model used can be found in section 2.6, p values of <0.05 were considered statistically significant.

3.3 Results

3.3.1 Isolation and peri-operative data.

Within this series, islet purification was initially restricted to larger pancreata, 72.25g (53.00-127.55) vs. 62.18g (22.75-109.00) and later phased out. Consequently, although islet losses during islet purification were substantial, the IEQs recovered and transplanted were similar in the purified and non-purified groups (2076 IEQ/kg (406-20385) vs. 2022 IEQ/kg (509-10020), p=0.7342), Table 3.1, Figure 3.1). Consequently the number of IEQs procured per gram of pancreas was on average 20% higher when purification was omitted (1455 IEQ/g (249-7445) vs. 1828 IEQ/g (304-12271), p=0.8574, Table 3.1). This result however did not reach significance, possibly as a result of heterogeneity between pancreata where varying levels of fibrosis, which has been well characterised in the late stages of chronic pancreatitis, influenced the efficiency of tissue digestion and possible beta cell depletion, (189,190).

Outcome	Purified	Non-purified	*p value*
Patient number	13	18	*n/a*
Pancreas weight (g)	72.25 (53.00-127.55)	62.18 (22.75-109.00)	*0.1406*
Islet Purity %	53 (10-75)	<5%	*n/a*
Transplant Volumes %	No data *	11.5 (1-20)	*n/a*
Total islet count	213000 (44600-380079)	269750 (33500-879000)	*0.4385*
IEQ/g pancreas	1455 (249-7445)	1828 (304-12271)	*0.8574*
IEQ/kg bodyweight	2076 (406-20385)	2022 (509-10020)	*0.7342*
Blood loss (ml)	500 (200-1000)	400 (200-1000)	*0.1917*
CVP pre (cm H_20)	6 (4-8)	5.5 (4-12)	*>0.999*
CVP post (cm H_20)	8 (8-10)	8 (6-15)	*0.4671*
HPP pre(cm H_20)	8.5 (4-16)	9 (6-14)	*0.4315*
HPP post (cm H_20)	17 (12-34)	17(13-33)	*0.9158*
BMI (weight (kg) / height (m^2)	20.7 (16.4-25)	20.3 (15.2-25.2)	*0.8129*
Operation Length (hours)	**8.5 (7.5-11.0)**	**7.5 (5.5-11.5)**	***0.0496***
ITU Stay (days)	2 (1-4)	3 (1-9)	*0.0941*
Total Hospital stay (days)	*18 (10-40)*	*19 (13-52)*	*0.8222*

Table 3.1 A comparison of the islet isolation data and peri-operative data for patients receiving either purified or non-purified islets. *IEQ represents islet equivalents normalised to diameter of 150µm. CVP and HPP have been used as abbreviations of central venous pressure and hepatic portal pressure. Due to inadequacies in data recording, accurate packed cell volumes were not recorded for the majority of purified islet grafts. P values of <0.05 are considered statistically significant.*

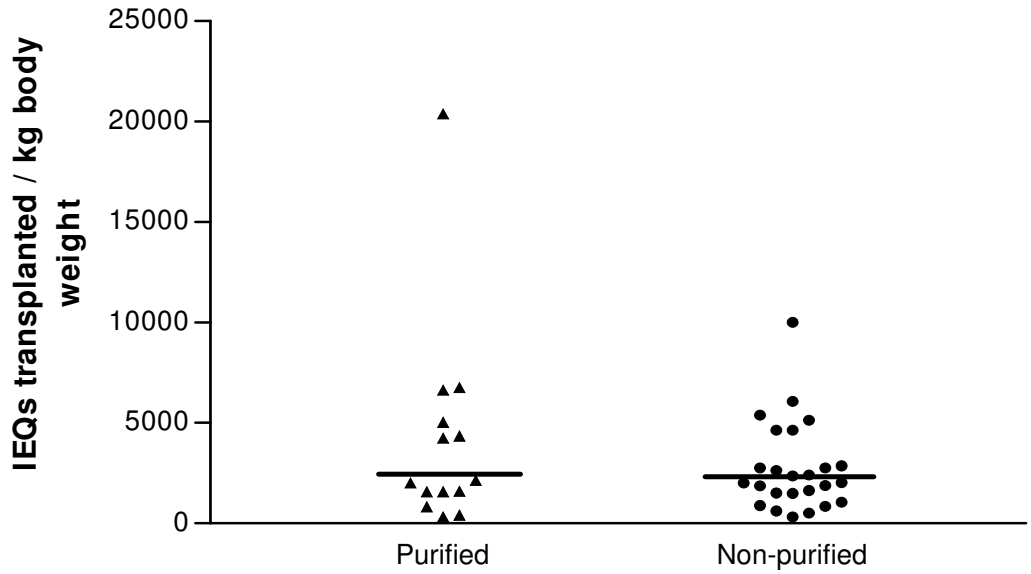


Figure 3.1 Scatter plot comparing the IEQs transplanted in both groups (purified vs. non-purified islet transplants). *Analysis using the Mann-Whitney test shows that there is no statistical significant difference between the means of each group.*

Analysis of perioperative data revealed that there were no marked differences in central venous or portal venous pressures during transplantation (Table 3.1). One significant finding of this study was that the total operation time of patients receiving purified islets was, on average, one hour longer and this difference reached statistical significance (8.5 hr (7.5-11.0) vs. 7.5 hr (5.5-11.5), p=0.0496). This result is important as it has been previously demonstrated that duration of surgery is a major risk factor for complications, in particular post-operative thrombosis (191,192).

3.3.2 Post operative liver function

An asymptomatic, self-limited transaminitis has been shown to occur ubiquitously following transplantation of both purified and non-purified islets (193-195). Typically alkaline phosphatase (Alp) and aspartate aminotransaminase (Alt) peak at up to 5 times the upper limit of normal, following surgery but resolve completely by week 4 post-transplant (194,195). As short-term derangement of liver function post transplant has been extensively reported, this study sought to detect any medium-term changes in liver function tests, (6mth and 12mth post-transplant), taking into account that non-purified islets may present a more profound insult to the liver than purified islets.

For both groups (purified and non-purified), median pre-and post transplant liver function results (Tables 3.2a & b) largely lay within normal limits at 6 and 12 months post transplant. This is despite the fact that alcoholism (a major cause of chronic pancreatitis) and narcotic usage (prescribed to treat the intractable pain in this patient group) have been demonstrated to significantly derange LFT scores (196,197). However, it must be noted that both ALP and ALT levels were notably elevated in the non-purified islet group compared with pre-transplant readings, possibly indicating that transplantation of high levels of non-islet tissue was

associated with a mild disturbance of heptocellular integrity. ALP was significantly elevated in the non-purified islet group at both 6 and 12 months compared with pre-transplant levels (71 iu/L (41-148) pre-transplant, 134 iu/L (61-424), at 6 months post transplant p=0.0001, and 95.5iu/L (65-306) at 12 months p=0.0063). ALT exhibited a near significant increase at 12 months post transplant compared with pre-transplant levels in the non-purified group (27 iu/L (9-64) vs. 33 iu/L (14-73), p=0.0658). Importantly, within the non-purified group there was no evidence that albumin or bilirubin synthesis was compromised and ALP and ALT levels were lower at 12 months compared with 6 months suggesting a resolution of the injury.

Derangement of liver function was also observed in the group receiving purified islets with significantly increased ALT levels at 6 months compared with pre-transplant levels (18.5 iu/L (11-60) vs. 47 iu/L (24-136), p= 0.0496), however, this had reduced to some extent by 12 months post transplant (18.5 iu/L (11-60 vs. 31iu/L (17-91), p=0.1812). In addition, a significant decrease in albumin levels could be seen at 12 months in the purified islet group (43g/L (38-45) vs. 35 g/L (24-40), p=0.0117. Indeed comparisons of purified versus non-purified islets at 12 months showed that albumin levels in the purified group was significantly lower (35.77+/-1.75 vs. 40.25+/-1.42, p=0.0047). However from a clinical prospective these reduced levels were well within the normal clinical reference range and of no apparent clinical significance.

a

	Purified pre-transplant (n=6)	**Purified post-transplant** (n=9)	**p value**	**Non-purified pre-transplant,** (n=19)	**Non-purified post-transplant** (n=13)	**p value**
Albumin (35-55g/L)	43 (38-45)	41 (22-47)	*0.8134*	40 (30-46)	35 (27-47)	*0.2194*
ALP (40-130 iu/L)	74 (32-102)	95 (51-223)	*0.1810*	71 (41-148)	134 (61-424)	***0.0001***
ALT (2-53iu/L)	18.5 (11-60)	47 (24-136)	***0.0496***	27 (8-64)	35 (12-62)	*0.1201*
Bilirubin (3-17 µmol/L)	10 (8-14)	9 (7-13)	*0.7223*	9 (2-27)	10 (2-21)	*0.8029*

b

	Purified pre-transplant (n=6)	**Purified post-transplant** (n=8)	**p value**	**Non-purified pre-transplant,** (n=19)	**Non-purified post-transplant** (n=14)	**p value**
Albumin (35-55g/L)	43 (38-45)	35 (24-40)	***0.0117***	40 (30-46)	41.5 (31-48)	*0.2508*
ALP (40-130 iu/L)	74 (32-102)	91.5 (56-225)	*0.1419*	71 (41-148)	95.5 (65-306)	***0.0063***
ALT (2-53iu/L)	18.5 (11-60)	31 (17-91)	*0.1812*	27 (9-64)	33 (14-73)	***0.0658***
Bilirubin (3-17 µmol/L)	10 (8-14)	10.5 (6-15)	*0.8972*	9 (2-27)	9 (5-22)	*0.7900*

Tables 3.2a & b Liver function test results at 6 months (Table 3.2a) and 12 months post-transplant (Table 3.2b*). Analysis was carried out using the Mann-Whitney test. Pre-transplant levels were defined as values measured upon hospital admission immediately before the operation. Due to patients living outside of the area and non-compliance the data is not complete. The p values represent T-tests comparing pre-transplant and post transplant data. Comparisons of post transplant values for the purified group vs. the non-purified group revealed one statistically significant result (albumin was statistically lower at 12 months in the purified group (35 (25-40) vs. 41.5 (31-48), p=0.0024, analysis not shown). The local references ranges for each LFT are in parenthesis.*

3.3.3 Markers of islet graft function

The glucose status of each patient represented by fasting glucose, stimulated glucose (both 30 min and 2 hours after an oral 75g glucose load) and HbA1c levels was used as an indicator of islet graft function. Additionally C-peptide, which is released in equimolar amounts with insulin, was used as a direct measure of insulin release during conditions of fasting and stimulation.

Annual patient follow-up data revealed that, in the majority of cases, transplantation of non-purified islets was often associated with superior glucose status year on year and in some cases superior insulin secretion (Figures 3.2 & 3.3). Most notably, non-purified islets were associated with significantly lower fasting glucose values compared with purified islets (6.15 mmol/L (3.70-10.50) vs. 9.60 mmol/L (7.40-20.50), p=0.0049) at 2 years post transplant. Additionally, at this 2 year time point, near significant results were observed for 2 hr stimulated glucose (13.00 mmol/L (5.80-26.40) vs. 24 (8.00-33.00), p=0.0702) and 2hr stimulated C-peptide levels (2.25 mmol/L (0.31-9.63) vs. 0.89 mmol (0.26-1.65), p=0.0545). When assaying glucose related data (Figure 3.2) with two exceptions (GTT120 year 5 and HbA1c at year 2) non-purified islet patients showed lower blood glucose values (fasting and stimulated) and lower HbA1c % scores year on year for the entire 5 follow-up period. Comparisons of fasting and stimulated C-peptide secretion were not so clear cut, with non-purified islets being associated with non-significant but superior C-peptide secretion in 6 out of 12 cases (Figure 3.3a-c). Assessments of daily insulin requirements were again not clear cut with insulin requirements being notably lower in the purified group during year 1 and 2 post-transplant. However as figure 3.4 illustrates, whilst insulin requirements within the non-purified group remain remarkably constant over the 5 year period, the purified group show a marked increase and significance is discussed below.

Comparing each outcome over the entire 5 year follow up period using a repeated measures multiple regression model confirmed that non-purified islets were largely associated with superior outcomes (Table 3.3). In particular fasting glucose levels and HbA1c results were significantly lower in the non-purified group (7.59+/-0.64 vs. 9.68, p=0.0282 and 7.50+/-0.31 vs. 8.7+/-0.39, p=0.019 respectively), suggesting superior long-term control of blood glucose levels. Upon the assessment of C-peptide secretion, non-purified islets were seen to be comparable to purified islets when assessing fasting C-peptide and C-peptide 30, however, C-peptide 120 was shown to be statistically higher in the purified group. This last finding however, has to be balanced with the fact that over the 5 year follow-up period, the purified group showed a year-on-year fall in recorded C-peptide 120, with values falling from a mean of 3.72ng/ml at year 1 post transplant to 0.87ng/ml at year 5. Interestingly, during the same period the non-purified group showed a reciprocal increase in values from 1.58 to 2.98ng/ml and the interaction between the 2 groups (i.e. the fall in the purified group and rise in the non-purified group) was shown to be significant using the regression model described (p=0.014). Therefore, despite the higher mean C-peptide 120 value calculated using the regression model, delineation of the year on year data suggests that islet function, in terms of response to glucose, is superior in the non-purified group over the long-term.

A further important finding of the regression model used, was the gradual deterioration of all markers of graft function (except C-peptide 120) across both groups showing a linear pattern, in particular, the significant increases in stimulated glucose score with time post transplant and the significant fall in fasting C-peptide values. GTT30 showed a 1.00mmol/L increase per year (p=0.0063) and GTT120 increased by 1.01mmol/L (p=0.002), whilst fasting C-peptide fell by a mean of 0.15ng/ml per year post transplant.

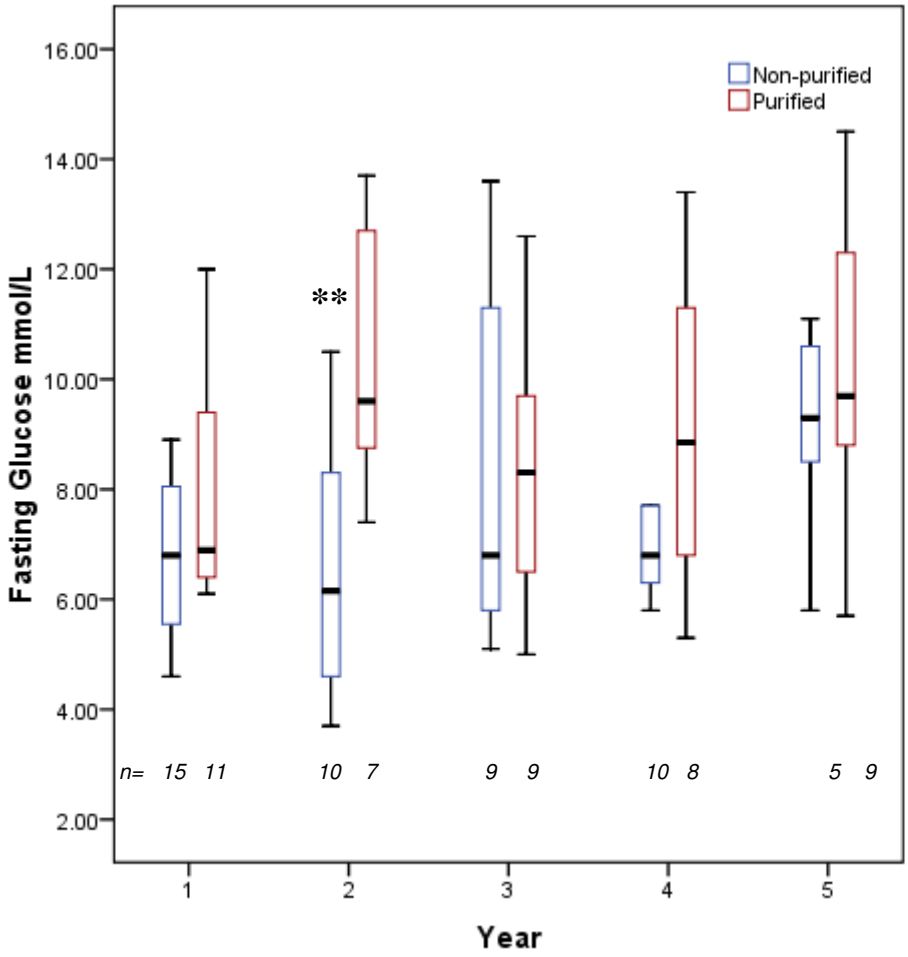


Figure 3.2a Fasting glucose following the transplantation of purified and non-purified islets. *Analysis of each timepoint using the Mann-Whitney test showed that non-purified islets (blue bars) were associated with lower fasting glucose values year-on-year and significantly lower values at 2 years post transplant (6.15 (3.70-10.50) vs. 9.60 (7.4-20.50), p=0.0046). Multiple regression analysis assessing the entire 5 year follow-up period agreed with the t-test results showing that non-purified islets were associated with significantly lower mean fasting glucose levels (7.59+/-0.64 vs. 9.68+/-0.74, p=0.028, table 3.3). The regression analysis also revealed that within both groups, glucose levels rose by a mean of 0.43mmol/L per year, however this trend did not reach significance (p=0.095, Table 3.3).*

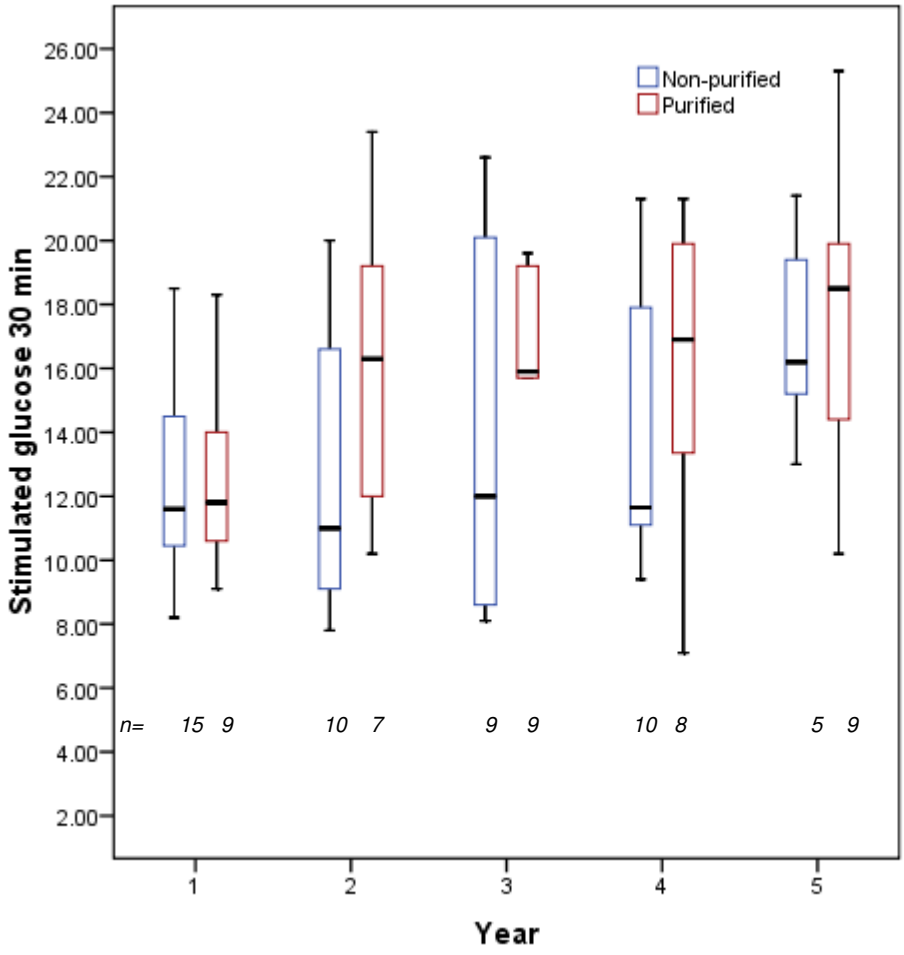


Figure 3.2b Stimulated glucose (GTT30) following the transplantation of purified and non-purified islets. *Analysis of each timepoint using the Mann-Whitney test showed that non-purified islets (blue bars) were associated with lower stimulated serum glucose (i.e. 30 min after a 75g glucose bolus), although this did not reach significance. Similarly, multiple regression studies assessing the entire 5 year follow-up period revealed lower mean glucose values within the non-purified group although again this trend did not reach significance (14.2+/-0.92 vs. 15.5+/-1.09, p=0.321, table 3.3). The regression analysis also revealed a significant rise in glucose levels in both groups over the follow-up period equating to approximately 1mmol/L per year (p=0.006, Table 3.3).*

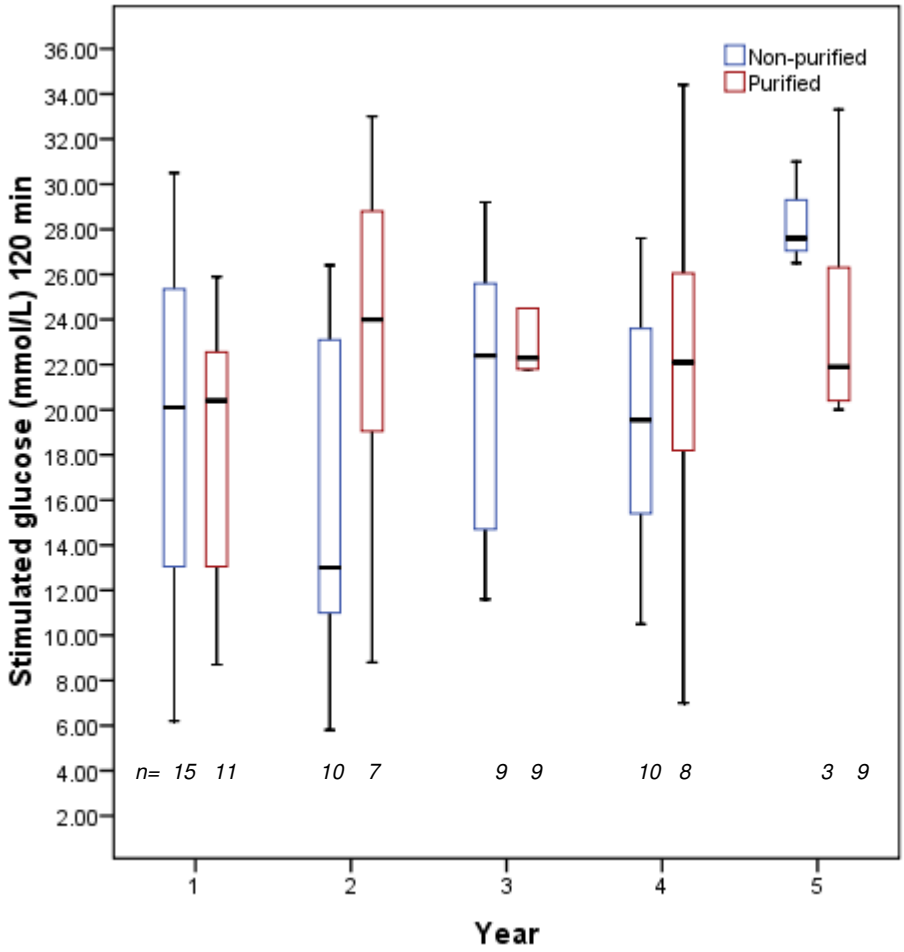


Figure 3.2c Stimulated glucose (GTT120) following the transplantation of purified and non-purified islets. *Analysis of each timepoint using the Mann-Whitney test showed that, non-purified islets (blue bars) were to be associated with lower stimulated serum glucose (i.e. 120 min after a 75g glucose bolus) up to year 4 post transplant, although none of these results reached significance. Similarly, multiple regression studies assessing the entire 5 year follow-up period revealed lower mean glucose values within the non-purified group, although again this finding did not reach significance (19.4+/-1.6 vs. 20.1+/-1.9, p=0.789, table 3.3). The multiple regression studies revealed that in both groups there was a significant rise in glucose levels each year equating to approximately 1.01mmol/L per year (p=0.002, Table 3.3).*

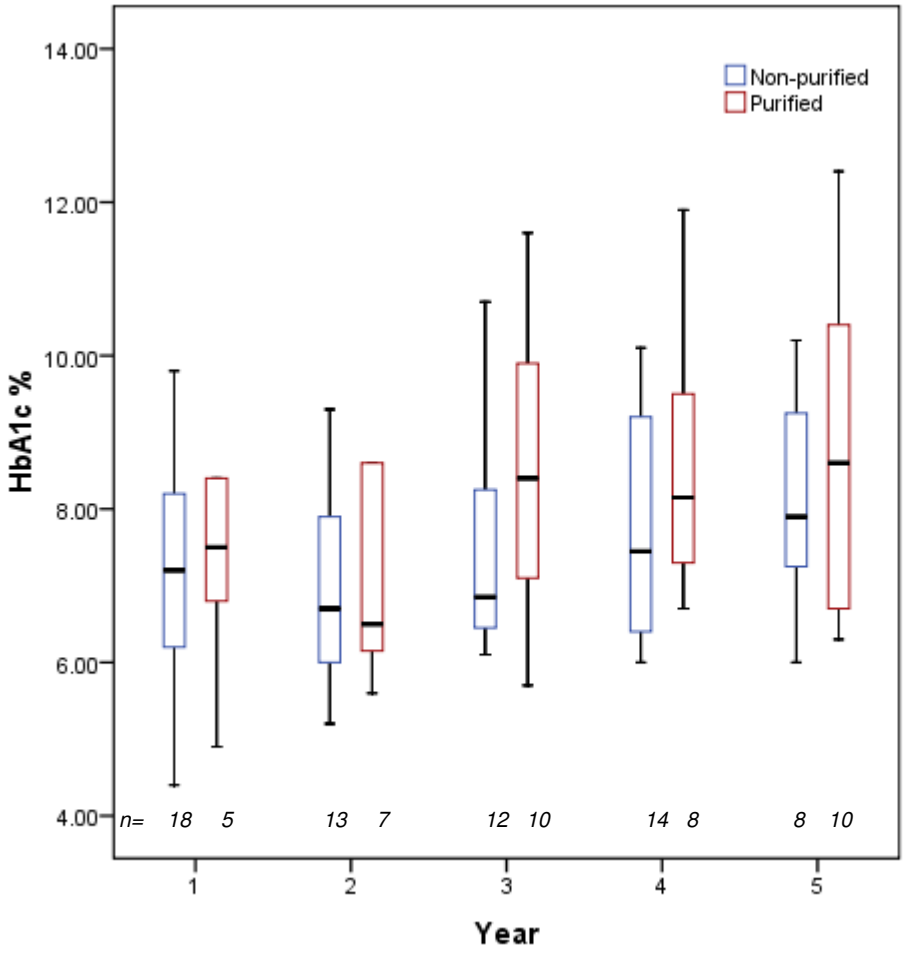


Figure 3.2d HbA1c values following the transplantation of purified and non-purified islets. *Analysis of each timepoint using the Mann-Whitney test showed that non-purified islets (blue bars) were associated with lower HbA1c values year-on-year, although none of these results reached significance. However, additional multiple regression analysis showed that non-purified islets were associated with significantly lower HbA1c when taking into account the entire 5 year follow-up period (7.5+/-0.31 vs. 8.7+/-0.39, p=0.019, Table 3.3). Multiple regression studies also revealed that HbA1c values rose across both groups at a mean rate of 0.18% per year, however, this trend did not reach significance (p=0.245), Table 3.3.*

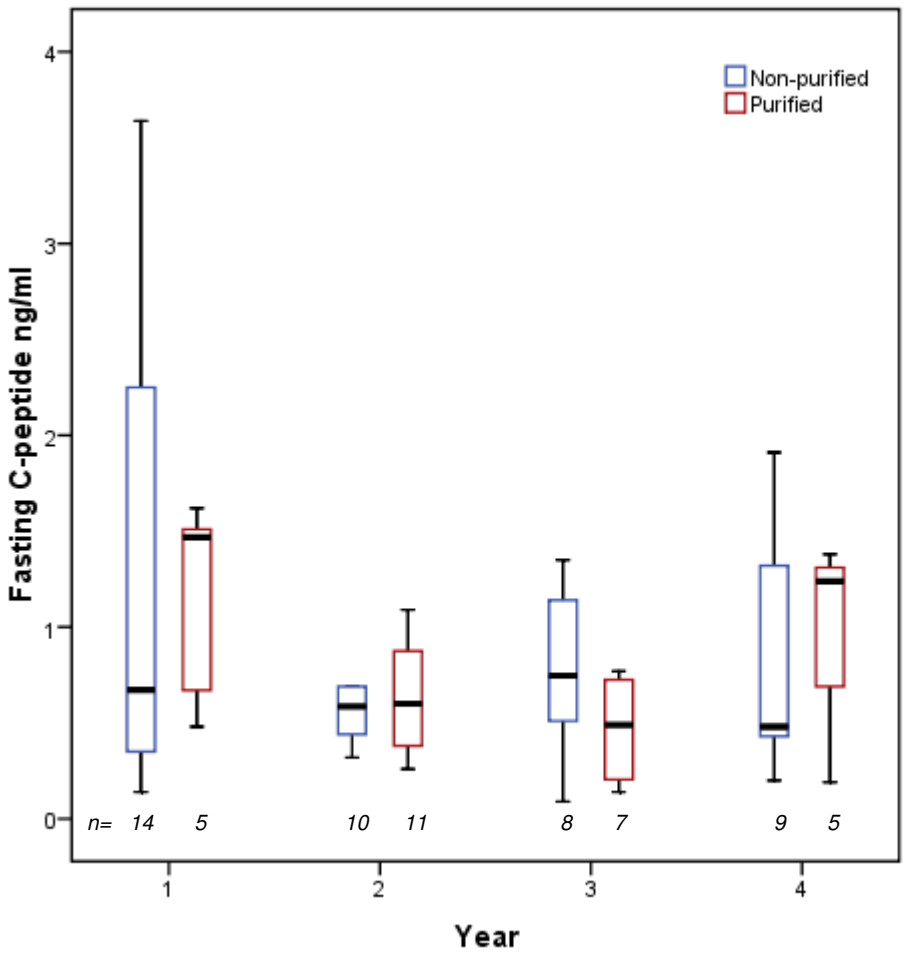


Figure 3.3a Fasting C-peptide (C-peptide 0) following the transplantation of purified and non-purified islets. *Analysis of each timepoint using the Mann-Whitney test showed that, fasting C-peptide levels in the non-purified (blue bars) and purified groups were statistically comparable. Multiple regression studies also confirmed that both groups were statistically comparable (0.85ng/ml +/-0.09 vs. 0.96ng/ml+/-0.10, p=0.371, for non-purified and purified islets respectively). However, the regression studies did show a significant fall in fasting C-peptide across both groups, equating to a mean of 0.15ng/ml per year (p=0.042 Table 3.3). Year 5 data were not included in t-test analysis due to insufficient data.*

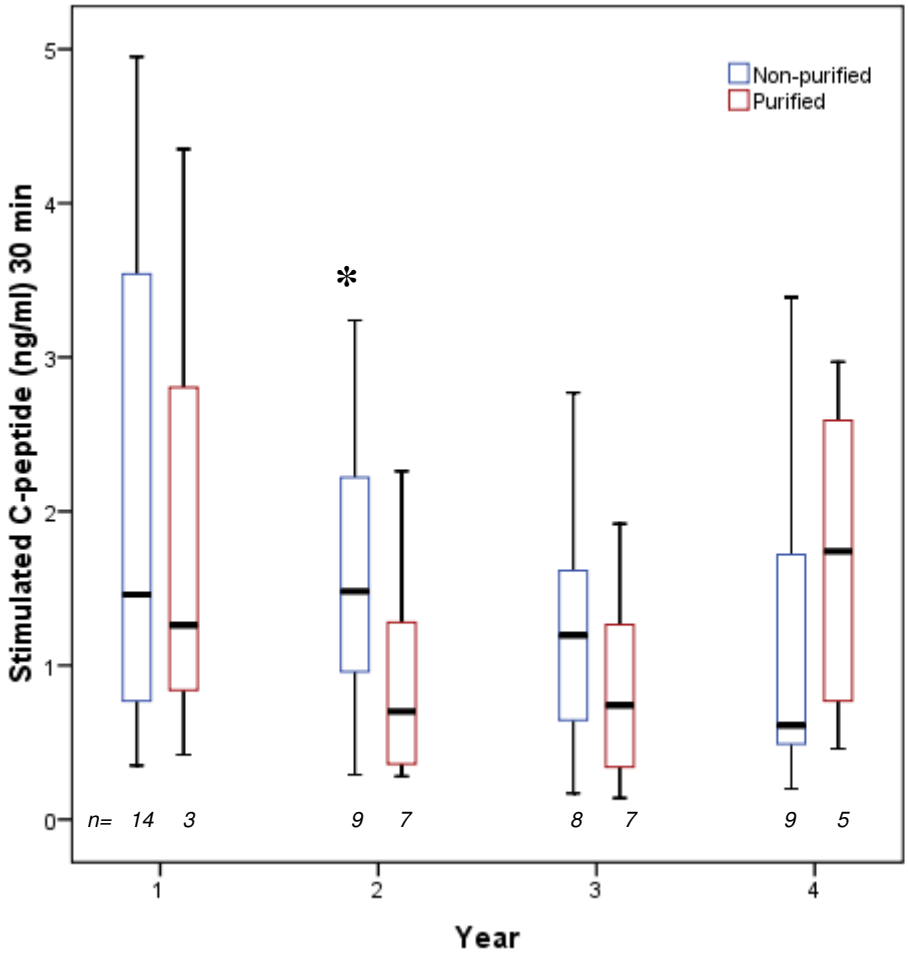


Figure 3.3b Stimulated C-peptide (C-peptide 30) following the transplantation of purified and non-purified islets. *Analysis of each timepoint using the Mann-Whitney test showed that stimulated C-peptide levels (taken 30 minutes after a glucose bolus). Up to year 3 post transplant non-purified islets were associated with superior C-peptide levels and at year 2 this reached significance (2.25 (0.31-9.63) vs.0.89 (0.26-1.65), p=0.018). Similarly, multiple regression analysis showed that mean stimulated C-peptide was higher within the non-purified group, although this did not reach significance (1.62ng/ml+/-0.28 vs. 1.32ng/ml+/-0.36, p=0.522). Multiple regression analysis also showed that mean C-peptide levels within both groups decreased by a mean of 0.21ng/ml per year although this trend did not reach significance (p=0.066, Table 3.3). Year 5 data were not included in t-test analysis due to insufficient data.*

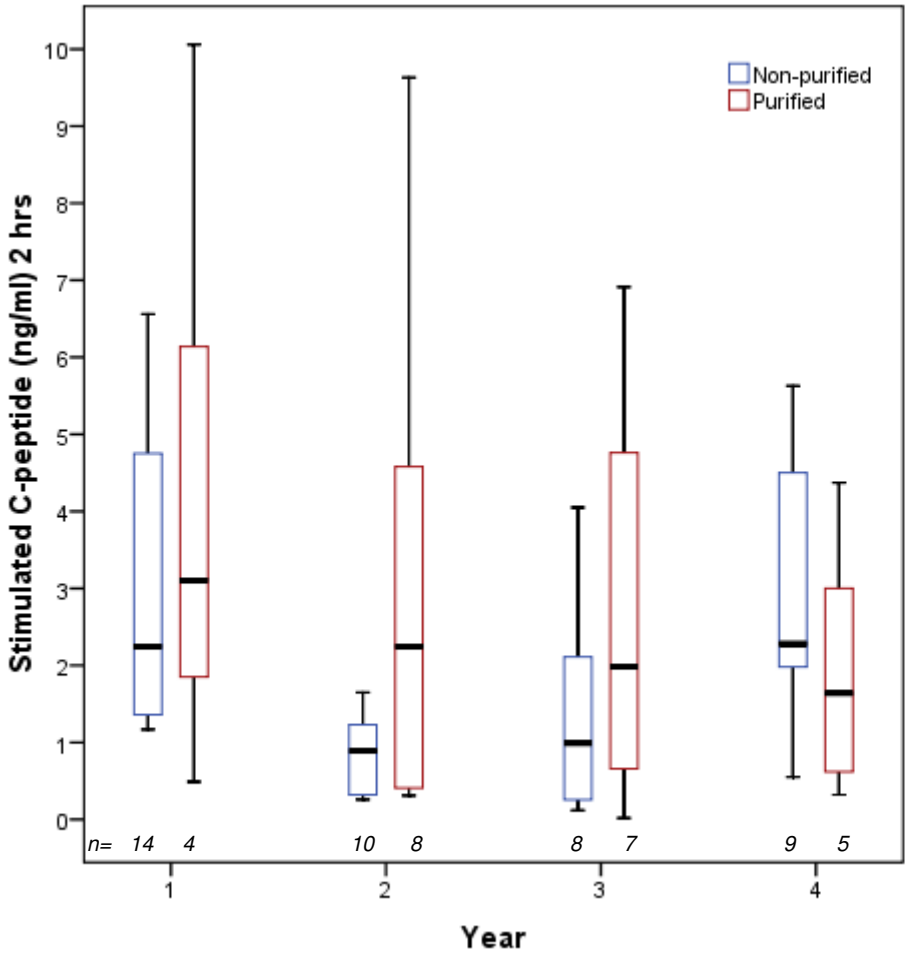


Figure 3.3c Stimulated C-peptide (C-peptide 120) following the transplantation of purified and non-purified islets. *Stimulated C-peptide levels (120 min after a glucose bolus) fluctuated year-on-year. Based on t-test data (the Mann-Whitney test), non-purified and purified islets secreted comparable levels of C-peptide at each time point. However, based on Multiple regression analysis, non-purified islets were associated with significantly lower stimulated C-peptide values (1.82+/-0.63 vs. 2.94ng/ml+/-0.49, p=0.008). Further analysis using the regression model described in section 2.6 showed an interaction between the purified and non-purified groups, in that, the purified group fell by a mean of 0.57ng/ml per year whilst the non-purified group showed an increase of 0.27ng/ml per year. Using the model described in section 2.6 the interaction between these 2 groups reached significance (p=0.014). Year 5 data were not included in t-test analysis due to insufficient data.*

	Purified (LS means)	Non-purified (LS means)	p (purified vs. non-purified)	Effect of time (mean increase/ decrease per year)
GTT0 mmol/L	9.68 +/- 0.74	7.59+/-0.64	***0.028***	+0.43mmol/L p=0.095
GTT30 mmol/L	15.5+/-1.09	14.2+/-0.92	*0.321*	+1.00mmol/L **p=0.006**
GTT120 mmol/L	20.1+/-1.9	19.4+/-1.6	*0.789*	+1.01mmol/L **p=0.002**
HbA1c %	8.7+/-0.39	7.5+/-0.31	***0.019***	+0.18% p=0.245
C-peptide 0 ng/ml	0.96+/-0.10	0.85+/-0.09	0.371	-0.15 **p=0.042**
C-peptide 30 ng/ml	1.32+/-0.36	1.62+/-0.28	0.522	-0.21 p=0.066
C-peptide 120 ng/ml	2.94+/-0.49	1.82+/-0.63	**0.008**	***** -0.57 (purified) +0.27 (non-purified) **p=0.014**

Table 3.3: Repeated measures multiple regression analysis of clinical markers of islet function over a 5 year follow up period. *The data displayed represents the mean glucose, HbA1c and C-peptide values of all patients tested within the non-purified and purified groups (assessed by least square means +/- standard error), and taking into account 5 years worth of data. The regression model used, primarily analysed the affect of year on each clinical graft outcome (purified and non-purified patients pooled together) and subsequently compared the Least square means (LS Mean) of the purified and non-purified groups, which equated to the mean value over the 5 year period following an adjustment for other affects of the model, i.e. time (years).*

When assessing the affect of time on the results, GTT30 and GTT120 showed a significant increase with time, whilst fasting C-peptide showed a significant decrease. When comparing the LS means of each group, non-purified islet patients showed significantly lower mean fasting glucose and HbA1c than purified islet patients. Interesting C-peptide 120 showed significantly higher values in the purified group however, the regression model also revealed that there was a significant interaction between each group, in that, values within the purified group where shown to fall whilst the non-purified group showed a mean increase in values. Raw data relating to the regression model used can be found in Appendix II.

3.3.4 Insulin Requirement

Analysis of Insulin requirement data, which is undoubtedly an important marker of long-term graft function showed a similar pattern of data as reported for C-peptide 120. Regression analysis of the 5 year period showed that the purified islets were associated with significantly lower insulin mean requirements (20.3+/-5.4 vs. 25.6+/-4.1, p=0.0229). However, when using the same regression model to look at the interaction between the 2 groups, the purified group showed a significant year on year increase in insulin requirements (from 11.4 at year 1 to 30.5 at year 5) whilst the non-purified group remained stable during the same period (26.5 at year 1 and 27.9 at year 5). The interaction between these 2 groups was shown to be significant (p=0.0046) and show a gradual derangement of islet function in the purified group.

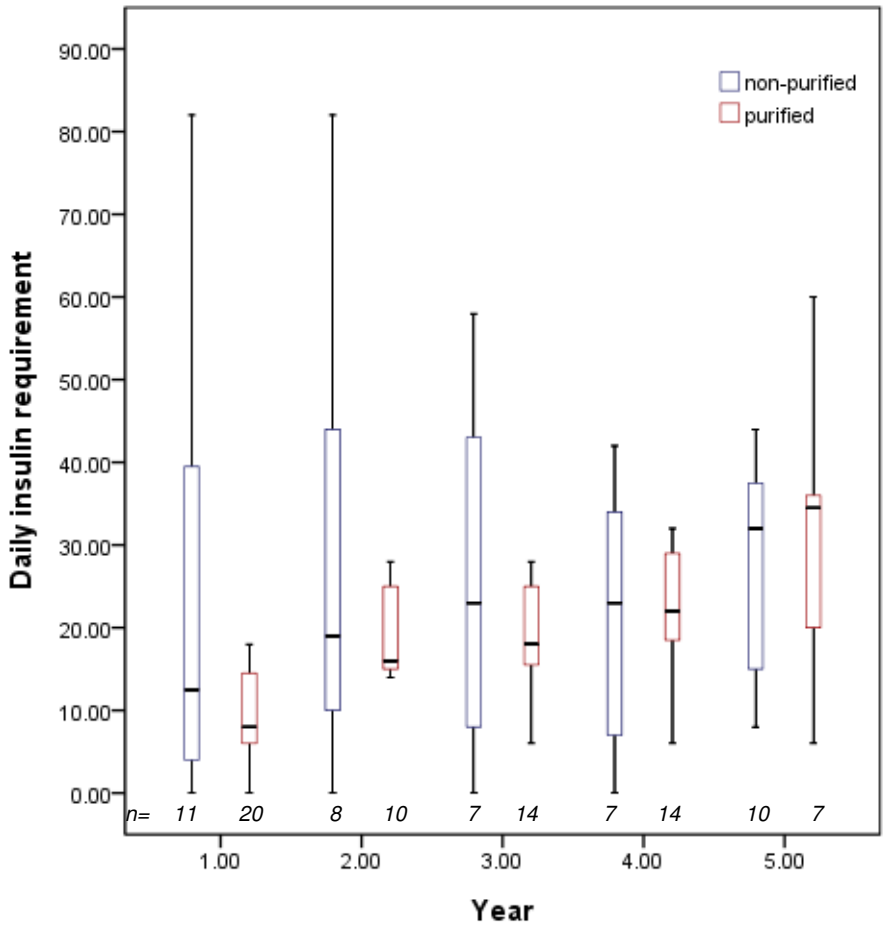


Figure 3.4 Daily Insulin dose following the transplantation of purified and non-purified islets. *Based on t-test data (the Mann-Whitney test), patients within the non-purified and purified islets groups required comparable levels of insulin at each time point. However, based on Multiple regression analysis, purified islets were associated with significantly lower insulin requirements* (20.3+/-5.4 vs. 25.6+/-4.1, p=0.0229). *Further analysis using the regression model described in section 2.6 showed an interaction between the purified and non-purified groups, in that, the purified group showed a substantially increase in insulin intake year on year (4.7 units/year) whilst insulin intake within the non-purified group remained relatively stable (average increase of 0.35 units/year). Using the model described, the interaction between these 2 groups reached significance (p=0.0046).*

3.4 Discussion

Auto transplantation of islets compared with allo-transplantation has many fewer confounding factors to consider and can therefore provide an uncomplicated clinical model to assess islet function post transplant. Issues of patient immunosuppressive regimens particularly toxicity and discontinuation of therapy (198) are avoided, cold ischaemia times are consistent and relatively short as organ procurement and processing is generally completed on one site, and islets are usually transplanted directly following isolation without a period of transportation or culture (76). In addition, tissue processing is completed within a shorter period (less than three hours (76)) largely due to the excision of the pancreas free of fat and the fact that simple syringe driven perfusion is primarily utilized (75).

The results of the current study are largely in line with the findings of other auto transplantation groups, most notably the publications of the Minneasota group (101,104), in that it provides evidence that transplantation of purified islets confers no advantage in the auto-transplant setting; indeed, the data of this study suggests the contrary. Analyses of post operative markers of graft function indicate that non-purified islets were associated with stable long-term function whilst peri-transplant venous pressures were shown to be comparable in each group. This study did highlight a mild derangement of ALP and ALT post transplant in both groups, particularly the patients receiving non-purified islets, however the data indicated a resolution of these changes by 12 months post transplant. Interestingly a significant drop in albumin levels was evident at 12 months post transplant in the purified group, however in the light of the studies of Peavy et al 1985 (199) and others (200,201) it is possible that this is an indication of insulin insufficiency within the purified group rather than a direct heptocellular insult. Peavy's study indicated that diabetes induction leads to rapid reduction of liver protein synthesis,

particularly the synthesis of albumin and other plasma proteins (12.7 to 2.2% of total protein synthesis during the first 3 days after the induction in rodents) (199). As such, the significant reduction in albumin levels at 12 months may reflect the relatively low C-peptide levels seen at year 1 post transplant within the purified islet group (Figures 3.3a & b).

The most interesting finding of this study was that within the Leicester series, non-purified islet transplants were not out-performed by purified islet transplants. Both fasting glucose and HbA1c were shown to be significantly lower within the non-purified group over the 5 years of follow-up (Table 3.3) additionally, stimulated glucose (30 and 120 min after a 75g glucose bolus) was lower and stimulated C-peptide (30 min after a glucose bolus) was higher in the non-purified group, although these results did reach significance (Table 3.3). The finding that the purified group showed significantly higher mean stimulated C-peptide 120 scores (over the 5 year period) initially appeared contradict the previous data, however the secondary finding that C-peptide values within the purified group fell over the 5 year period whilst, within the non-purified group values showed a increase year on year, again suggested that over the longer-term non-purified islets were associated with more stable long-term function. This pattern was reflected by insulin requirement data, although mean requirements over the 5 year period were significantly lower in the purified group, this group also showed a sharp increase in daily requirements year on year, whilst the non-purified group remained unchanged This finding suggests that whilst transplantation of purified islets may confer an initial advantage, non-purified islet graft has superior stability and potentially longevity, however, further studies analysing data over a longer follow-up period are required to prove this assumption.

An explanation for the arguably superior stability of non-purified islets is inevitably going to be multifactorial and will include not only patient and isolation factors but also technological advances and particularly those related to collagenase production.

Over the past decade, ductal tissue, which is largely removed during islet purification, has been shown to confer an advantage to islets in the *in-vitro* and clinical settings (110,112,116). *In-vitro* studies by Ilieva (110) have shown that co-culture of ductal cell and islets prevents central necrosis of the islet clusters possibly through the action of IGF-II, whilst later studies by Movahedi and colleagues (116) have suggested that ductal epithelial cells secrete the angiogenic factors VEGF and IL-8 at levels 3 and 10 fold higher than islets respectively, and as such ductal cells may hasten the re-establishment of a vascular supply post-transplant. Clinically, the study of Street *et al* (112) reported that co-transplantation of high levels of ductal epithelial cells with islets resulted in superior metabolic outcomes, here measured as the response to an intravenous glucose tolerance test post transplant. A relationship nearing statistical significance was found between graft success or failure and the total number of ductal cells infused. It is likely that it is the findings of Ilieva, Movahedi and Street have initiated the current interest in ductal cells, with several groups in this field now studying the impact of ductal epithelia on islet graft outcome (202) in the hope of improving the currently poor long-term results of islet allo-transplantation.

A second explanation may lay in the fact that standard islet purification methods have been shown to be detrimental to islets by initiating loss of insulin stores (52). As such it is possible that the full impact of the widely utilized Euro-Ficoll–sodium diatrizoate-based islet purification method using the COBE 2991 processor has more of a profound effect on long-term islet survival than initially believed.

A further explanation may be that non-purified islet patients were transplanted with a higher proportion of mantled islets largely due to the fact the purification gradient process is governed by the density of each tissue group and as such mantled islets are often not collected in islet rich layers. As discussed in section 1.2.4, the mantled islet may have a survival advantage due to the fact that the islet micro-environment and peripheral cells are protected (53,125-127). Indeed, preliminary work for this study looking at the overall performance of islet grafts within the Leicester series highlighted that approximately 65% of patients that exhibited insulin independence had been transplanted with >40% mantled islets (75).

A final and perhaps more contentious explanation is the possible transdifferentiation of non-islet cells into the beta cell phenotype post transplantation. To date there is no direct clinical evidence to support this theory however, there is extensive evidence of transdifferentiation *in-vitro* (150) and *in-vivo* under pathological and non-pathological conditions (148,162,163). Indeed, ductal transdifferentaition events post transplant may, in part, explain the positive impact of ductal tissue on transplant outcomes (112). In the context of the present study, the increase stimulated C-peptide 120 within the non-purified islet transplant over the follow-up period (Table 3.3) may suggest an increase in islet secreting mass several years post transplant and this finding is consolidated by the superior graft stability reported within the non-purified group. However, in the face of the fact that the markers of graft function largely diminish with time within both groups (Table 3.3), this assumption cannot be substantiated.

Despite the possible benefits of transplanting non-purified islets, the increased risk of portal vein thrombosis must be considered. The potential lethal outcome of transplanting large volumes of non-purified islets was highlighted by Walsh *et al* (1982) (203) who published a report detailing a case of severe portal hypertension

following the embolisation of non-purified islets into the liver. The patient subsequently developed extensive hepatic infarction and died of liver failure.

Recent data reported by the CITR (74) revealed that of 649 allo-transplants carried out in North America and Europe between 1999 and 2007, 30 (4.6%) have resulted in non-fatal portal vein thromboses or haemorrhage and one would expect that transplantation of larger volumes of non-purified islets would further increase this rate of morbidity. However, in the Leicester islet auto-transplantation series (75), transplant volumes of up to 32ml (median 13 (range 1-32) were found to be well tolerated and safe (when transplanted into the liver), although infusion rates and volumes are carefully adjusted to compensate for transient rises in portal and central venous pressures. Additionally, all infusions were given whilst the abdomen was still open which may be advantageous as evidence provided by Bucher *et al* (2004) has suggested that this is a safer practice than transplanting islets percutaneously (204). Further strategies such as transplanting sub-therapeutic amounts of tissue over a prolonged period, has been associated with superior islet graft outcomes in animals (144) and may further increase the safety of transplanting large volumes of tissue in the future.

3.5 Conclusions

This study is based on a detailed analysis of the clinical results obtained of the largest islet auto transplant centre in Europe. It provides evidence that non-purified islet transplantation is associated with comparable glucose control and insulin secretion in the long-term as using purified islets. Indeed there is some evidence that there is enhanced glucose control in these patients based on statistically enhanced fasting glucose values and HbA1c levels over the 5 year follow-up period.

The following sections will question why non-purified islet preparations may be associated with superior graft function. The aim of the *in-vitro* studies described in Chapter 4 were to directly analyse viability, integrity and function of purified versus non-purified islets over the short-term, whilst a histology-based study presented in Chapter 5 assessed whether transplantation of non-islet cells, particularly islet precursors, had a long-term effect on graft function.

Chapter 4: An evaluation of beta cell function in purified and non-purified islet preparation; an *in-vitro* study

4.1 Introduction

The preliminary study presented in Chapter 3 provided clinical evidence that transplanting non-purified islets does not adversely affect islet function post-transplantation. In fact, the findings indicated that non-purified islets are associated with enhanced graft stability. Although such clinical data often presents the endpoint of a study, the following investigation explores whether the superior islet function observed was associated with bio-trophic support provided by the non-islet tissue, whilst the final results chapters will look at the possibility of transdifferentiation of non-islet tissue into an islet phenotype post-transplantation, thus increasing the potency of the non-purified islet graft.

In-vitro studies have proved to be a necessary pre-requisite to both experimental animal models and clinical trials in the field of islet transplantation. As such the aims of this chapter were two-fold. The primary aim of this study was to define a method for the successful culture of non-purified islets. With few exceptions (205,206) successful culture of non-purified islets has proved difficult, largely due to the fact that culture conditions designed for purified islets have been applied and the potentially damaging affects of acinar tissue have not been accounted for, leading to reports of poor islet viability (96,207). Thus, when handling non-purified islets, techniques that largely focus on the 'maintenance' of the tissue such as cold storage and utilising protease inhibitors, have been used (208,209). Although non-purified islets can be relatively easily maintained in the short-term, these conditions are do not mimic the post-transplant milieu where the majority of acinar cells have been shown to die within days of transplantation (97).

The second aim of this investigation was to determine whether non-purified islets were associated with comparable islet cell function and viability compared to highly purified islets. This hypothesis is based on the assumption that although acinar secretions may be deleterious to viability/function, the inclusion of acinar and ductal tissue may allow the re-establishment of regulatory factors that support islet viability/function (111,210) and this point has been discussed in Section 1. 2.

4.1.1 An *in vitro* model for the study of non-purified islets.

Successful non-purified islet culture presents a myriad of problems to overcome. In itself, culture of solid tissue is fraught with problems of central necrosis due to inefficient mass transfer of nutrients, oxygen and waste (211). Moreover, the very nature of collagenase delivery during pancreas digestion (via the ductal tree with each ductal element terminating within an acinus) coupled with the well-documented fragility of acinar cells (157) leads to substantial acinar cell death following pancreas digestion; causing the release of enzymes which are potentially damaging to cell membrane integrity. Numerous studies have reported poor islet viability rates following culture of non-purified islets (Table 1.2.1) and these findings have been largely accepted within the field of islet transplantation (72).

However, it is equally well accepted that a period of culture directly after islet purification effectively increases islet purity whilst islets remain of transplant quality (3). Additionally, earlier studies by Matas and colleagues (205) and Weber et al (206) in the 1970s, showed that under simple *in-vitro* conditions, non-purified islets could survive and maintain potency in culture whilst cell death and damage was almost exclusively restricted to acinar cells, as shown by a total loss of amylase secretion (Appendix III, Tables i & ii).

The Clinical study presented in Chapter 3 was able to show that transplantation of non-purified islets resulted in excellent beta cell function which was shown by data relating to insulin secretion (fasting and stimulated C-peptide) and glucose homeostasis (low fasting and stimulated blood glucose and low HbA1c). In order to mimic *in-vivo* conditions, the present section describes the establishment of an *in-vitro* model for the culture of non-purified islets. Following an in-depth literature search, the culture conditions discussed Table 4.1.1 and set out in Table 4.1.2 were used as starting basis for further in-house optimisation.

Media type	Low glucose (5mmol/L) culture media such as CRML is associated with superior islet function and viability (212,213). Traditional media of 11mmol/L have been shown to be both toxic and induce beta cell exhaustion (214).	**Tissue was cultured in RPMI supplemented with 5.5mmol/L glucose**
Tissue culture vessels	Monolayer culture in tissue culture treated flasks is associated with loss of differentiated beta-cell function (215,216). Although islet structure can be maintained in 3D matrices (99), recovery of islets post culture is difficult.	**Suspension culture (in hydrophobic petri dishes or High Aspect Rotating Vessels (HARVs) was employed.**
Temperature of culture	Low temperatures inhibit the lysosomal activation of zymogens that can occur following acinar cell damage (217). However this was not seen to mimic post-transplant conditions as there is evidence that acinar tissue quickly necroses post-transplant (62,97).	**Culture at 37°C was chosen**
Delivery of oxygen and nutrients	Hypoxia and central necrosis are a particular problem of islet (or spheroid) culture (211,218). Shallow media depths of (< 2mm) facilitate islet survival and prevent central necrosis (219,220). Additionally, the HARV has been shown to facilitate mass transfer of both nutrients and oxygen within solid tissue structures (221)	**Tissue was either: cultured at a depth not exceeding 2mm in hydrophobic dishes or cultured in the HARV.**
Protection from endogenous enzymes	Endogenous trypsin released from acinar cells activates other endogenous serine pro-enzymes during pancreas digestion and possibly culture (222). Although reagents such as soybean trypsin inhibitor have been shown to effectively inhibit acinar cell autolysis (223), inhibition of acinar cell death was not perceived to mimic the acinar cell death associated with post-transplant conditions (72). Serum proteases (present in human serum and FCS contains an array of protease inhibitors).	**Culture media was changed frequently (at least every 48 hours) to prevent islet damage from proteases. Media was supplemented with FCS.**
pH	In concordance with later studies, Brunstedt and colleagues (224) showed that a difference of just 0.4 pH points (7.2 vs.7.6) greatly diminished islet function.	**Media was buffered with HEPES solution.**

Table 4.1.1. An assessment of optimal conditions for the culture of non-purified islets based on the literature.

Media type	*Glucose free RPMI supplemented with glucose (5.5mmol/L).*
Media supplementation	*10% FCS, 2mM L-glutamine, 1mM sodium pyruvate, 71.5µm β-mercaptoethanol, 8nm hydrocortisone and 20µm HEPES*
Tissue culture conditions	*Standard Hydrophobic bacteria grade petri dish and High Aspect rotating culture vessels were used with media not exceeding 2mm depth Tissue was cultured at 37℃ and tissue replenished at least every 48 hours.*

Table 4.1.2. Standard tissue culture conditions derived from the literature and summarised in table 4.1.1. *The culture conditions outlined were used as a starting basis for further experiments. Previous experience showed that RPMI media supplemented with L-glutamine, sodium pyruvate, β-mercaptoethanol, hydrocortisol supported islets viability and function in culture.* (225-227).

4.1.1 Rudimentary Optimisations

4.1.1.1 Selection of islet species

Preliminary optimisation experiments (n=6) were carried out on pancreata procured from 6-month-old, market weight pigs. Pancreas digestion and islet isolation was based on the method described by O'Neil and colleagues (2001) (183) and is fully described in section 2.1.6. However, following pancreas digestion islets were consistently fragile, fragmented and glucose non-responsive. (Appendix III, Table iii & Figure i). Since these studies were carried out, a literature search revealed that that islet fragility is frequently reported in pigs of this age (228). Thus, experiments using porcine tissue were terminated and the results presented here were carried out on rodent (rat) pancreatic tissue.

4.1.1.2 Selection of seeding densities

A work-up was undertaken to identify the range of seeding densities that best supported islet viability (based on dithizone staining of cultured tissue and insulin

staining of fixed tissue). The seeding density of each culture was expressed in terms of packed cell volume (PCV) per ml of media. In line with the clinical islet isolation program within the University Hospitals of Leicester the packed cell volume equates to the tissue pellet volume following centrifugation of tissue at 200G for 2 min in UW preservation solution. Seeding densities of between 3µl and 100µl of tissue per 1ml of culture media were initially assayed and quickly revealed that seeding densities above 30µl/ml resulted in widespread tissue necrosis (refer to figure 4.1.1 a&b). Conversely, densities of less than 5µl/ml encouraged tissue to flatten and form monolayers (refer to figure 4.1.1c). Apart from the loss of islet structure associated with monolayer culture and the reported loss of beta cell function (229), fibroblast overgrowth has also been proven to be a potential problem (230). Thus, tissue densities of 5, 10 and 20µl/ml were taken forward for further assessment as they appeared to support the maintenance of tissue viability.

4.1.1.3 Selection of feeding methods

Based on the literature discussed in Table 4.1.1 and previous experience of islet cell culture (225,226) the following basic culture conditions were used: Glucose free RPMI supplemented with: 5.5mmol/L glucose, 10% FCS, 2mM L-glutamine, 1mM sodium pyruvate, 71.5µM β-mercaptoethanol, 8nM hydrocortisone and 20mM sodium bicarbonate. Cultures were incubated at 37°C with 95% air and 5% CO_2 and fed every 48 hours by centrifuging cells at 200g for 2 minutes and removing spent media. However, characterisation of islets through dithizone staining showed that islet numbers diminished over the course of the culture, indeed by day 6, no islets could be recovered in n=8 preparations (Figure 4.1.2a). However, by changing the mode of feeding (centrifugation was replaced with sedimenting the tissue for 15 min) viable islets could be recovered throughout the entire culture period (Figure 4.1.2b).

4.1.1.4 Simple suspension culture versus the HARV

The NASA built high aspect rotating vessel (HARV) mimics zero gravity sending tissue into constant freefall, improving the transfer of oxygen and nutrients with cultured tissue in comparison with static suspension culture (221,231,232). A previous study be Rutzky and colleagues has also shown that islets cultured in HARVs contain large channels, which not only facilitate the transfer of nutrients and oxygen in-vitro but may also prove important in promoting angiogenesis post-transplant (221). Moreover, previous experience of culturing cells derived from neo-natal non-purified islets in the HARV resulted in viable hormone expressing tissue following 30 days of HARV culture (226). Through H&E assessment and insulin immunohistochemistry, it was shown that HARV-non-purified islets were largely necrotic (Figure 4.1.3a). It can be assumed that the wide spread cell death was due to acinar protease activity as to date only purified islets (111,221) and neonatal pancreatic cells that had been rendered undifferentiated through monolayer culture (226) have been successfully cultured in the HARV (Figure 4.1.3b). Due to these results, simple suspension culture in hydrophobic petri dishes was used for all further experiments.

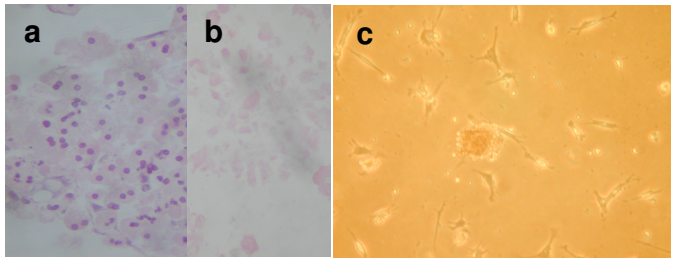


Figure 4.1.1 Seeding densities of non-purified islets. *Seeding densities of more than 20µl/ml/ml resulted in widespread tissue cell death. Pyknosis (nuclear shrinkage preceding cell death) and karyolysis (complete dissolution of chromatin characterised by even eosin staining following H&E) were evident throughout the cultures (a & b respectively). Seeding densities of 5µl/ml resulted in attachment of islets to the surface and flattening of islet structures (c). Photographs a & b x 400 and c x 200 magnification.*

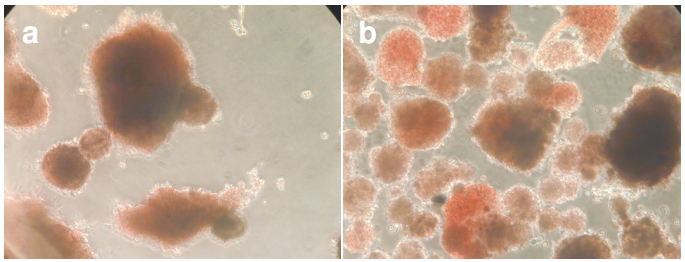


Figure 4.1.2 Centrifugation of non-purified islets during media replenishment. *Centrifugation of non-purified islets (2 min at 200g) encouraged the tissue to cluster into large aggregates which were completely insulin negative by day 8 of culture as characterised by lack of dithizone staining (a). However, sedimentation of tissue supported the recovery of dithizone-positive islets (b). Photographs a & b photographed at x 200 magnification.*

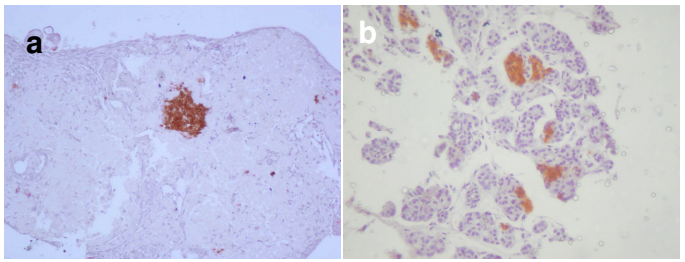


Figure 4.1.3 HARV culture of non-purified islets. *Non-purified islets cultured within a high aspect rolling vessel (HARV). Non-purified islets rapidly clustered into large tissue masses (a). By day 3 of culture, the tissue masses were largely a-cellular (a). Previous studies showed that passaged human pancreatic cells derived from non-purified islets procured from patients with persistent hyperinsulinaemic hypoglycaemia of infancy remained viable following 14 days of culture (b). Original Magnifications for a & b were x 40 and 100 respectively.*

4.1.2 Fine-tuning islet culture conditions

Following the crude optimisation experiments described in 4.1.1, experiments aimed at further optimising the culture protocol were carried out, with the aim of trying to prevent the build up of toxic levels of released protease enzymes. Six conditions were assessed in n=7 experiments. The culture protocol published by Weber and colleagues (replenishing media at day 2, 4, 6 and 8) was compared with a modified protocol whereby tissue was fed more intensively based on the assumption that acinar secretions are likely to be highest in the 24hours post pancreas digestion (replenishing media at 6hrs, 24hrs, day 3, 5 and 7). Additionally seeding densities of 5, 10 and 20µl of tissue per 1ml of media were compared for their impact on: islet hormone expression (assayed using immuno-histochemistry, section 2.4), insulin response to glucose (section 2.3.2) and levels of intracellular insulin (section 2.3.4), both immediately after isolation and following 8 days of culture. The experiment design of the fine-tuning experiments are outlined in Figure 4.1.4

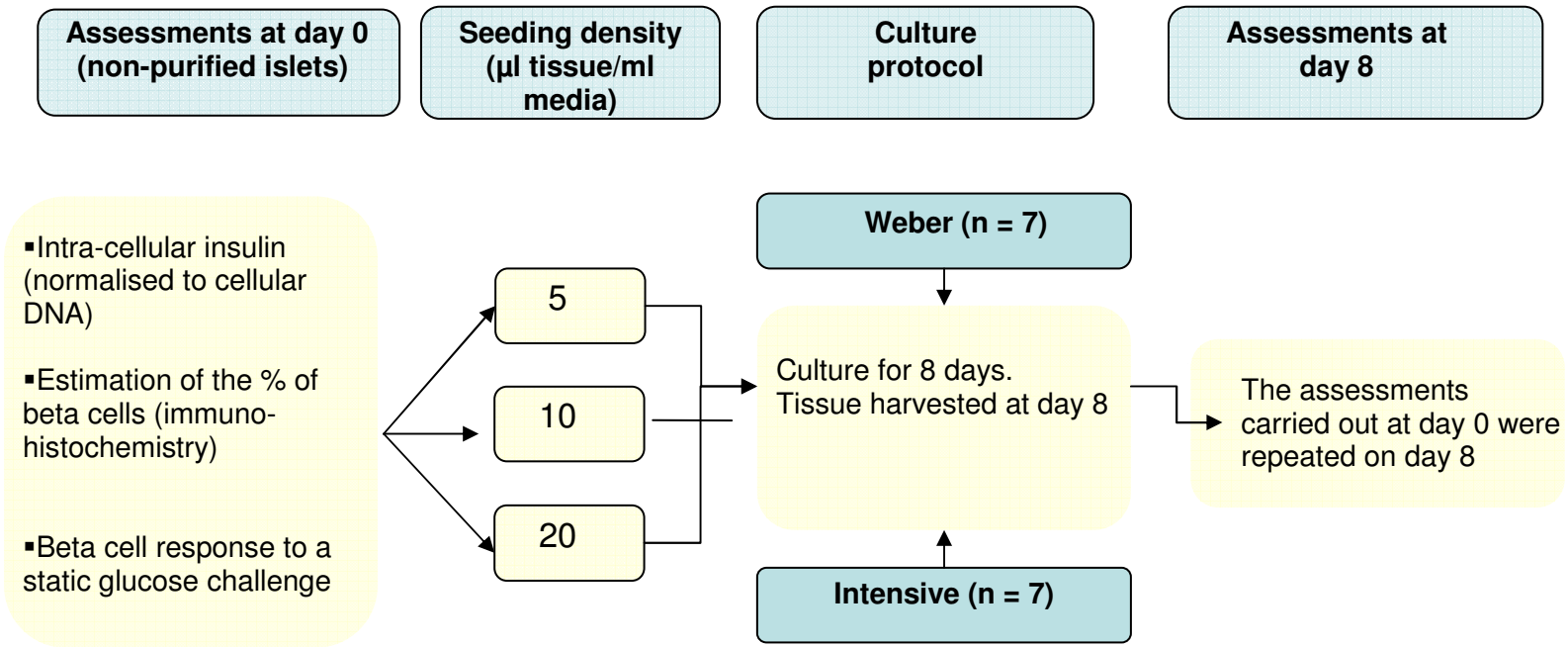


Figure 4.1.4 ***Schema of experiments carried out in order to optimise the culture of non-purified islets.*** *Using Webers culture protocol, cultures were fed at days 2, 4, 6 & 8, whereas with the intensive protocol takes account of the fact that high levels proteolytic enzymes may be released from acinar cells in the initial period (first 24 hours) of culture, therefore, cultures were fed at 6hrs, 24hrs and days 3, 5 & 7.*

4.1.2.1 Comparisons of the Weber and Intensive culture protocols

In line with the findings of Weber and colleagues (206), using Weber protocol, intracellular insulin increased over the 8 days of culture, additionally dithizone stained islets were successfully recovered (Figure 4.1.5a). However based on the results presented in table 4.1.2, it was concluded that the Intensive protocol was superior. Tissue cultured under the intensive protocol showed higher intracellular insulin at all seeding densities. Furthermore, due to the diminished insulin levels and arguably the cell damage sustained by tissue cultured under the Weber protocol, fixed tissue was shown to be insulin negative using standard immuno-histochemistry protocols (Section 2.4, Figure 4.1.5b). Conversely non-purified islets cultured using the intensive protocol remained insulin positive.

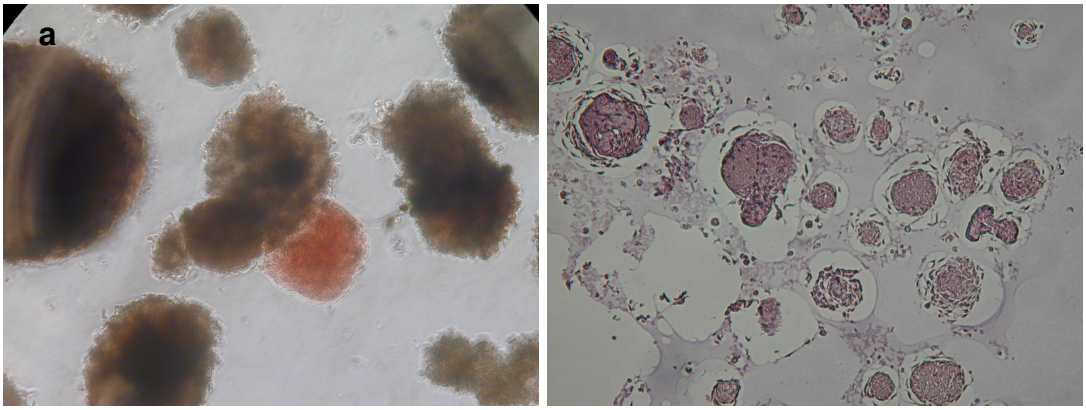


Figure 4.1.5 Typical morphology of non-purified islets cultured under Weber's protocol. *In agreement with Webers findings, islets could be detected through dithizone staining (a) and cellular insulin could be detected (refer to Table 4.1.3). However insulin staining could not be detected using standard immunohistochemistry techniques (b), additionally tissue spheroids were generally a-cellular.* Figures a & b photographed at magnifications of x 200 and 40 respectively.

		Seeding Density 5µl/ml			Seeding Density 10µl/ml			Seeding Density 20µl/ml		
	Day 0 data	Weber	Intensive	*Wilcoxin matched pairs test*	Weber	Intensive	*Wilcoxin matched pairs test*	Weber	Intensive	*Wilcoxin matched pairs test*
Insulin content (ng/µg DNA)	6.54 (0.10-32.00)	13.07 (0-48.42)	33.19 (1.81-400)	***p=0.0313***	5.17 (0.69-44.46)	30.09 (8.67-383)	*p=0.3125*	4.17 (0.183-23.54)	7.5 (0.13-108)	*p=0.4688*
Insulin positive cells %	1.38 (0.615-1.715)	No staining	8.09 (2.34-17.9)	*ND*	No staining	5.63 (2.87-8.69)	*ND*	No staining	11(4.09-11.9)	*ND*
Stimulation index to a glucose challenge	1.16 (1-11.69)	1.13 (1-14.7)	1.05 (1-11.7)	*p=0.7235*	1 (1-1.97)	1.310 (1-6.6)	*p=0.1563*	1.64 (1-5.71)	1 (1-8.190)	***p=0.036***

Table 4.1.3 A comparison of Weber's culture protocol vs. the Intensive culture protocol. *In this experiment rat islets were cultured at 3 seeding densities (5, 10 and 20 µl/ml) using 2 different culture protocols (Weber vs. Intensive). Insulin content, % of insulin positive cells and glucose stimulation index data was used to assess islet viability and function following 8 days of culture. A comparison of the Weber and Intensive protocol indicated that the Intensive protocol supported superior islet insulin content at all seeding densities and significantly at a seeding density of 5µl/ml. Additionally, insulin positive cells could only be detected in tissue cultured under the Intensive protocol when using standard immuno-histochemistry techniques. The median response to a glucose challenge under all conditions was low (below 2) and widely varied within each group and within test duplicates (Appendix III, Table iv).*

4.1.2.2 Comparison of seeding densities

Analysis of the results immediately highlighted that a seeding density of 20µl/ml was associated with poor viability as assessed by H&E and was excluded from further statistical evaluation (Figure 4.1.6 d and Table 4.1.4). Wilcoxon matched pair analysis showed no statistical difference between the intracellular insulin levels of tissue seeded at 5µl/ml and 10µl/ml following 8 days of culture, (33.19ng/ug of DNA (1.81-400) vs. 30.08ng/ug DNA (8.67-383), p=0.230, respectively). Additionally, the percentage of insulin positive cells at day 8 of culture was statistically comparable at both seeding densities (8.09% (2.34-17.9) vs. 5.63% (2.87-8.69), p=0.875, for 5µl/ml and 10µl/ml respectively). However, it is of note that an estimated 75% (55-90) of tissue cultured at 5µl/ml tended to form monolayers, distorting islet structure and providing a less physiological model of islet and non-islet cell interactions (Table 4.1.4).

	Day 0	Day 8 5µl/ml	Day 8 10µl/ml	Day 8 20µl/ml	Day 8 5 vs. 10µl/ml
Insulin content ng/µg DNA	6.54 (0.1-32)	33.19 (1.81-400)	30.08 (8.67-383)	7.5 (0.13-108)	*p=0.2031*
Insulin positive cells	1.3 (0.61-1.72)	8.09 (2.34-17.9)	5.63 (2.87-8.69)	10.97(4.09-11.9)	*p=0.875*
Stimulation index to a glucose challenge	1.16 (1-11.69)	1.05 (1-11.7)	1.31 (1-6.6)	1 (1-8.19)	*p=0.556*
Estimated % of adhered tissue	0%	75%	<10%	negligible	*n/a*

Table 4.1.4 A comparison of islet viability and function following culture at various seeding densities under the Intensive culture protocol following 8 days *in-vitro.* *Assessment of tissue cultured at 20µl/ml immediately highlighted that it widespread tissue death and therefore, the 20µl/ml data was excluded from further statistical evaluation. Seeding densities of 5µl/ml and 10µl/ml were compared using Wilcoxin matched pairs and revealed that both were associated with statistically comparable insulin content values, % of insulin positive cells and stimulation index to a glucose challenge, however at the seeding density of 5µl/ml an estimated 75% (55-90%) of tissue was adherent to the petri dish.*

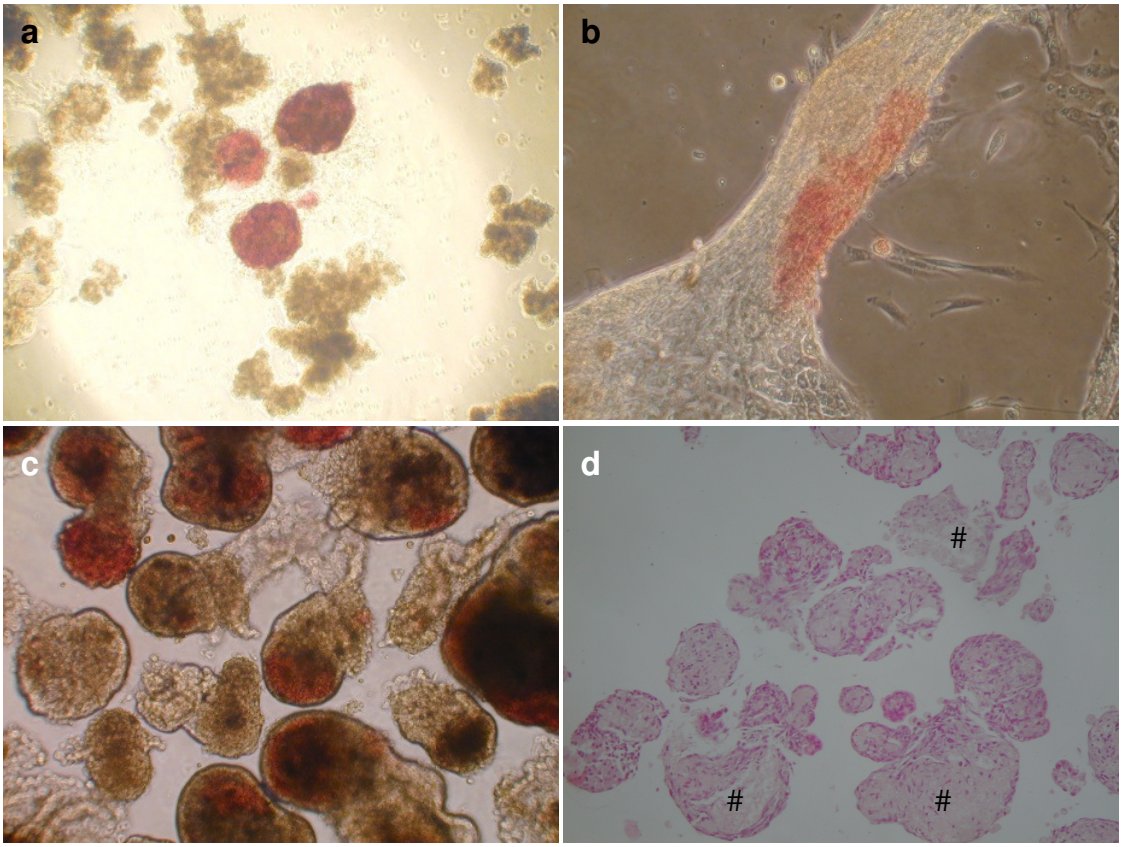


Figure 4.1.6. Tissue morphology at varying seeding densities. *Figure a shows the appearance of non-purified islets directly after digestion. Figure b shows that non-purified islets cultured at 5µl/ml often formed monolayers when cultured under the Intensive protocol for 8 days. Conversely, non-purified islets cultured at 10µl/ml largely remained in suspension and retained a similar morphology to freshly isolated islets (c), although it must be noted that islets were seen to form attachments to non-islet tissue in-vitro (this fact will be discussed in section 4.2.2.5). Figure d is an H&E of tissue cultured at 20µl/ml and highlights marked areas of a-cellular tissue (#) following 8 days of cultures. Photographs were taken at a magnification of x 100, 200, 200 and 100 for figures a, b, c and d respectively.*

4.1.4 Optimised Culture conditions

Based on the preliminary experiments described, an optimal culture protocol was defined for the culture of non-purified rat islets and is described in table 4.1.5.

Media type	*Glucose free RPMI supplemented with glucose (5.5mmol/L).*
Media supplementation	*10% FCS, 2mM L-glutamate, 1mM sodium pyruvate, 71.5µm β-mercaptoethanol, 8nm hydrocortisol and 20µm HEPES*
Seeding density	*Tissue seeded at a density of 10µl tissue per ml culture media*
Tissue culture conditions	*Standard hydrophobic bacteria grade petri dish, with media not exceeding 2mm depth and a culture temperature of 37℃. The Intensive culture protocol was used i.e. media was replenished at 6hr, 24hr, day 3, 5 and 7 by sedimenting tissue for 15 min at room temperature.*

Table 4.1.5 Optimised conditions developed for the successful culture of non-purified islets.

4.1.3 Serum Free Media

In light of the results presented in sections 4.1.2.1 and 4.1.2.2, attempts were made to further improve the protocol outlined in table 4.1.5 by testing the efficiency of using the specialised serum free islet media (Memphis media) which was first described by Gaber and Fraga *et al* in the late 1990's (reviewed in (212)). They were able to show maintenance of islet structure and function for up to 6 months *in-vitro*, additionally, following 1 month *in-vitro*, showed recovery rates of between 85-88%, which is far superior to recovery rates of between 39% and 66% that have been reported following the use of serum containing media (233,234).

The design and results of the studies carried are outlined in Appendix III (Figure ii and Table v). Interestingly, culture of non-purified islets in Memphis was associated with 100% disintegration of cellular material within the first 2-3 days of culture and

indeed no islet viability, response to glucose or intracellular insulin could be detected following 8 days of culture. Therefore, this line research was discontinued and the optimised culture conditions set out in table 4.1.5 were used the proceeding studies which compared islet viability and function of purified and non-purified islets.

4.2 In-vitro outcomes of non-purified versus purified islets

Section 4.1 sought to ascertain a set of optimal cell culture conditions for non-purified islets and by using the conditions set out in table 4.1.5, viable and functional islets could be successful recovered following 8 days of culture. The main aim of the following study was to compare islet function, viability and integrity of non-purified versus purified islets using the culture protocol described in table 4.1.5 (experimental design described in Figure 4.2.1), while the secondary aim was to define the in-vitro characteristics of cultured non-purified islets, as to date, there is a paucity of data in this area.

4.2.1 Methods

The subsequent experiments were carried out on non-purified and purified rat islets procured using methods outlined in section 2.1.5, using the culture conditions outlined in Table 4.1.5. The purified islets used were on average 85% pure (70-95) as assessed by dithizone staining directly after isolation (refer to sections 2.1.5.2-3). A schema of the experimental protocol used can be found in Figure 4.2.1. All experiments were repeated x7, however histologically fixed specimens could not be recovered for 1 experiment due to loss of tissue during paraffin embedding. Methods used can be found in sections 2.1.5, 2.2, 2.3, 2.4 and 2.6. Median values and ranges or mean values +/- SEM were used as descriptive statistics.

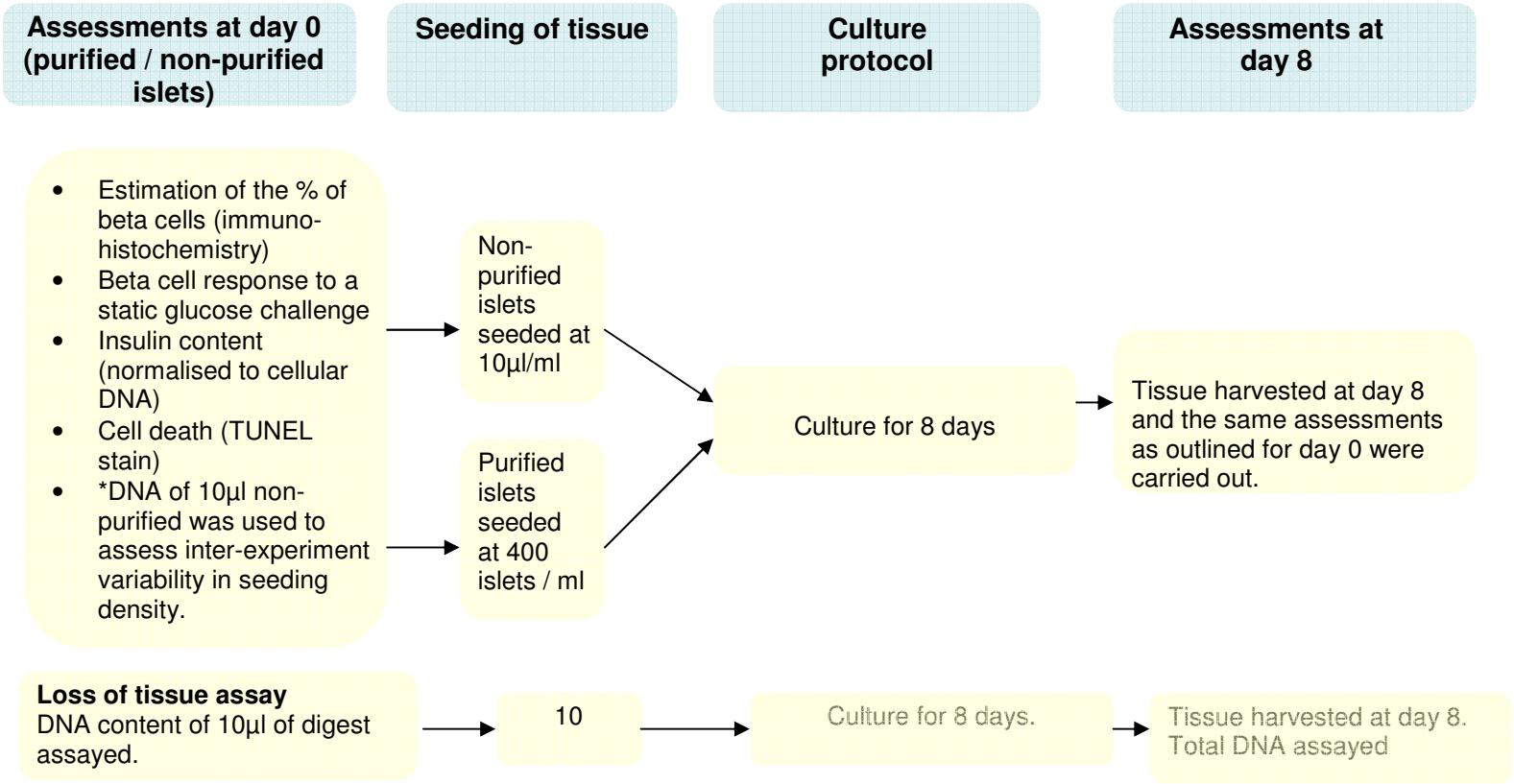


Figure 4.2.1 Schema of experiments carried out to compare the function and integrity of non-purified and purified islets. **Not carried out on purified islets.*

4.2.2 Results, non-purified islets vs. cultured islets

4.2.2.1 Cell death and insulin expression of non-purified islets.

The most striking observation upon culturing non-purified islets was the marked loss of tissue over 8 days of culture. Loss of DNA during culture was used as an indicator of cell death as unlike cell protein, cellular DNA levels remain relatively consistent and provide a good measure of cell number. Immediately post digestion and following centrifugation in UW for 2 min at 200g, 1µl of non-purified islets (packed cell volume) was shown to contain 4.19µg (4.05-5.50) DNA. However, following 8 days in vitro, the DNA content of the culture decreased considerably to 0.51µg (0.21-0.83) µg DNA per 1µl of non-purified islets originally seeded ($p<0.0001$, $n=7$), representing a 90% drop in cell mass (Figure 4.2.2a).

Concomitant with extensive cell death was a significant increase in the intracellular insulin levels of the cultured tissue suggesting selective death of non-islet tissue. Intra-cellular insulin (ng/µg DNA) increased from 6.54 (0.10-32.00) to 30.09 (8.67-383.00) by day 8 of culture, representing a 4.6 fold increase ($p=0.002$, $n=10$, Figure 4.2.2b). Similarly, immuno-histochemistry analysis revealed that the occurrence of insulin positive cells had increased 5 fold, from 1.138 (0.615-1.715) to 5.630 (2.87-8.691) by day 8of culture, $p=0.021$, $n=5$ (Figure 4.2.2c).

Analysis of purified islets showed that there was significant decrease in intracellular insulin between day 0 and day 8, 372.22 (53.38-2083.32) at day 0 vs. 25.26 (2.36-711.73) at day 8, $p=0.0313$, $n=6$. Similarly, over the 8-day culture period there was evidence of a significant increase in insulin positive cells from 59.05 (54.8 – 65.1) to 69.75 (66.8 – 75.4), $p=0.0313$, $n=6$ (Figure 4.2.2 d & e).

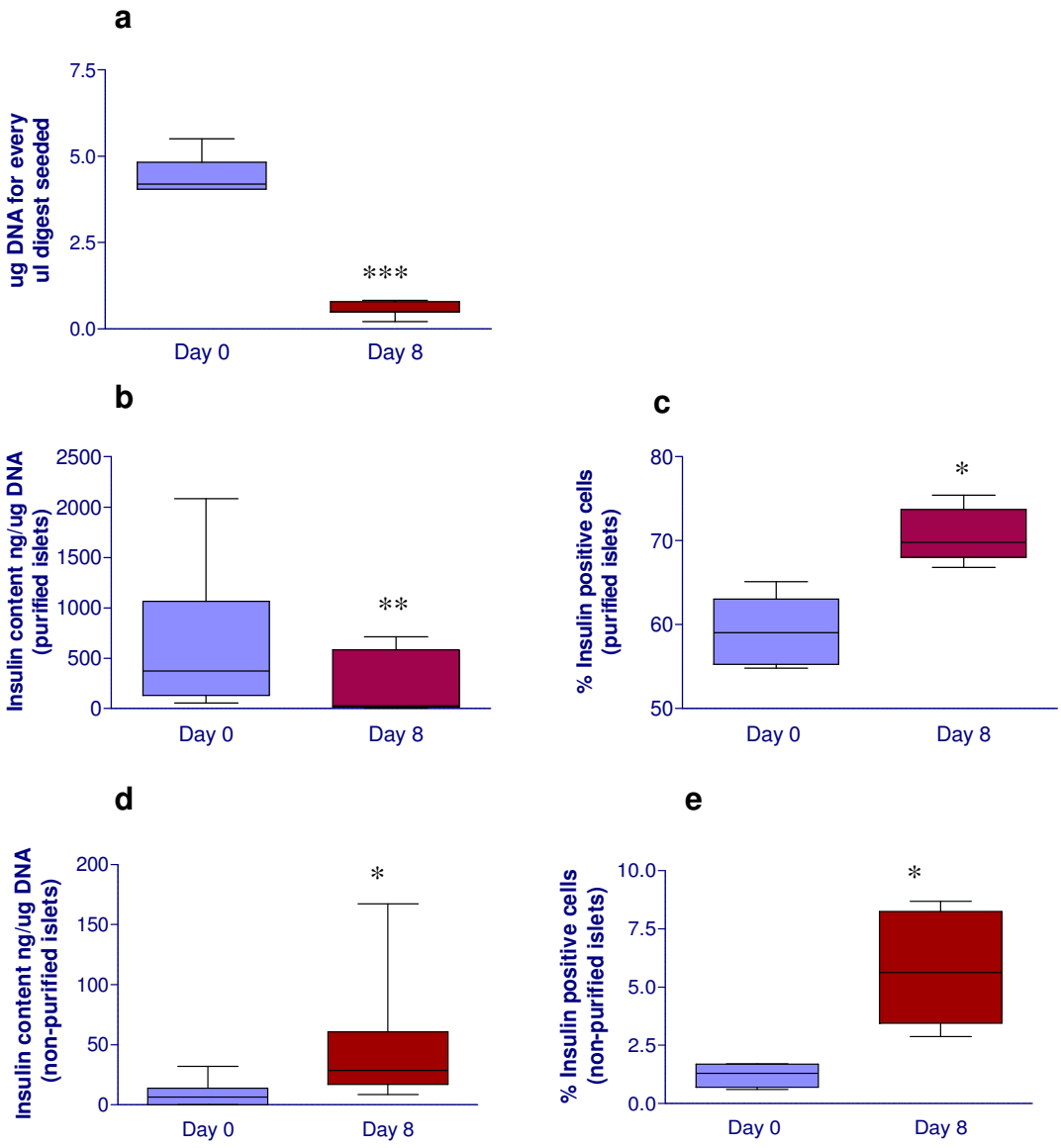


Figure 4.2.2 a-e *Culture of non-purified and purified islets over 8 days*. *All analysis was carrid out using the Wilcoxin matched pairs test. Culture of non-purified islets resulted in a 90% loss of cell mass (a) (p<0.0001), this was accompanied with an increase in intracellular insulin levels (b) (p=0.0002) and an increase in the percentage of insulin positive cells detected using immuno-histochemistry (c) (p=0.021). Similarly, culture of purified islets also resulted in a significant increase in intracellular insulin levels (p=0.013) (d) and a significant increase in the % of insulin positive cells (p=0.013) (e).*

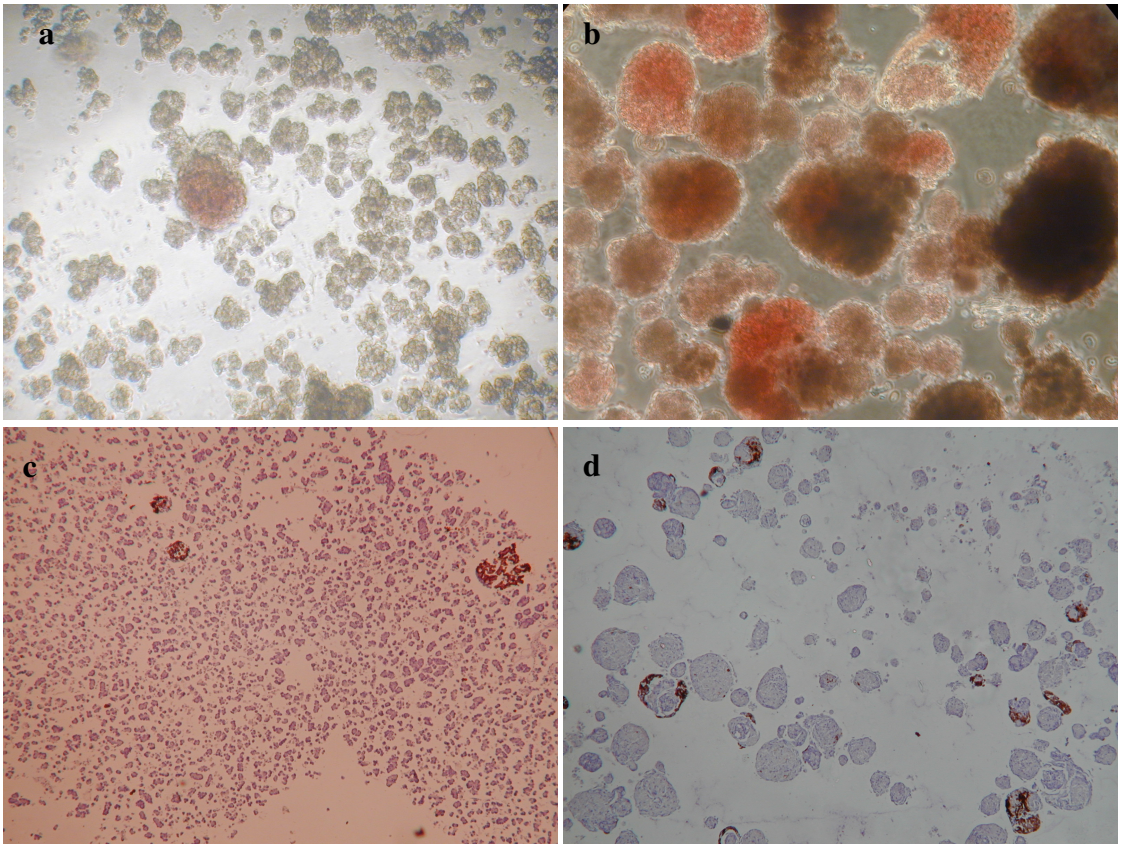


Figure 4.2.3 Non-purified islets at Day 0 and at Day 8 of culture. *Figures a & b show typical levels of islets at day 0 (a) and day 8 (b) of culture as demonstrated by dithazone staining, whilst Figures c&d show levels of insulin staining at day 0 (c) and day 8 (d). Figure a was photographed at an original magnification of x 100, b at x 200 and c&d at x40*

4.2.2.2 Insulin content per beta cell

To investigate whether culture had a detrimental effect on intracellular insulin stores, the insulin content was normalised against the percentage of insulin positive cells that had been characterised using immuno-histochemistry, thus allowing an estimate of ng of intracellular insulin per µg of beta cell DNA (Figure 4.2.4, raw data tabulated in Appendix III, Table vi) Intracellular insulin was seen to be comparable in fresh non-purified and purified islets 770 ng/µg DNA (22-3683) vs. 661 (82–3761) p=0.9372 respectively and following culture, non-purified islets showed a notable, although non-significant, increase in insulin stores 770 ng/µg DNA (673-3683) vs. 1370 (187-4915), p=0.1985. This result became more noteworthy on the finding that purified islets cultured under identical conditions exhibited a significant reduction in insulin levels by day 8 (661 ng/µg DNA (82-3761) vs. 34 (3.5-1034), p=0.0313). Importantly from a transplantation point of view, non-purified cultured islets were shown to contain comparable (indeed, marginally higher) insulin stores than freshly purified islets 1370 ng/µg DNA (187-4915) vs. 661 (82 – 3761), p=0.6991.

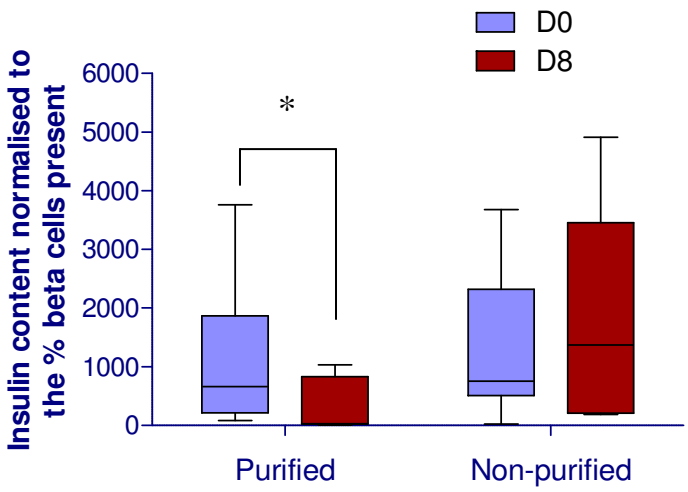


Figure 4.2.4 Intracellular insulin stores normalised against the percentage of insulin positive cells. *Day 0 vs. Day 8 values were analysed using the Wilcoxin matched pairs test. Purified islets showed a significant decrease in insulin stores over following 8 days of culture (p=0.0313) whilst non-purified islets exhibited a marginal increase (p=ns).*

4.2.2.3 Islet Recovery

Additional experiments assessing islet recovery were carried out by seeding a known number of islets into islet depleted tissue acquired from the pellet found at the bottom of the purification gradient. An assessment of the islet depleted tissue showed that it contained just 0.35 ng insulin /µg DNA (0.02-2.07) which was approximately 20 (18.7 fold) fold less than D0 non-purified islet preparations (6.54 (0.10-32.00) ng insulin / µg DNA). Following 8 days of culture, paired analysis showed that cultured non-purified islets exhibited marginally higher (although non-significant) recoveries than purified islets 63.93% (41.22-75.22) vs. 60.84% (12.17-80.46), n=8, P=0.461), although this may be explained by the low levels of islet contamination in the islet depleted tissue.

4.2.2.4 Islet Function

Islet function was measured by exposing islets to low (2.5mmol/L) high (16.7mmol/L) followed by a second exposure to low glucose (Section 2.3.2), with the stimulation index (SI) representing insulin released in response to high glucose divided by the mean insulin release in response to low glucose. Within this study the stimulation index was highly variable and largely poor, with median SI's falling below 2 in all groups (Table 4.2.1). Interestingly both purified and non-purified islets showed a marginally improved SI following culture and this fact has been previously reported (227).

	Stimulation indices	P Value
Purified islets islet D0 vs. D8	1.22 (1-3.58) vs. 1.77 (1-3.24)	P=0.6410
Non-purified islets D0 vs. D8	1.16 (1-11.69) vs.1.310 (1-6.60)	P=0.7646
D0 Purified islets vs. Non-purified islets	1.22 (1-3.58) vs. 1.16 (1-11.69)	P=0.7088
D8 Purified islets vs. non-purified islets	1.77 (1-3.24) vs. 1.310 (1-6.60)	P=0.5986

Table 4.2.1 Islet function of purified and non-purified islets immediately after isolation and following 8 days of culture. *All data was analysed using the Wilcoxin matched pairs test.*

4.2.2.5 Morphological analysis of purified and non-purified islets

Following digestion of the pancreas both non-purified and purified islets were seen to be well cleaved (mantled islets were infrequent) with a loose structure, intense hormone staining and a similar cellular arrangement to native islets within the intact pancreas (Figure 4.2.5 a-c). However, following culture, purified islets frequently displayed weak and heterogeneous insulin staining (Figure 4.2.5 d-f) whilst insulin staining within the cultured non-purified preparations remained intense (Figure 4.2.5 g-i). However, culture of non-purified islets was accompanied with several structural changes. Firstly, the islet tissue showed a high propensity to attach to non-islet tissue and form stable structures. In fact, within the non-purified islet cultures, 78% (55-90) of islets formed attachments (Figure 4.2.5 g-i).

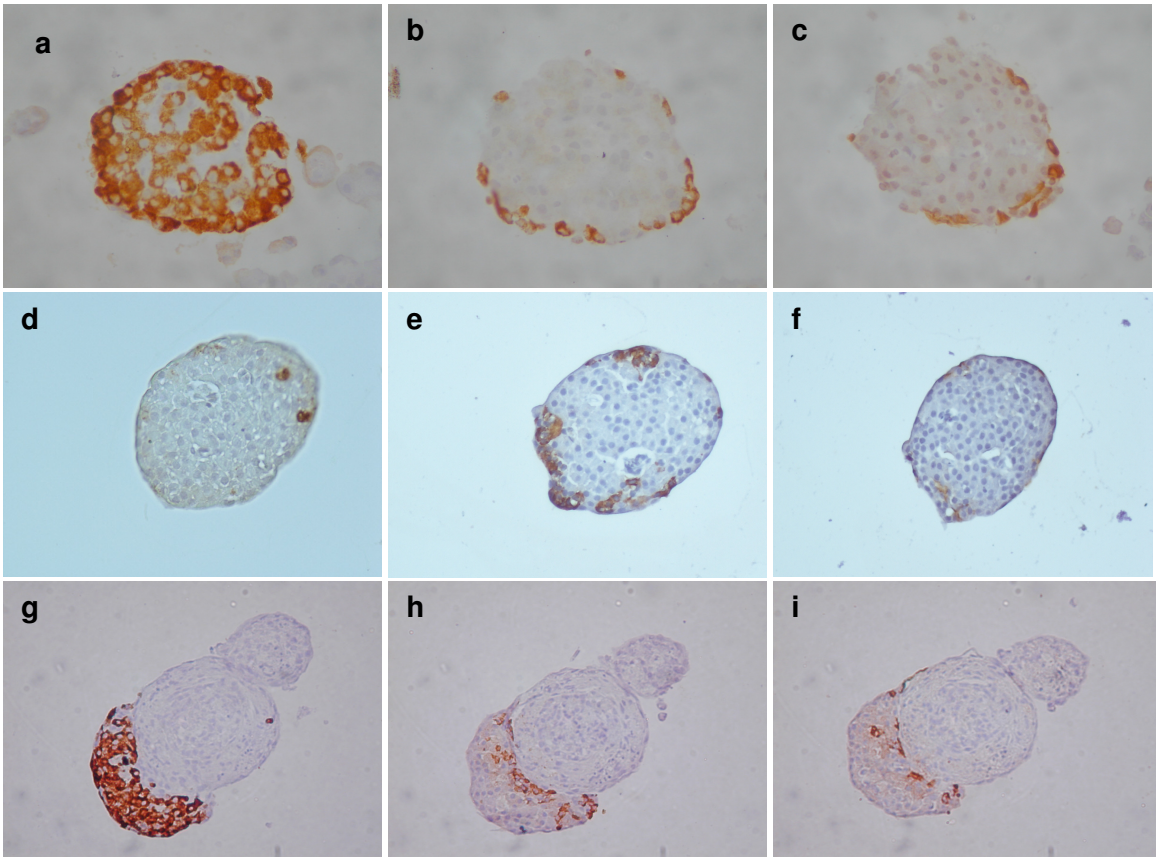


Figure 4.2.5 Hormone staining of non-purified and purified islets. *Using immuno-histochemistry, fresh non-purified and purified islets had identical morphology and exhibited intense insulin (a), glucagon (b) and somatostatin (c) staining (images of non-purified islets are shown in figures a-c). Following 8 days of culture, hormone staining for insulin, glucagon and somatostatin within non-purified islets remained intense (g-i respectively), conversely within some samples, insulin staining within purified islets was greatly diminished (d), although glucagon (e) and somatostatin (f) expression was largely unaffected (e&f). Figures a-f photographed at a magnification of x 400, figures g-i were photographed at x 200.*

4.2.3 Characterisation of non-purified islets

The following section describes experiments further characterising non-purified islets, largely using immuno-histochemistry. The following section includes data on daily samples collected throughout the 8 day culture period.

4.2.3.1 Structure of non-purified islets

Assessment day-8 tissue sections stained with insulin showed that within the non-purified islet cultures, 78% (55-90) of islets were found attached to the non-islet tissue, and interestingly, when attached to non-islet tissue, no islet central necrosis was observed (n=5). Conversely, paired analysis showed that more than half of the free islets within the non-purified islet cultures (53% (50-67)) showed evidence of central necrosis. However, it is noteworthy that the majority of islets attached to non-islet tissue lost their spherical morphology, flattening into structures that were typically 4 to 5 cells deep (Figure 4.2.6 a&b), potentially abrogating cell hypoxia. Conversely free islets remained spherical. Raw data relating to central necrosis in attached and free islets can be found in Appendix III Table vii. Preliminary studies analysing the mode by which islet and non-islet tissue adhered to one another proved inconclusive although involvement of connexion 32 an acinar derived gap junction protein (Appendix III, Figure iv). Additionally, in an attempt to corroborate the finding that islets attached to non-islet tissue exhibited the lowest levels of central necrosis, sections were stained for vascular endothelial factor receptor 1 (VEGF R1 / Flt-1) which has been shown to be expressed at elevated levels by cultured islets in response to hypoxia (235). However within this set of studies, purified islets and non-purified islets (both free and attached islets) exhibited statistically similar levels of VEGF (Appendix III, Figure v).

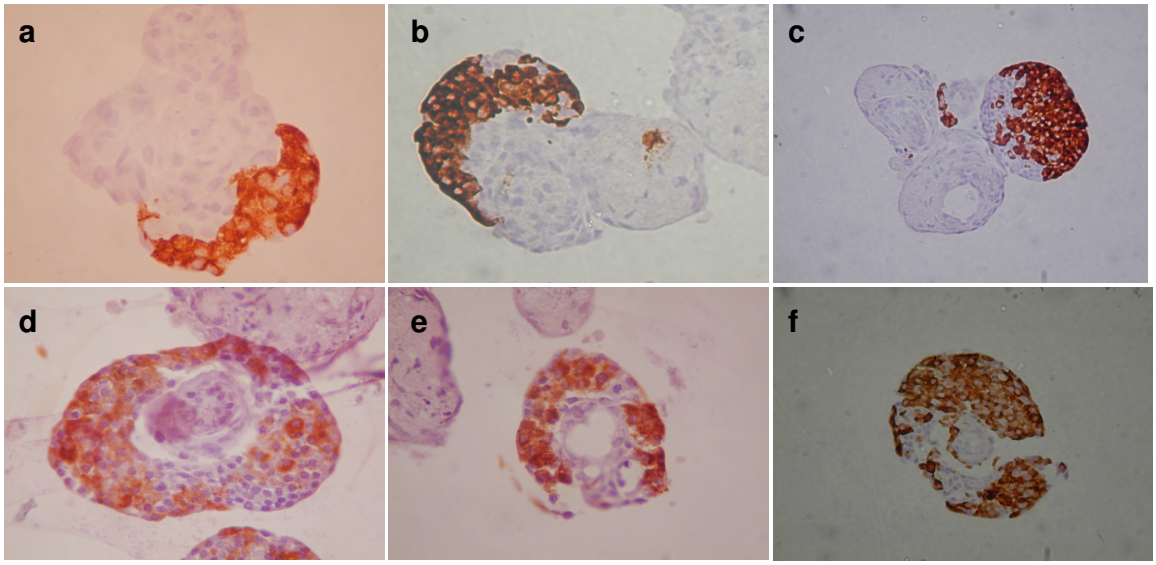
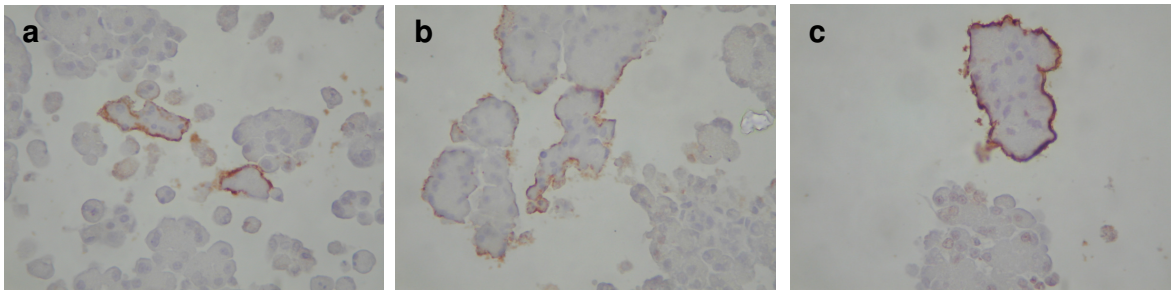


Figure 4.2.6. Insulin staining of non-purified islets following 8 days in culture. *Three examples of islets attached to non-islet tissue (a-c) and 3 examples of free islets (d-f). Islet tissue attached to non-islet tissue exhibited no central necrosis; possibly due to the fact that islet tissue was often flattened to 4-5 cells in diameter (a&c), although some attached islets retained their spherical morphology (c). Free islets within the non-purified islet culture typically showed marked central damage (d-f) as evidenced by lack of insulin staining within the core (d&f) and in some cases absence of cellular material at the core (e). Figures a, b d, e, f, photographed at a magnification of x400, Figure c was photographed at x200.*

4.2.3.2 Glucagon and somatostatin expression of non-purified islets

Following 8 days of culture, the alpha, beta and delta cell ratio was found to be 72.425% (44.68-100), 13.895%(0-48.9) and 6.285% (0-40) respectively (Figure 4.2.5 g-i), with beta cells levels being marginally lower than fresh islets 76.82 (15.28-100), raw data presented in Appendix III, Table viii. This data suggests that the alpha and delta cells (which are largely found on the outside surface of the islets, Figure 4.2.5 b & c) were well preserved during culture and enzyme release from dying acinar tissue was adequately controlled for.

Interestingly, within the first 2 days of culture, the somatostatin hormone was seen to attach to the surface of the digested acini (Figure 4.2.7 a-c). Although this was an unexpected finding, acinar cell membranes have been shown to be rich in somatostatin receptors (236,237). However, further analysis of this phenomenon was deemed outside the scope of this study. The potential implications of somatostain action are discussed below.



4.2.7 Somatostatin staining of acini. *Examples somatostatin staining on the surface of pancreatic acini at 6 hours (a), 24 hours (b) and at day 2 of culture.*

4.2.3.3 Characterisation of non-islet tissue

Based on immunohistochemistical assessment, 41.8% (34.6-47.5) of non-islet tissue post pancreas digestion was ductal as characterised by Cytokeratin 19 (CK19) staining (Figures 4.2.8 a&b). However, due to the well documented fragility of acinar tissue

(Figure 4.2.8 c-f), CK19 was seen to increase over the course of the culture (Figure 4.2.9 a-d), although accurate quantification of CK19 positive cells was not possible due to the fact that unlike islet tissue, non-islet tissue was typically composed of both viable cells and a-cellular areas (Figure 4.2.9 d). The increased predominance of ductal cells was thought to be due to acinar cell death rather than acinar to ductal cell transdiffereniation, indeed the scale acinar specific cell death was evident directly after pancreas digestion as shown by the high levels of acini specific cell death (Figures 4.2.8c-f and 4.2.8a). Between days 0 and 8 of culture, amylase expression had dropped considerably (Figure 4.2.9 e&f) and this was mirrored by the significant reduction in both amylase secretion (10.09IU+/-1.38 vs. 0.09+/-0.05, P=0.0003) and intracellular amylase levels (278+9.6IU / µg DNA vs. 1.70+/-0.28), p=<0.0003) by day 8 of culture (Figure 4.2.10).

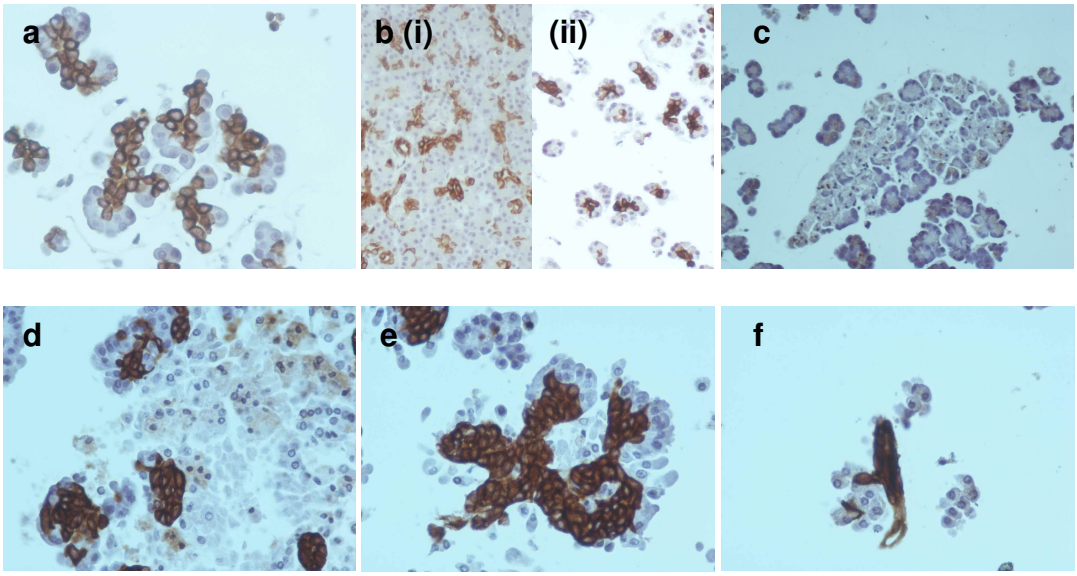


Figure 4.2.8 Non-islet tissue directly after islet isolation. *Analysis showed that non-islet tissue was composed of CK19 (41.8% (34.6-47.5)) and corresponded to the centroacinar / intercolated duct position of each acinus (a). Analysis of corresponding intact pancreas tissue and digested tissue revealed a higher proportion of ductal cells in digested tissue (41.8% (34.7-47.5) vs. 34.4% (28.3-46.9), p=0.456) (b) Acinar tissue is specifically damaged during pancreas digestion (c-f). In particular, acinar tissue (CK19 negative tissue) often exhibited pyknotic nuclei (c) and a-nuclear areas (d). Additionally, certain ducts were seen to lack associated acinar cells (e-f). Figures a, d, e & f photographed at a magnification of x400, figures b & c photographed at x100 magnification.*

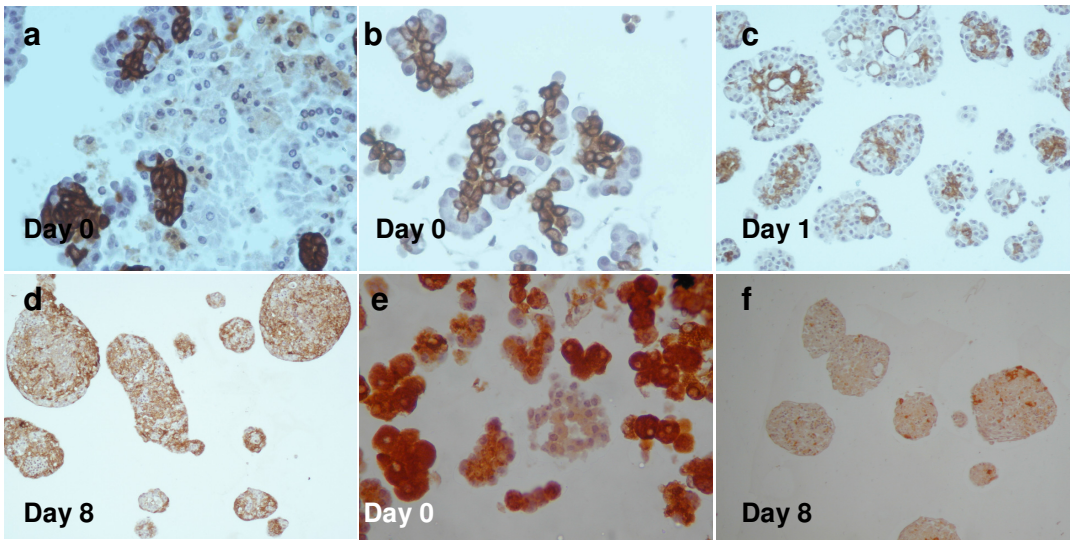


Figure 4.2.9 Analysis of the composition of non-islet pancreatic tissue. *Non-islet tissue was shown to be composed of 41.8% (34.6-47.5) CK19 positive cells directly following pancreas digestion (a&b), with this ratio gradually increased during culture (c & d). Concomitantly, there was evidence of acinar cell death directly following pancreas digestion as evidenced by karyolitic acini (a), and a marked decrease in amylase expression to little above background levels (e&f). Figures a & e photographed at a magnification of x200, figure b at x400 and figures c, d & f at x100.*

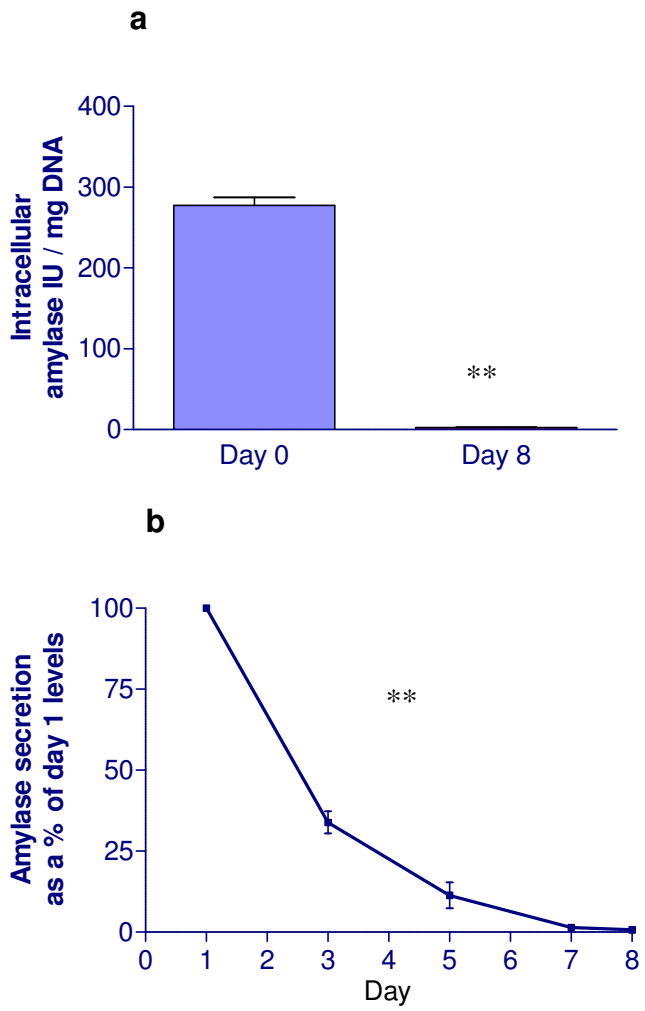


Figure 4.2.10 Amylase secretion and intracellular amylase expression of non-purified islet preparations. *Non-purified islets were assessed for both intracellular (a) and secreted amylase (b). The intracellular amylase levels had fallen significantly by day 8 of culture (10.09IU+/-1.38 vs. 0.09+/-0.05, p=0.0003). Similarly, secreted amylase showed a gradual diminution with time (278+9.6IU/µg DNA vs. 1.70+/-0.28), p=<0.0003). Data analysed using the Wilcoxin matched pairs test.*

4.2.4 Discussion

The culture of non-purified islets forms an important area of study, as to date there are few studies describing the fate of islets that are co-localised with non-islet, particularly acinar, tissue. Additionally, of the studies carried out, most report that islets show poor viability when co-localised with non-islet tissue (97,238), despite the fact this finding conflicts with clinical data reporting excellent islet function following auto transplantation of non-purified islet grafts (76,77,98). Therefore, the main aims of this study were to: define a criterion for the successful culture of non-purified islets and to use the culture system developed to directly compare the viability and function of purified and non-purified islets over a period of 8 days. A further aim of this study was to define the *in-vitro* characteristics of cultured non-purified islets as to date there is a paucity of published data in this area.

In setting up the culture conditions one key consideration was to control for the build up of acinar secretions, (e.g. through low seeding densities, regular media replenishment and inclusion of mild protease inhibition in the form of fetal calf serum). In some ways this culture model directly mimics the transplant situation where the vast blood flow of the hepatic portal vein (the route of islet transplantation) has the effect of diluting potentially deleterious acinar secretions whilst serum protease inhibitors (including alpha-2 macroglobulin, alpha-1 anti-trypsin, antichymotrypsin, serum trypsin inhibitor and c1 esterase inhibitor) actively inhibit proteolytic enzymes with resulting complexes being cleared through reticuloendothelial system. The optimal cell culture conditions defined are outlined in Table 4.1.5 and were shown to give rise to viable islets following 8 days of culture however, islet stimulation indices (analysed using static incubations, Section 2.3.2) were highly variable and this finding is discussed below.

Having optimised the culture conditions and subsequently culturing purified and non-purified islets for 8 days, recovery rates were marginally higher in the non-purified group compared with the purified group, although both groups showed recovery rates that were comparable with previously published data (220,234). The use of serum free medium, developed for use in clinical islet transplantation programs, (239) proved particularly unsuccessful for non-purified islets culture and was associated with 100% cell death by day 8 of culture (Appendix III, Figure ii, Table v). This data may in part explain why some previous attempts of culturing non-purified islets have resulted in poor islet viability rates (141).

The intra-cellular insulin levels of non-purified islets were seen to increase following the culture period (Figure 4.2.4), suggesting that the culture protocol developed was able to support insulin synthesis. However, it was also clear that the intracellular insulin levels of purified islets fell significantly using the same culture conditions. The reason for this are not clear but may be related to the purification process itself (52) or to the loss of trophic support from non-islet cells and this currently constitutes an active area of study (110,111).

Histological studies of non-purified islets showed that although insulin positive cells became more predominant over the culture period, CK19 cells (ductal cells) became the dominant cell type (Figure 4.2.9.a-d) whilst amylase positive cells (acinar cells) markedly diminished (Figure 4.2.9 e-f). Interestingly, within the non-purified islet culture, islets were largely seen to attach to non-islet tissue by day 8 of culture and appeared to exhibit a resistance to central necrosis and retained strong expression of insulin. Although mitigation of central necrosis may have been associated with the fact that islet tissue generally became flattened therefore allowing improved diffusion of oxygen and nutrients, previous work by Ilieva and

colleagues has suggested that ductal secretions (particularly IGF I) directly reduce central necrosis, although the mechanism is unclear (110).

One major criticism of the culture model set up is that it failed to support highly functional islets, indeed stimulation indices recorded during the optimisation studies and subsequent studies comparing purified and non-purified islets, frequently showed stimulation indices of less than 2 which is poor compared with the average stimulation indices of between 3-5 reported in islets isolated for transplant purposes (112,240). Due to the fact that freshly isolated islets showed equally poor SI values, it can be assumed that the islet isolation protocol used in the study and described in section 2.1.5 had adversely affected islet function. Additionally, the typically loose structure of islets directly following digestion (Figure 4.2.5) supports the notion that islets were over-digested and further optimisation of the isolation protocol may be required before future studies are undertaken. Additionally, although the stimulation index has long been accepted as the gold standard for analysis of islet function *in-vitro*, it has been noted by some that SI data does not predict subsequent islet performance post-transplantation (112,241) and therefore may not accurately reflect the function of the islets within this study.

An unexpected observation of this study was that somatostatin released during culture was seen to bind to acinar cell membranes and this phenomenon was observed within the first 2 days of culture (coinciding with the highest secretions of amylase, Figure 4.2.10b). Previous work has demonstrated that somatostatin plays a major role in modulating acinar secretion of zymogens (237), primarily by inhibiting the action of insulin which potentiates cholecytokinin and carbamycholine stimulated amylase release. However, there is a body of evidence that somatostatin has the ability to directly suppress acinar secretions based on the finding that acini cell membranes are particularly rich in somatostatin receptors

(236,237,242). As such this phenomenon is likely to represent a protective reaction to the acinar autolysis. As somatostatin has been suggested to reduce the production acinar secretions by reducing acinar cell amino acid uptake (243) and/or suppressing amylase release (244), this mode of action is unlikely to have any inhibitory impact upon autolytic cells, but may serve to control excess enzyme secretions from viable acinar cells.

Perhaps one of the most important findings of the culture protocol developed supported the enrichment of insulin positive cells within the non-purified islet preparations (Figure 4.2.2 c). Over the 8-day culture period, the tissue volumes were shown to reduce by a median of 90%, whilst the percentage of insulin positive cells increased 5-fold (Figure 4.2.2 a-b). Two of the main issues known to be important in successful islet allo transplantation are a) maximising the number of islet transplanted and b) minimising the volume of tissue transplanted. Data from the Collaborative Islet Transplant Registry indicate that islet yields can reduced by a mean of 40% (74) following purification, thus, the application of the culture system developed here could greatly improve islet yields and could theorectically improve the success of islet transplantation.

4.2.5 Conclusions

The pancreas represents an integrated organ with cells of the endocrine, exocrine and ductal compartments both physically and humourally connected (33,147,245,246). Following pancreas digestion this study has shown that islet integrity can be maintained when islets are co-cultured with other pancreatic elements, further this study suggests that non-purified islet show superior function to purified islets as shown by significantly superior retention of insulin. Although concerns that the proteolytic secretions from acinar cells has focused attention on

the necessity of islet purification, this study suggests that the presecence of non-islet tissue and possible trophic (cell-cell and soluble survival factors) effects obtained, actually greatly enhance the survival of functional, although structurally altered, islets. Additionally, the culture protocol developed for the present study supports significant enrichment of islet tissue and based on the data presented here it would be possible to significantly enhance islet transplant mass by using the Intensive culture protocol on the tissue normally discarded from the lower levels of the density gradient.

Chapter 5: The impact of non-islet endocrine cells, pre-endocrine and ductal cells on islet graft outcomes

5.1 Introduction

It is well accepted within the field islet transplantation that the number of islet equivalents transplanted is positively linked with graft success, with a critical mass of 10,000 IEQ /kg bodyweight shown to alleviate insulin dependence following allo transplantation (247) and approximately 2,500 IEQ/kg following auto transplantation (reviewed in (98)). However, within the Leicester islet auto transplantation series, no such cut-off point was established (75), indeed one patient showed sustained insulin independence following the transplantation of less than 1000 IEQ/kg (187). Additionally, the results presented in Chapter 3 showed that, non-purified islet transplantation was associated with superior long-term graft stabilty compared with the purified islet group, despite the fact that equivalent IEQs were transplanted. These findings therefore suggest that other factors, rather than pure islet mass, affect the insulin secreting potential of the islet graft post-transplantation. This could be due to a range of factors, such as; the inclusion of non-islet cells that provide bio-trophic support (110,111) or cells that give rise to/transdifferentiate into a beta cell phenotype post-transplantation (150) (although the detrimental effect of islet purification methods may also be of issue (52)). One of the key findings of the results presented in Chapter 4 was that co-culture of islets with non-islet pancreatic tissue supported the maintenance of intracellular insulin stores whilst highly purified islets showed a significant decrease over the 8-day culture period, suggesting that immediately post-transplant and beyond, non-islet tissue may play an important role in maintaining islet function. To compliment the work presented in Chapter 4, the current study will explore the possibility of beta cell genesis post-transplant as this represents a second major mechanism by

which non-purified islet grafts may have an advantage over purified islet transplants.

As discussed in Chapter 1C, there are a plethora of animal models and *in-vitro* studies evidencing beta cell genesis from non-islet sources (113,150,159,248). Furthermore, numerous histological studies of both normal and pathological pancreata, describe the occurrence of cells displaying beta cell characteristics located outside of the islets of Langerhans (161,162), the occurrence of pluripotent cells which have the potential to convert into islet cells (249,250) and disease states that cause the up-regulation of ductal epithelial cells, which are accepted to be a source of islet neogenesis (150).

Therefore the frequency of potential endocrine precursors was assessed in the pancreata of chronic pancreatitis patients that had undergone total pancreatectomy with concomitant (non-purified) islet auto transplant (n=23). Subsequently, the prevalence of each endocrine precursor was correlated with islet graft performance over 5 years post-transplant to ascertain whether their inclusion was associated with superior long-term islet graft outcomes.

5.1.1 Ductal epithelia cells

To assess the prevalence of ductal epithelial cells, two markers were utilised. Cytokeratin 19 (CK19) is a member of the cytokeratin family of intermediate filaments and is uniformly expressed throughout the entire ductal system (251). Conversely, CK7 shows a gradient of expression, being intensely expressed in large ducts and absent in the most proximal cells of the duct epithelium i.e. centroacinar cells and intercalated ducts (251) thus, subtraction of CK7 positive cells from CK19 positive cells provides an indication of the prevalence of small duct cells; which is important as these are most commonly associated with beta cell

neogenesis (113,115). Additionally, with the progression of the ductal epithelium from the centroacinar cells, to the intercalated ducts, intralobular ducts, interlobular ducts and finally the main pancreatic duct, there is a gradual increase in the amount collagen invested within the epithelium (particularly from the intralobular duct onwards, (145). For this reason, the larger duct epithelium is more resistant to enzymatic digestion, remaining intact within the Ricordi chamber at the end of pancreas digestion (Figure 2.1.4); it therefore follows that for transplantation purposes, it is the cells of the small duct that are included in the final non-purified islet preparations.

5.1.2 Non-islet endocrine cells

The term non-islet endocrine cells (NIECs) is used here to describe hormone positive cells found outside the islets of Langerhans and includes intermediate cells (162) (pancreatic cells displaying both endocrine and exocrine phenotypes) and hormone positive cells within the ductal epithelium (148). For this study both insulin and glucagon positive NIECs were analysed. Although hormone positive ductal cells can be clearly identified, the identification of intermediate cells within the acinar parenchyma is slightly more contentious due to the argument that they may simply represent the tip of an islet of Langerhans. Thus, cells were only categorized as NIECs within the parenchyma if they were located within an acinus and this was verified by double staining sections with insulin/glucagon and laminin which forms a continuous border around each acinus.

5.1.3 Endocrine precursor cells / stem cells

Several markers have been shown to be expressed by endocrine precursors during embryogenesis and development including: pancreatic and intestinal hormone YY, tyrosine hydroxylase, acid beta-galactosidase, neurogenin, betaceullin, PDX-1, Glut-2 and vimentin. Interestingly, the transcription factor PDX-1 does not merely

serve as a marker but has been shown to play a central role in regulating pancreas development, insulin gene transcription and islet neogenesis. Although PDX-1 is transiently expressed in all fetal pancreatic epithelial cells, in the adult, its expression is restricted to cells within the islets of Langerhans; thus, in the adult pancreas, non-islet PDX-1-positive cells are presumed to be latent endocrine progenitor cells. Similarly, the expression of vimentin in ductal cells is normally only expressed during embryogenesis and may again represent latent progenitors (252).

5.2 Methods

5.2.1 Tissue assessment

Intact samples of CP pancreata were collected as a routine part of the pancreatectomy procedure. Out of the 56 patients that have been transplanted as part of the Leicester islet auto transplantation series, only 44 (those collected prior to the 29th August 2006) were eligible for use due to ethical constraints. Of these, 13 patients were excluded as the pancreatic tissue was purified prior to transplantation, partial pancreatectomy patients (n=4) were excluded due to the influence of the residual gland and finally samples could not be recovered for 4 patients; therefore, samples from n=23 CP patients were available. Eight control pancreatic samples were obtained from non-heart beating donors and these control pancreata were subsequently digested for the collection of non-purified islets (Section 2.1.4.1). Ethical approval was obtained for all tissues used.

Histological sections were stained using standard immunohistochemistry techniques (section 2.4.1) and analysed using Axiovision imaging software with the exception of glucagon. The Axiovision data presented here represents the percentage positive area (PPA) which equates to the % of positive pixels per field. However it is recognized that the actual percentage of positive cells will exceed the

Axiovision figures quoted due to the fact antibody staining is either restricted to the nucleus or cytoplasm. Glucagon and vimentin analysis was carried out manually as positive cells associated with the ductal epithelium were counted. For nine CP patients and 1 control patient multiple samples were assessed (between 2-7 tissue blocks) and in these cases all specimens were analysed and median values were used for statistical analysis. Analysis of positive cells within digested tissue versus intact tissue was also evaluated manually as digested tissue contained variable levels of dead space. The evaluation of all antigens excluded staining within the islets of Langerhans.

5.2.2 Assessment islet function post-transplantation

Assessment of islet function was undertaken by C-peptide assay (ELISA, DRG diagnostics, Nottingham, UK) as a part of normal patient care. The lowest limit of sensitivity was 0.04ng/ml. Follow-up assessments were made at 6 and 12 months post-transplantation and annually thereafter, using the 75g OGTT. Prior to the assessment, patients were fasted overnight and had their insulin stopped for at least 12 hours. Blood samples were taken and processed for the evaluation of plasma glucose and serum C-peptide levels at 0 (fasting), 30 and 120 minutes; additionally blood samples were taken for assessment of serum glycosylated haemoglobin (HbA1c) (refer to section 2.1.2 for plasma and serum collection). Post-transplantation, the stimulation index was calculated as stimulated C-peptide level divided by the basal level. Since many patients were recruited from centers at some distance from Leicester, a significant number of patients failed to attend an assessment every year, although in these cases some limited data i.e. HbA1c and fasting glucose was obtained from Diabetic Assessment follow-up forms completed by the patient's local Diabetes Care Team. Another reason for incomplete data was due to patients not completing their OGTT assessments due to feeling unwell.

5.2.3 Statistics

Median values and ranges or mean values +/- SEM were used as descriptive statistics. The levels of antigen detected in chronic pancreatitic samples versus control pancreata were measured using the Mann-Whitney U test. Correlations between each antigen and metabolic markers of graft function were made using Pearson and Spearman Rank correlations. All statistics were calculated on SPSS version 16 and graphs were created on both SPSS version 16 and Prism graph pad. P values of <0.05 were considered statistically significant.

5.3 Results

5.3.1 CP pancreata versus control tissue

Markers associated with the ductal epithelium and endocrine phenotype were higher in the CP tissue group, indeed insulin positive NIECs, PDX-1 positive cells and Cytokeratin 7 and 19 positive cells were found at significantly higher levels in the CP tissue (Figure 5.3.1 and 5.3.2). Levels of glucagon positive NIECs and the sub-set of the glucagon positive ductal epithelial cells were also shown to be marginally higher in CP tissue; however, this did not reach significance (Figure 5.3.1). Vimentin positive ducts were found too infrequently to carry out any formal analysis and only 5 CP samples and 3 control samples had vimentin positive ducts.

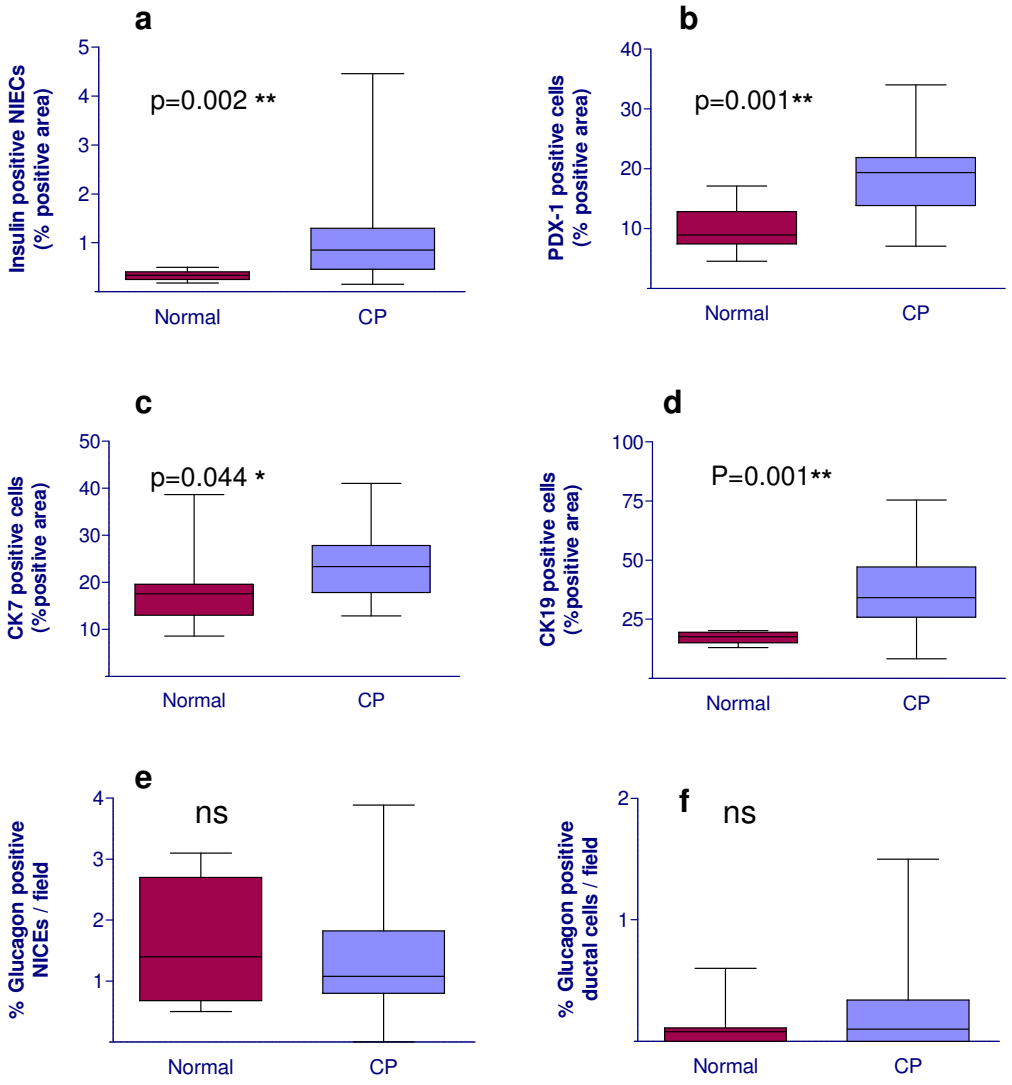


Figure 5.3.1 a-f Proportions of sub-populations of potential precursor cells within the normal and chronic pancreatitic pancreas. *Expression of insulin positive NIECs and PDX-1 was significantly higher in CP patients compared with normal pancreata, 0.85 (0.15-4.46) vs. 0.34 (0.18-0.50), p=0.002 and 19.40 (7.07-34.00) vs. 8.99 (4.57-17.10), p=0.001 respectively (a & b). Additionally, both CK7 and CK19 expression were significantly higher in CP tissue, 23.40 (12.90-41.00) vs. 17.60 (8.66-38.70), p=0.044 and 34.20 (8.35-75.40) vs. 17.70 (13.10-20.30), p=0.001 respectively (c&d). No significant difference was found between CP and normal tissue with regards to glucagon positive NIECs, 1.081 (0-3.889) vs. 1.40 (0.50-3.10), p=0.475 or glucagon positive ducts, 0.10 (0-0.15) vs. 0.08 (0-0.6), p=0.688 (e&f), although levels were seen to be marginally higher in normal tissue. All analysis was carried out using the Mann-Whitney test.*

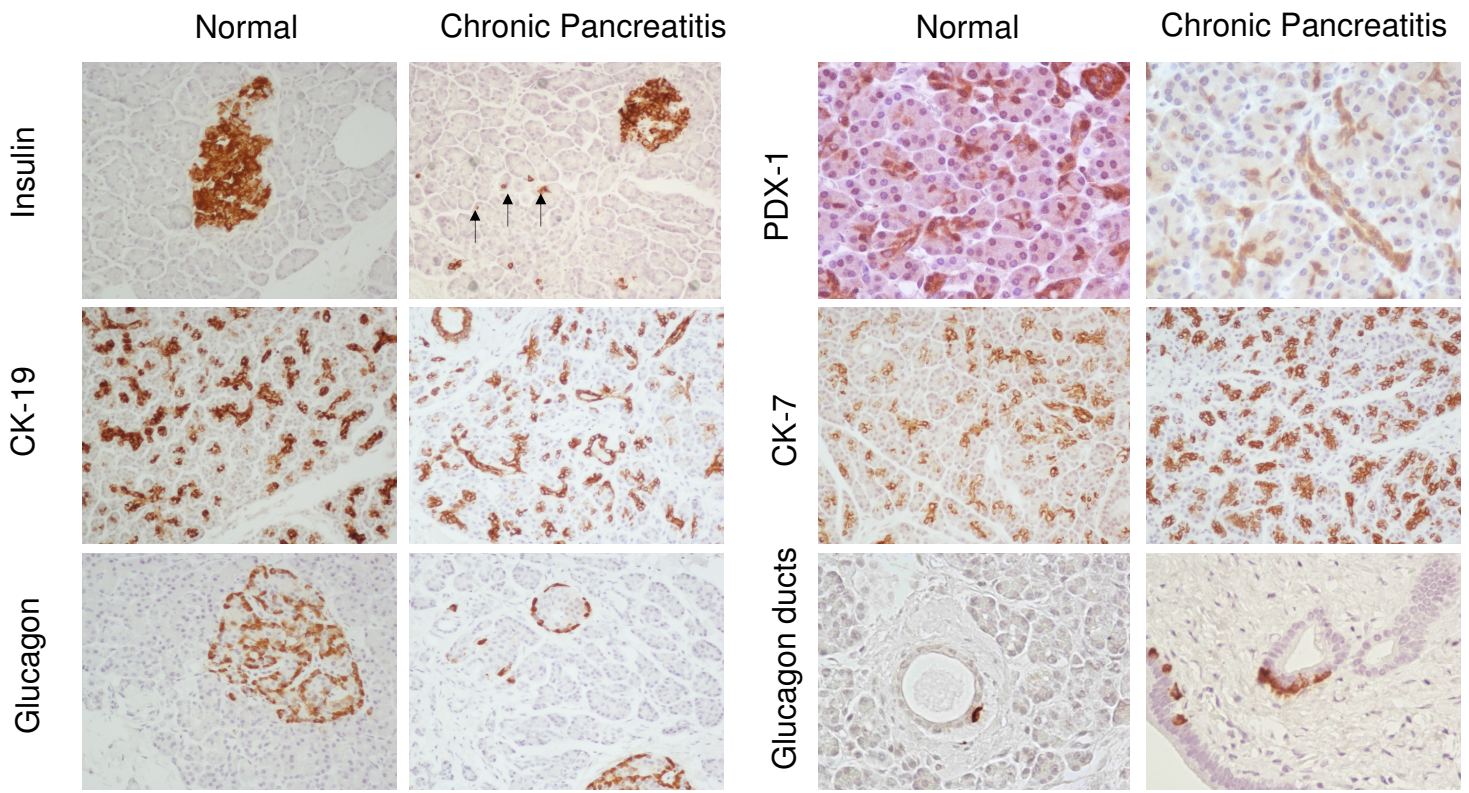


Figure 5.3.2 a-f. Typical examples of endocrine and ductal markers within the normal and chronic pancreatitic pancreas. *Expression of insulin positive NIECs, PDX-1 and CK7 and CK19 was statistically higher in CP tissue. No significant difference was found when assessing glucagon positive NIECs or glucagon positive ducts. All photographs were taken at an original magnification of x 200 with the exception of PDX-1 staining and Glucagon ducts which were taken at x400.*

5.3.2 Potential islet precursors vs. islet graft outcomes

A schema of the correlations carried out can be found in Figure 5.3.3 and the full list of correlation results can be found in Appendix IV (Tables i – vi). In total, 378 correlation analyses were carried out, and both significant and near significant correlations (p=<0.1) will be discussed. Figure 5.3.4 relates to results presented in sections 5.3.3 to 5.3.9.

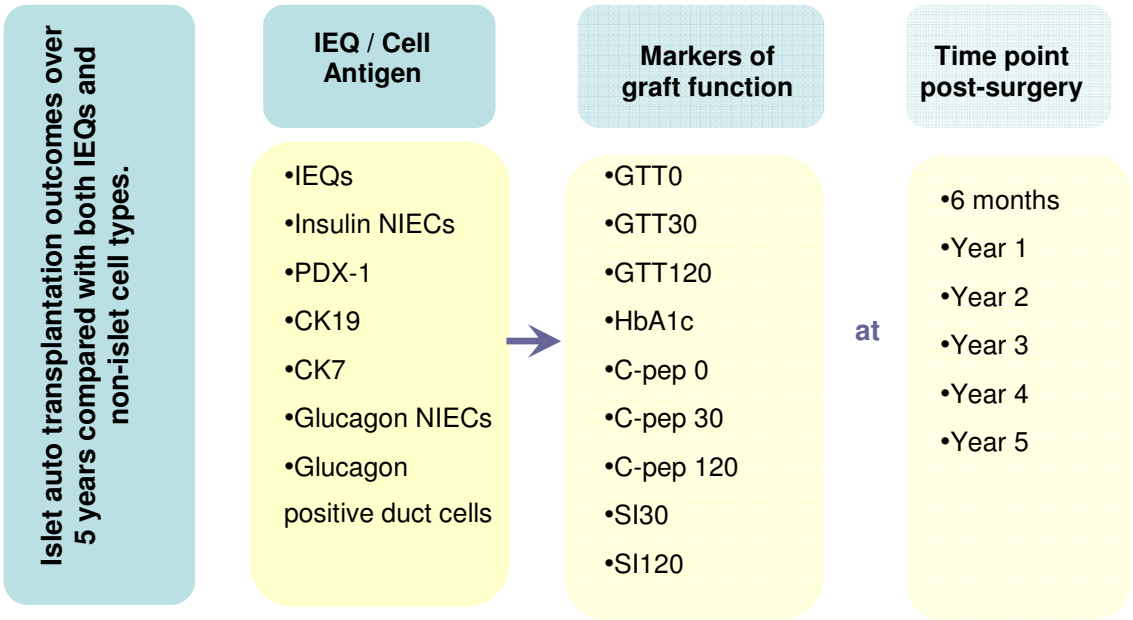


Figure 5.3.3. An overview of the correlations made between histological data and markers of islet graft function at 6 separate time points. *GTT 0, 30 and 120 and C-pep 0, 30 and 120 refer to the serum glucose or serum C-peptide levels 0, 30 or 120 min after a 75g oral glucose load. Similarly, SI30 and 120 (stimulation index 30 and 120) refer to the fold increase in C-peptide release either 30 min or 120 min after a 75g oral glucose load. For statistical analysis histological markers (and islet IEQ) were cited as the independent variables. Markers of graft function were not monitored past Year 5 as there was a decrease in available data.*

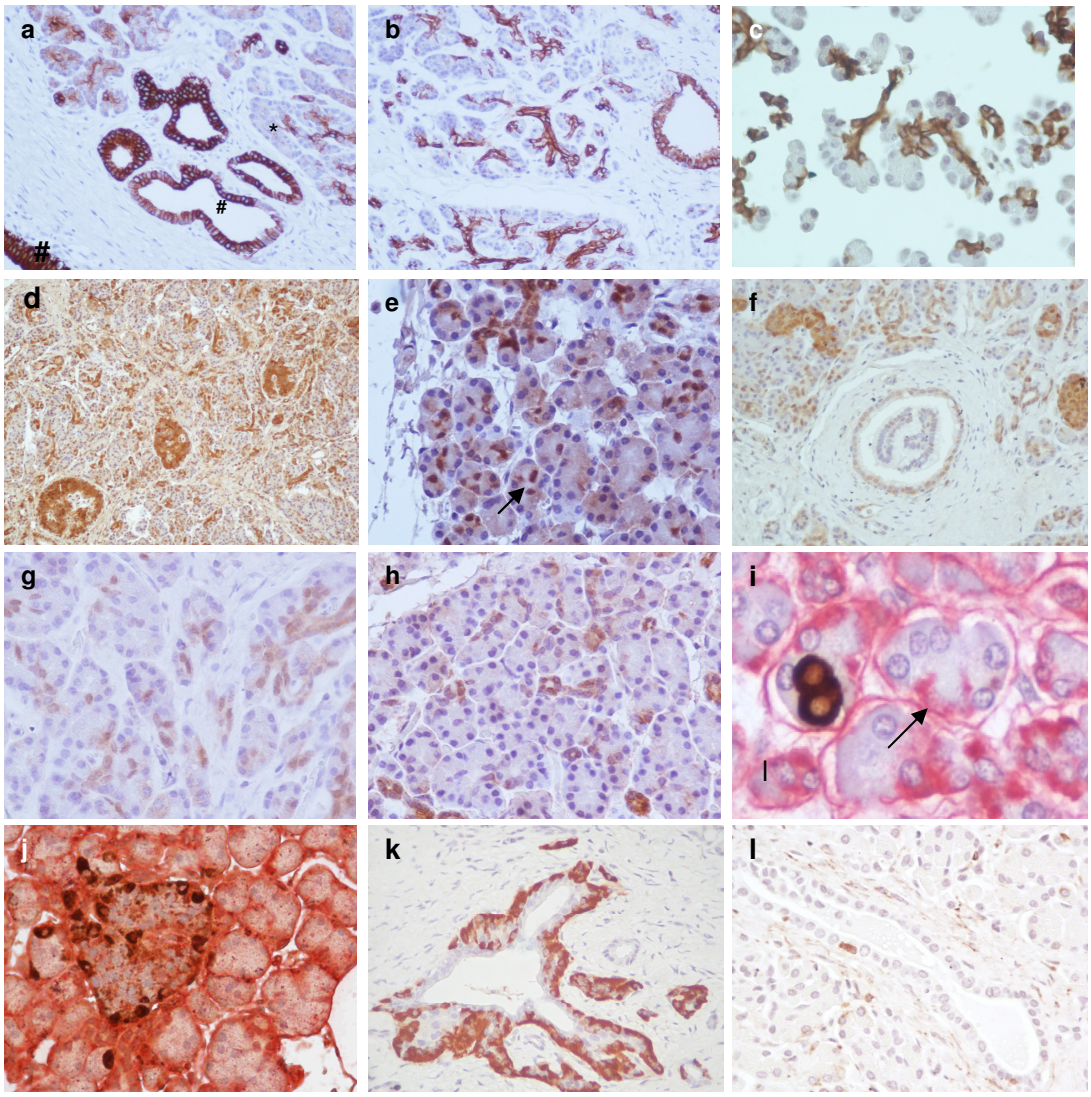


Figure 5.3.4 Immuno-histochemistry staining of potential islet precursors cells. *CK7 staining showed a gradient of staining with centro-acinar (*) cells exhibiting weak staining compared with larger ducat epithelia (#), Figure a. CK19 staining conversely was consistent throughout the ductal epithelia (b). CK19 staining showed that large ductal structures were largely absent from non-purified islet preparations (c). The intensity of PDX-1 staining varied between patient samples (d-g) and PDX-1 staining was either diffuse within the cytoplasm (note the duct in Figure f) or intense nuclear staining (e). Sections stained using PDX-1 antibodies targeted towards either the N-terminus (g) or Carboxyl-end (h) of the PDX-1 molecule produced a near-identical staining patterns. The location insulin NIECs (i) and glucagon NIECs (j) NIECs within the acinar parenchyma were confirmed by double staining each hormone (brown) with laminin (red). The majority of glucagon NIECs were closely associated with mid-size (intra and interlobular) ducts (k). Vimentin positive ductal cells were infrequently observed (l). Figures a, b,d & f photographed at a magnification of x100, figures c, e, g, h, j, k and l photographed at x200 and figure l photographed at x400.*

5.3.3 Ductal epithelial cells

As expected, CK19 staining was largely restricted to the pancreatic ducts and within each acini at the centroacinar position (Figure 5.3.4b), whilst CK7 was either absent or weak at the centroacinar position (Figure 5.3.4a). The frequency of CK19 positivity within CP tissue was approximately double that observed in normal tissue (34.20 (8.35-75.40) vs. 17.70 (13.1-20.3), p=<0.001), whilst CK7 positivity was more modestly up-regulated (23.40 (12.90-41.00) vs. 17.60 (8.66-38.70), p=0.044). This data suggests that chronic pancreatitis causes a significant increase in the proportion of small ductal epithelial cells within the pancreas.

Correlation studies showed that CK7 expression was associated with significantly lowered HbA1c at one year post-transplant, r=-0.559, p=0.025, n=16 (Figure 5.3.5) and was also associated with lower GTT30 and GTT120 at 6 months post-transplant, GTT120 at 2 years post-transplant and HbA1c at 3 years post-transplant, although these values did not quite reach significance (Table 5.3.1). In line with the CK7 results, CK19 was also associated with significantly lower HbA1c at one year post-transplant (r=-0.544, p=0.029, n=16); additionally, at 1 year post-transplant, fasting C-peptide secretion, perhaps the most pertinent marker of background islet function, was significantly higher with increased numbers of CK19 positive cells detected (r=0.635, p=0.015, n=14) (Figure 5.3.6). Furthermore, high levels of CK19 positive cells were also associated with higher fasting C-peptide at 6 months, higher C-peptide 30 at 1 year and lowered Hba1c at year 4 although these values did not quite reach significance (Table 5.3.1).

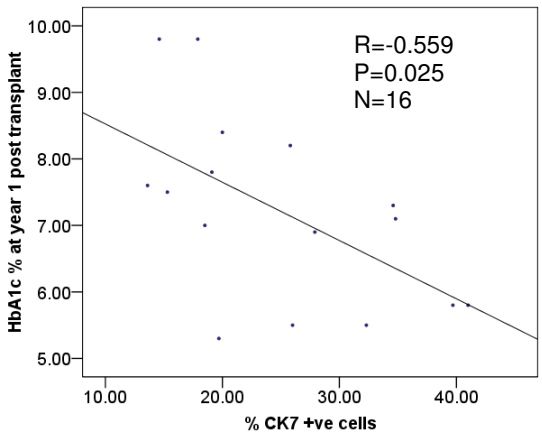


Figure 5.3.5 Graft function vs. CK7 antigen expression, significant correlation. *Scatter plot displaying a significant correlation between the percentage of CK7 staining and low HbA1c % at 1 year post-transplant.*

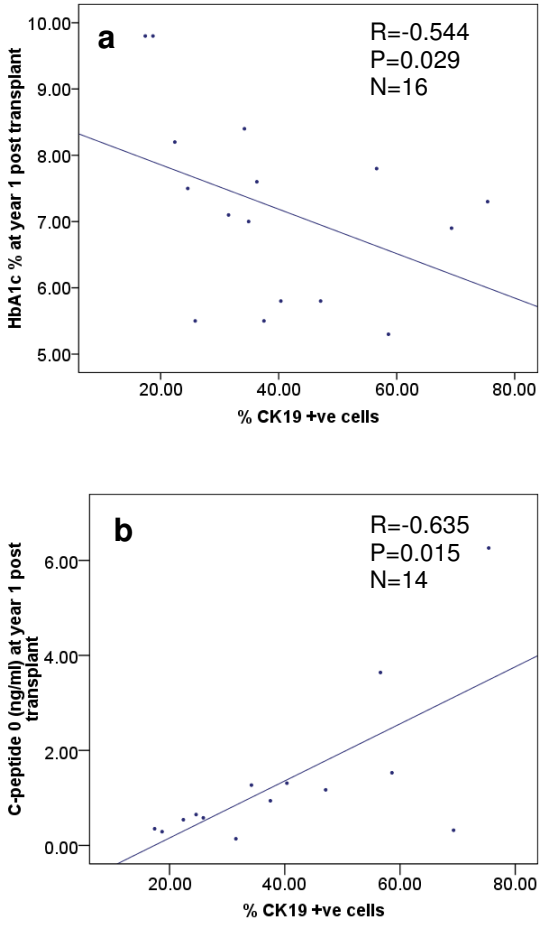


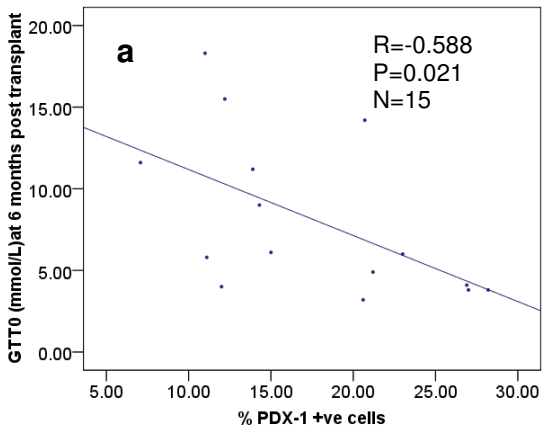
Figure 5.3.6 a&b Graft function vs. CK19 antigen expression, significant correlations. *Figure 5.3.6a has the same patient population as 5.3.5, data is expressed in terms of the the Spearman correlation coefficient. Scatter plots displaying significant correlations between levels of CK19 staining (%) and islet graft outcomes. CK19 was observed to be correlated with significantly low HbA1c % (a) and high fasting C-peptide (ng/ml) values at 1 year post-transplant (b).*

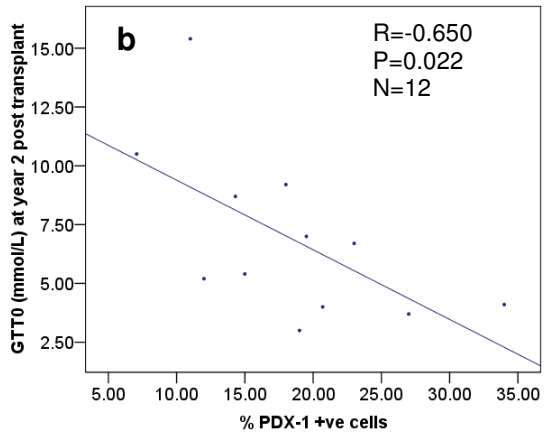
Graft Outcome	r	p	n
CK19 vs. C-pep 0 at 6mth	0.552	0.098	10
CK19 vs. C-pep 30 at year 1	0.512	0.061	14
CK19 vs. HbA1c at year 4	-0.492	0.087	13
CK7 vs. GTT30 at 6 months	-0.475	0.074	15
CK7 vs. HbA1c at year 3	-0.571	0.084	12
CK7 vs. GTT120 at year 2	-0.557	0.060	12

Table 5.3.1. Further correlations (p<0.10) between cytokeratin expression and graft function. *Data is expressed in terms of the the Spearman correlation coefficient. Near significant correlations have been deemed noteworthy in this report due to the small patient cohort studied.*

5.3.4 PDX-1 positive cells

PDX-1 staining within both CP and normal tissue was observed as diffuse cytoplasmic staining within the islets (Figure 5.3.4d), acinar parenchyma (and sporadically within the ductal epithelia and pancreatic vasculature) with a sub-section of more intense nuclear staining in certain cells (Figures 5.3.4 e&f). Normal tissue displayed a median level 8.99% (4.57-17.10) PDX-1 positive staining which is higher than previously described (253,254). Moreover, in line with the findings of Song and colleagues 1999 (255), the number of PDX-1 positive cells was up-regulated in CP specimens, 17.70% (13.10-20.30). Increased PDX-1 expression was significantly correlated with lowered fasting glucose at both 6 months and 2 years post-transplantation (r=-0.588, p=0.021, n=15 and r=-0.650, p=0.022, n=12, respectively) and with significantly lower GTT 120 at year 2 (r=-0.587, p=0.045, n=12), Figure 5.3.7.





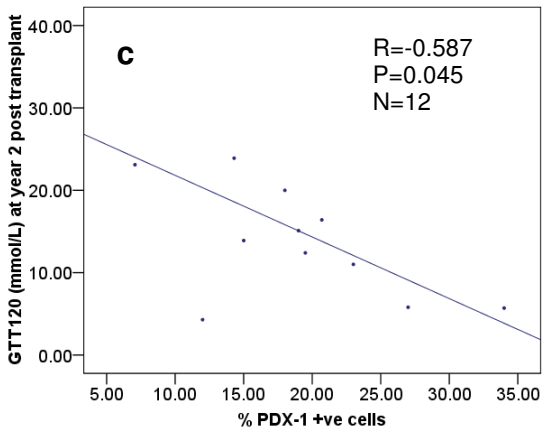


Figure 5.3.7 a-c Graft function vs. PDX-1 antigen expression, significant correlations. *Figures a & b represent the same patient cohort, data is expressed in terms of the the Spearman correlation coefficient. Scatter plots displaying the relationship between the % of PDX-1 staining and islet graft outcomes post-transplant. PDX-1 was observed to be significantly correlated with lower fasting glucose (mmol/L) at both 6 months and 2 years (a & b) and stimulated glucose at 2 years post transplant (c).*

5.3.5 The insulin positive NIECs

Within this study insulin positive NIECs were almost exclusively intermediate cells (i.e. located within the acinar parenchyma) with few insulin positive ductal cells, although insulin positive neo-islets emerging from the ductal epithelium were sporadically observed. Insulin positive NIECs were shown to occur at low frequency of 0.85% (0.15-4.46) in CP tissue (Figure 5.3.1 & 5.3.2). Although their occurrence of was not significantly associated with superior graft function, a near positive correlations were observed at 2 years post-transplant when comparing insulin positive NIECs with C-peptide 0 and C-peptide 30 ((r=0.565, p=0.089, n=10) and (r=0.503, p=0.097, n=10) respectively, Table 5.3.2).

5.3.6 Glucagon positive NIECs and glucagon positive ductal cells

The majority of glucagon positive NIECs were found within the acinar parenchyma but unlike insulin positive NIECs, an appreciable proportion was observed closely associated with the ductal epithelium (Figure 5.3.4k). Levels glucagon positive NIECs were low, 0.23 % (0-5.71), although one patient showed abberantly high levels (30 cells/field) and so was excluded as an outlier. Correlation analysis showed a positive correlation between glucagon positive NIECs and high SI30 levels recorded at 6 months post-transplant (r=0.648, p=0.043, n=10), Figure 5.3.8a, additionally a near significant correlation was observed between low GTT30 scores at 6 months post-transplant and low SI120 at 6 months post-transplant (Table 5.3.2)

Assessments of the subset of glucagon NIECs restricted to the ductal epithelium (glucagon positive ducts) found that although they were found at a low frequency) in CP cohort (0.019% (0-2.57, Figure 5.3.1 and 5.3.2), they were correlated with similar magnitude of improved outcomes as CK19 and PDX-1 (Figures 5.3.8 b-d). Notably, glucagon positive ductal cells were seen to be strongly correlated with:

reduced GTT30 at 6 months (r=-0.540, p=0.046, n=14), reduced GTT30 at Year 2 (r=-0.712, p=0.009, n=12), low HbA1c at Year 2 (r=-0.671, p=0.009, n=14) and a higher response to glucose (SI120) at 6 months post-transplant (r=0.729, p=0.026, n=9), (Figure 5.3.8 b-e). A further correlation was observed between high levels of glucagon positive NIECs restricted to the duct and lowered HbA1c values at year 3 year post-transplantation, although this did not reach significance (Table 5.3.2).

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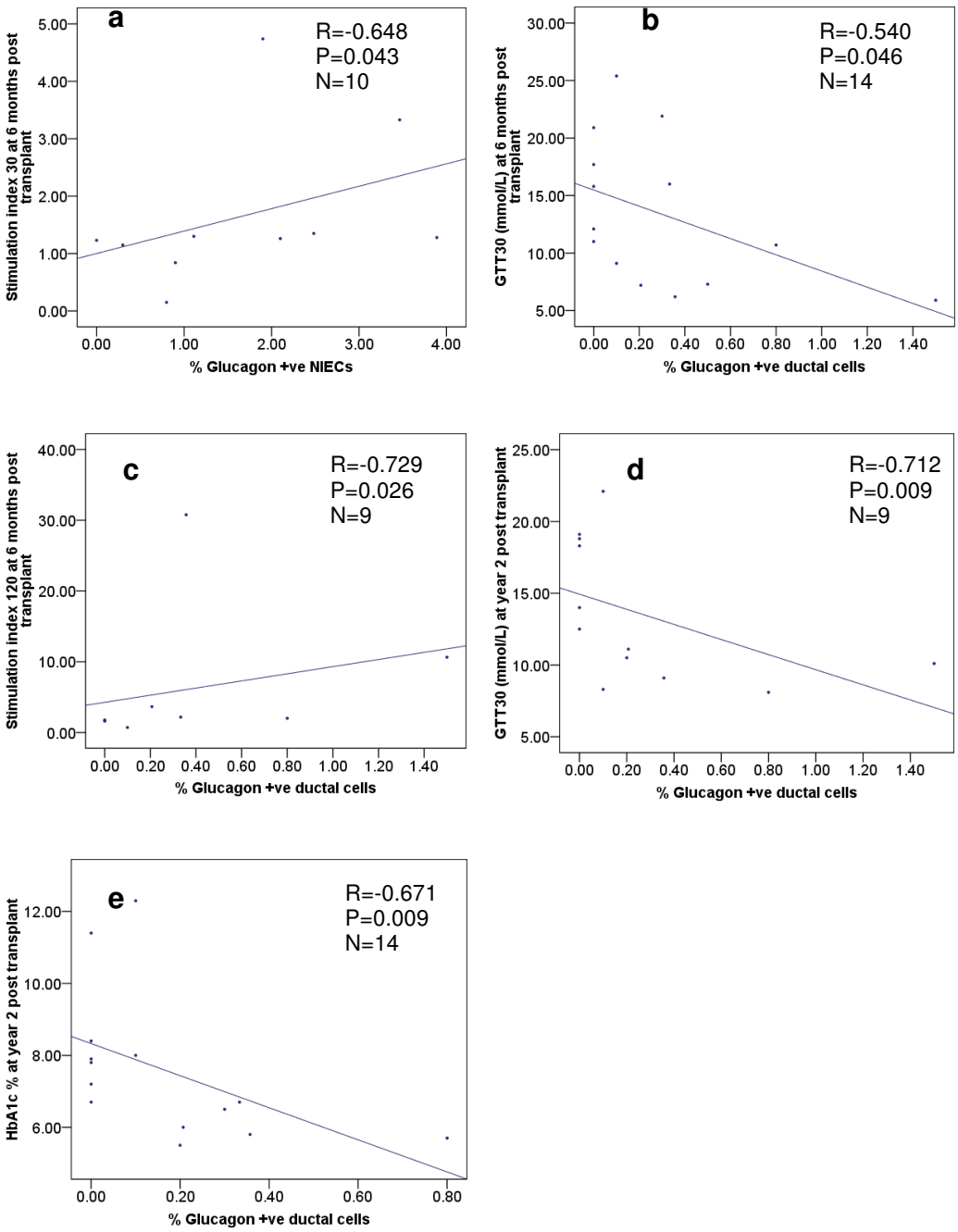


Figure 5.3.8 a-e Graft function vs. Glucagon positive NIECs, significant correlations. *All figures represent slightly different patient populations due to factors explained in Section 5.2.2, data is expressed in terms of the the Spearman correlation coefficient.. Scatter plot displaying the relationship between glucagon positive NIECs and islet graft outcomes post-transplantation. The % of glucagon positive NIECs were shown to be significantly correlated with a higher SI30 at 6 months post-transplant (a). Glucagon positive ducts were shown to significantly correlate with lower stimulated glucose levels (b) and an increased SI120 at 6 months post-transplant (c) and lower stimulated glucose (d) and lower HbA1c % (e) at 2 years post-transplant.*

Graft Outcome	r	p	n
Insulin vs. C-pep 0 at 2 years	0.565	0.089	10
Insulin vs. C-pep 30 at 2 years	0.553	0.097	10
Glucagon NIECs vs. GTT30 at 6 months	-0.486	0.078	14
Glucagon NIECs vs. SI120 at 6 months	0.633	0.067	9
Glucagon ducts vs. HbA1c at 1 year	-0.493	0.062	15

Table 5.3.2 Further statistical correlations (p<0.10) between expression of non-islet endocrine cells and graft function. *Data is expressed in terms of the the Spearman correlation coefficient. Near significant correlations have been deemed noteworthy in this report due to the small patient cohort studied.*

5.3.7 Vimentin

Vimentin positive ducts are a suggested marker for latent endocrine progenitor cells (252). Vimentin staining of CP pancreata showed several examples of vimentin positive cells associated with the duct (Figure 5.3.4l); however, in the majority of patients it could not be detected (only 5 CP pancreata and 3 normal pancreata expressed vimentin positive ductal cells).

5.3.8 Islet equivalents

For the CP patient group, islet IEQ was seen to be significantly associated with high levels of C-peptide 30 (r=0.5321, p=0.0412, n=15), Figure 5.3.8, whilst at year 3 a near significant correlation was observed between IEQ and increased levels of C-peptide 30 (r=0.5879, p=0.0806, n=10, Figure 5.3.9).

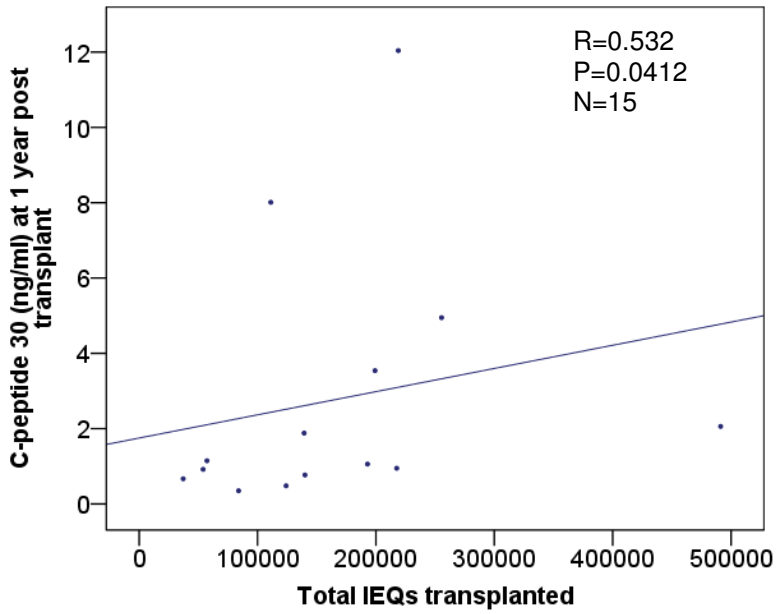


Figure 5.3.9 Graft function vs. IEQs, significant correlation. *Scatter plot showing a positive correlation between total IEQs transplanted and C-peptide 30 at year 1 post-transplantation, data is expressed in terms of the the Spearman correlation coefficient.*

5.3.9 Cell type correlations

Further correlations between each antigen marker (and islet IEQ) showed that levels of glucagon positive ductal cells were positively correlated with CK19 expression (r=0.512, p=0.015, n=22).

5.3.10 Patient demographic data vs. the prevalence of potential islet precursors

An analysis of donor data including: patient BMI, age and previous morbidities (Appendix IV, Table vii) versus the prevalence of potential precursors was carried to ascertain whether patient demographic factors affected the prevalence of islet precursors. This study was based on the previous reports that for example for example: obesity and Chrons disease are associated with pancreatic regeneration (245,249). Within the CP cohort no significant relationships were found between demographic data and the prevalence of potential precursor cells, possibly due to fact that chronic pancreatitis itself initiates pancreas regeneration. However, within

the control group, levels of glucagon NIECs were significantly reduced with increased age of the donor ($p=0.0067$, $r=-0.9286$, $n=7$) and this finding is discussed below. Raw data relating to these studies can be found in Appendix IV, Table vii).

5.3.12 Other cell types studied

For this study the following cell types were also analysed. CA19.9 represents a further ductal epithelial marker, however it was shown to display an identical (although less intense) staining pattern to CK19 and as such the results are not presented (Figure 5.3.10a). CD133 the haemopoetic stem cell marker (256) and putative cancer stem cell marker (257) has repeatedly been shown to stain the luminal side of ductal epithelial cells, (258), *and is reported to stain 95% of pancreatic ductal cells.* However within this patient cohort (both CP and normal), staining was restricted into infiltrating meschymal-like-cells with infrequent positivity in constitutive pancreatic cells (Figure 5.3.10b-d), as such the results were not reported. Glut-2 has previously been studied as an early marker of the endocrine phenotype (259). Within the liver, which served as a positive control for Glut-2 staining, the transmembrane location of Glut-2 was clearly identified on hepatocytes cells (e) however within the pancreas, non-islet Glut-2 expression was seen throughout the acinar parenchyma as small speckles and was deemed as background staining (Figure 5.3.10e-g). Levels of somatostatin positive NIECs were also studied however due to their infrequency (only 2 sections assessed showed positivity) this data was not presented (images not shown). Finally, insulin mRNA was analysed using in-situ hybridization, however the staining was deemed identical to insulin protein staining, although quality of tissue architecture were poorer (Figure 5.10 h & i), therefore results were not presented.

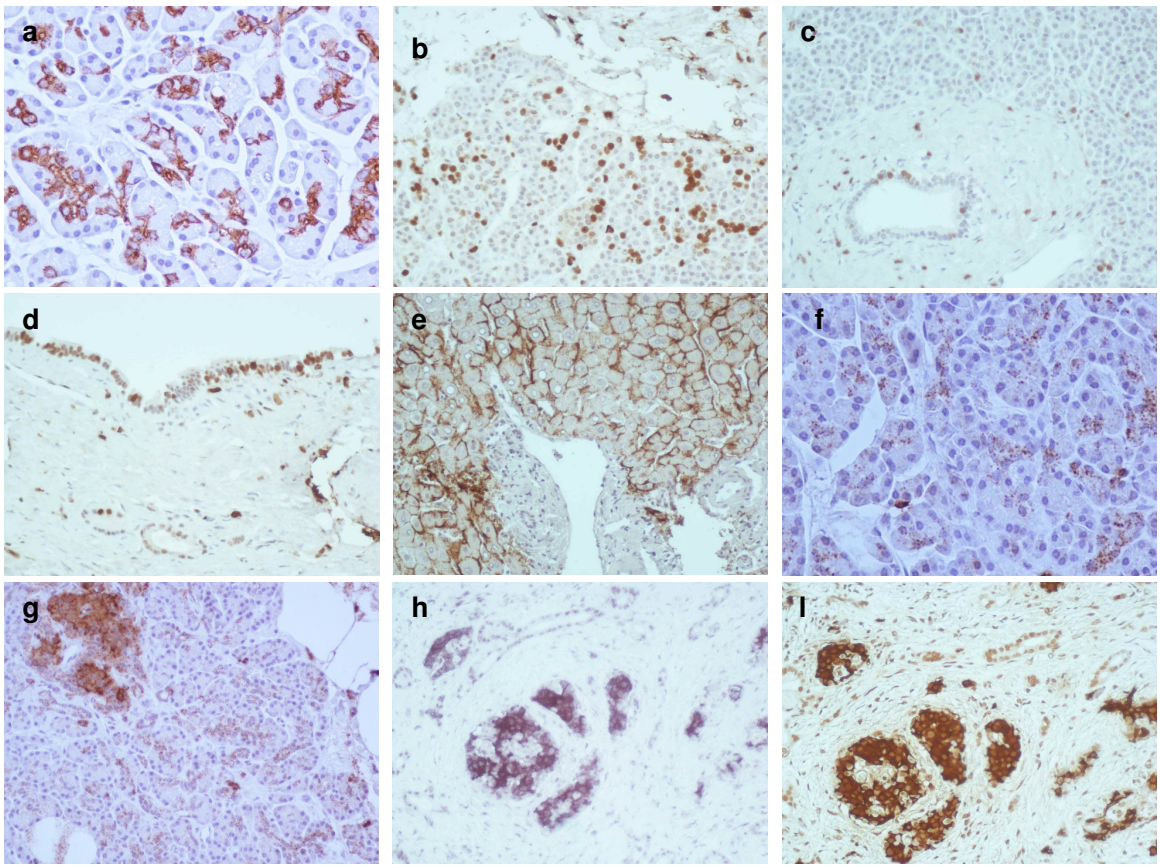


Figure 5.3.10 Further potential markers of islet precursor cells. *The ductal epithelial marker CA19.9 displayed an identical (although less intense) staining pattern to CK19 (Figure a, also refer to Figure 5.3.4b for an example of CK19 staining). CD133 appeared to stain infiltrating meschymal-like-cells (b) and some ductal cells (c &d). Within the liver, the transmembrane location of glut-2 on the surface of the hepatocytes was clearly observed (e) however within the pancreas, non-islet Glut-2 was deemed as background staining (f & g). In-situ hybridization for insulin mRNA (h) produced identical to insulin protein staining, although tissue architecture was poorer.* Figures a & f photographed at a magnification of x 200, figures b, c, d, e, g, h & I photographed at x 100.

5.4 Discussion

The main aim of the present study was to assess the impact of potential islet precursor cells and on islet graft outcomes, with the hypothesis being that such cells contribute to long-term insulin secretion post-transplantation. Although this study employs a less vigorous approach than certain *in-vitro* and lineage-based models that use either genetic or physical labeling of individual cell types, it's importance lies in the fact that data is based on transplantation of human tissue which has been under the influence of a natural (i.e. not experimentally induced) disease course (chronic pancreatitis) and takes into account the long-term function of the islet graft. This is in total contrast with experimental *in-vitro* models that rely on cell types with heightened pluripotency (i.e. ARJ cells ((159,160)) and lineage-based experiments which are carried out in lower vertebrates or developing mammalian systems (260,261). Although these approaches are essential for providing proof of principle they are unlikely to faithfully replicate what happens to the adult pancreatic epithelium post-transplant.

Essentially, this study, in line with earlier reports, demonstrates that ductal epithelial cells, PDX-1 positive cells and non-islet endocrine cells are up-regulated in response to chronic pancreatitis, (162,255,262). Secondly, due to the increased prevalence of precursor cells in CP pancreata, this study was able to successfully establish clear correlations between potential islet precursors and markers of islet graft function; this is in spite of the fact that islet equivalents transplanted should have the most profound effect on graft performance (51,263). Additionally, patient factors (i.e. BMI) and transplant factors (i.e. instant blood mediated inflammatory reaction) have also been recognized influence on graft performance (60,264). The significance of these results is also strengthened by the fact that all correlation

statistics were based on cohorts of less than 20 subjects due to incomplete patient follow-up.

Remarkably the results presented here suggest that transplantation of ductal epithelial cells (both CK19 positive and glucagon positive cells) and PDX-1 positive cells are associated with a similar magnitude of influence as total IEQs transplanted. Although these results are surprising, volume for volume, the numbers of ductal and PDX-1 positive cells transplanted may outweigh the number of islet cells transplanted. Additionally, post digestion, ductal structures and acini represent small tissue units (refer to Figure 5.3.4c) and the problems associated with central necrosis (211,265) would be reduced.

This study represents the first report of improved islet graft outcomes in association with high levels of PDX-1 positive cells transplanted, either in an experimental or clinical setting. This finding is potentially contentious, due to the fact that PDX-1 positive cells are widely believed to be restricted to the islets of Langerhans in the adult pancreas (266). However, some studies have suggested that the low-intensity staining of acini produced by PDX-1 antibodies represents specific staining due to the fact that the staining is non-uniform and often nuclear (254). By employing Axiovision image analysis in this study it was possible to objectively show appreciable levels of PDX-1 positive cells within normal tissue, indeed the lowest level recorded was 4.57 PDX1 positive pixels per field, additionally, significantly higher levels of PDX-1 positive cells were detected within CP patients and this data concurs with small animal models of pancreatic injury (267). Moreover, the validity of the staining is increased by the fact that PDX-1 antibodies directed to both the Carboxyl terminal and N-terminal regions of the PDX-1 molecule (refer to Table 2.4.1) produced near identical staining patterns, differing just in the levels of accompanying background stain (Figure 5.3.4 g & h).

Although it has proved difficult to study the conversion of adult non-islet PDX-1 positive cells into beta cells *in-vitro* or post-transplantation, transfection experiments carried out *in-vitro* have shown that PDX-1 uptake by ductal cells is accompanied with acquisition of beta cell characteristics including transcription of genes involved in insulin production (176). It is conceivable that the 'insult' of pancreas digestion and subsequent transplantation promotes a state of 'endocrine sparing' promoting beta cell genesis from embryonic islet precursors, although the exact mode of beta cell genesis is in doubt. Work carried out by Miyatsuka and colleagues (268) used a transgenic mouse model to show that sustained PDX-1 expression within the acinar parenchyma cells induced an acinar-ductal transition through Signal transducer and activator of transcription 3 (STAT3) in a cell-autonomous manner. The Miyatsuka study provides an attractive mode by which PDX-1 positive cells may convert into the beta cell phenotype, i.e. via the ductal phenotype and within this patient cohort a number of patient samples exhibited PDX-1 positivity which was largely reminiscent of CK19 staining refer to figure 5.3.4 g & h), supporting the supposition that PDX-1 positive cells may, in part, contribute the ductal cell mass post-transplant. However, when analysing the entire CP cohort (n=23), no significant concordance between PDX-1 expression and CK19 / CK7 expression was observed.

The role of ductal cells in islet transplantation is gaining momentum and in line with the experimental transplantation model used by Bogdani *et al* 2003 (248) and the clinical studies of Street *et al* 2004 (112) this study is the third to suggest spontaneous duct to beta cell transition post-transplant. Although there is a growing body of experimental data evidencing the trophic role of duct tissue (110,111,116), precursor-progeny relationships between ductal islet cells have also been demonstrated (refer to Chapter 1C) and confirmed using lineage based experiments (150). The data presented here show potential islet precursors are

associated with improved graft outcomes up 2 years post-transplantation whilst the islet equivalents transplanted were not correlated with improved graft outcomes past 1 year mark. It is therefore possible that there presence of the ductal cells in islet autograft goes beyond providing islets with bio-trophic support.

Whilst the small ductal cells of the pancreas represent a recognized source of endocrine precursors (115,269) glucagon positive cells associated with the duct arguably represent cells committed nesidioblastosis (145). Again this study is the first report of improved transplantation outcomes associated with glucagon positive cells associated with the duct, however, the mode by which these cells exert their support is of issue. Correlation analysis revealed a significant positive relationship between glucagon positive duct cells and CK19 positive cells, suggesting that glucagon positive cells are produced at a constant rate throughout the ductal epithelium and are therefore a surrogate marker for ductal cells. However this supposition does not hold fast in all circumstances. One patient with severe CP coupled with marked cachexia showed extensive and aberrant glucagon expression particularly associated with the ductal epithelia; indeed certain ducts were completely enveloped in glucagon positive cells. This data potentially suggests that in extreme circumstances all ductal epithelial cells have the ability to commit to nesidioblastosis, which one could hypothesize, is an evolutionary attempt to maximize glucose uptake at times of severe starvation.

The final finding to be discussed was the fact that regression analysis revealed that the number of glucagon NIECs (particularly glucagon positive intermediate cells) negatively correlated with the increased age of the donor within the cohort of control patients. In the first instance, this finding is consistent with the general assumption that cell types associated with regeneration diminish with age. These preliminary findings would suggest that young patients provides optimal donors for

the procurement of islet precursors cells and this preliminary data warrant further assessment upon a larger patient cohort.

5.5 Conclusions

To date islet allo transplantation of purified islets has failed to provide sustained insulin independence in the majority of transplant recipients and improvements to the islet isolation and islet transplantation protocols are needed. In line with the previous findings, this study provides convincing evidence that transplantation of potential islet precursor cells can significantly improves islet graft outcomes. Although the data presented here relates to the chronic pancreatitic pancreas, our analysis also shows appreciable levels of PDX-1 positive cells, glucagon positive ductal cells and most obviously ductal epithelial cells within the normal tissue. With islet allo-transplantation groups increasingly seeking methods of predicting the success/failure of islet graft , this study suggests that may prove informative for more islet transplant centres to correlate both islet IEQs and the prevalence of non-islet cells with graft outcomes and embrace the possibility that transplantation of highly purified islet preparations may not be best practice.

Chapter 6: Discussion and Conclusions

6.1 Introduction

Islet transplantation has the potential to produce insulin independence, stable glucose values, liberalization of diet, the prevention of long-term diabetic complications and freedom from potentially life threatening hypoglycaemia. Unfortunately, widespread implementation of islet transplantation is not currently possible due to the considerable shortage of donor pancreata, inefficient engraftment rates following transplantation (62) and issues surrounding graft rejection/recurrence of autoimmunity post transplant (66,70,270). Advances in immunosuppressive regimens, organ procurement techniques, isolation techniques and availability of defined collagenase blends have contributed to the continuing promise that islet transplantation will be the treatment of choice for type 1 diabetes mellitus in the future. However, the lack of insulin secreting tissue still remains a fundamental stumbling block.

The present batch release criterion for islets is 'relatively' inclusive:

'*The final criteria for islet product release included an islet infusion compatible with the ABO blood group, an islet mass of* ***5000 islet equivalents*** *per kilogram or more (on the basis of the weight of the recipient), an islet purity of* ***30% or more****, a membrane-integrity viability of 70% or more, a packed-tissue volume of less than 10 ml, negative Gram's staining, and an endotoxin content of 5 endotoxin units per kilogram or less (on the basis of the weight of the recipient)t'* (56).

However, in the attempt to reach 30% purity, (indeed a number of groups far exceed this target (3)) many preparations that would have achieved the required islet numbers pre-purification do not meet the required islet numbers post purification, on the basis that an estimated 40% of islets are lost during the purification process (3). This project set out to question whether islet purification

was necessary particularly in the light of evidence that auto transplantation of non-purified islets is associated with the most successful graft function rates within the field of islet transplantation, non-islet pancreatic tissue has been shown to provide islets with bio-trophic support and non-islet cells have the potential to convert into a beta-cell phenotype.

6.2 Discussion of study methods and results

6.2.1 Auto islet transplantation of non-purified islet vs. purified islets; a clinical study

This study began with an analysis of the long-term graft function of patients either transplanted with purified or non-purified islets within the University Hospitals of Leicester. To date, there are few clinical studies reporting allo transplantation of non-purified islets (271), indeed concerns over loss of islet function (96,97) and patient safety have focused attention on islet purity. Conversely, to date, the majority of data in the field of islet auto transplantation relates to the transplantation of non-purified islets based the fact that retention of islet mass is of paramount importance. Consequently, the islet auto transplantation series from the University Hospitals of Leicester provides a rare opportunity to compare the long-term outcomes of patients receiving purified or non-purified islet auto transplants.

For reasons that have been set out in Section 3.1 & 3.4, auto islet transplant patients represent good subjects for the study of islet transplantation outcomes due to the fact confounding influences related to recurrence of diabetes and immune suppression are avoided, (66,67,70,198) and pancreas procurement and processing is significantly shorter and relatively standardised compared with allo transplantation protocols (75,76).

Despite the potentially damaging impact of acinar tissue on both islet function and liver integrity, no significant benefits were associated with transplanting purified

islets. Indeed peri-operative factors including; intra-operative venous pressures and blood loss, operation length and hospital stay were largely comparable within both groups, although operation length, which has been associated with increased risk of thrombosis (191,192) was significantly longer in the purified islet group (Table 3.1) and this is undoubtedly due to the increased time required for islet purification.

Analysis of routine liver function tests (alkaline phosphatase, aspartate aminotransaminase, bilirubin and albumin synthesis) taken at 6 and 12 months post-transplant, again showed that purified and non-purified islets were associated with largely comparable liver function. Although the study highlighted a mild derangement of ALP and ALT post-transplant in both groups, particularly the patients receiving non-purified islets, the data also indicated a resolution of these changes by 12 months post-transplant transplant (Table 3.2 & Section 3.3.2). Interestingly, direct comparison of albumin synthesis at 1 year post-transplant in each group showed that transplantation of purified islets was associated with reduced albumin output. As induction of diabetes has been reported to rapidly reduce albumin synthesis (199-201) as such the reduction in albumin levels at 12 months potentially reflects the relatively low C-peptide levels also seen at year 1 post-transplant within the purified islet group (Figures 3.3a & b).

Taking into account 5 years-worth of islet graft data, the regression analysis revealed significantly superior glycaemic control (both fasting serum glucose and HbA1c) within the non-purified group (Table 3.3), despite the fact that IEQs transplanted were comparable in each group; and these finding are corroborated by early canine studies carried out by the Minneapolis islet auto transplant group (101,104). Although markers of glucose control were shown to be superior in the non-purified group C-peptide response to glucose (2 hours after an oral 75g bolus)

was on average superior in the purified group additionally mean insulin requirements were also lower n the purified group. However balanced with this data was the finding that Insulin requirement showed a significant increase year on year post transplant while stimulated C-peptide showed a significant year on year reduction. Reciprocally, both stimulated C-peptide showed an increase in the non-purified group and insulin requirement remained stable.

The study presented in Chapter 3 suggests that transplantation of non-purified islets is not associated with adverse peri-operative outcomes or inferior liver function post-transplantation, in fact, purified islets were associated with a significant increase in operation time and reduced albumin production by the liver. The findings also suggest enhanced long-term islet graft stability in the non-purified group, supporting the hypothesis that other factors, not related to pure islet mass, have an impact on long-term islet graft function. However the data potentially raised concerns as to whether non-islet cells are detrimental to islets in the shorter-term.

6.2.2 An evaluation of beta cell function in purified and non-purified islet preparations; an *in-vitro* study

Based on the finding that non-purified islets graft supported comparable islet function in the long-term but may have potentially affected insulin secretion in the shorter-term, a culture model was developed that allowed direct comparison of the survival and function of islets cultured with and without non-islet pancreatic tissue. The 8-day culture model was very revealing as it was apparent that whilst islet function and viability were comparable in both groups, retention of insulin stores was significantly enhanced in the non-purified group (Figure 4.2.4 and Section 4.2.2.2). This may have been due to the fact that the majority of islets were seen to form attachments with non-islet tissue (primarily ductal tissue) which appeared to

confer resistance to central necrosis (Figure 4.2.6 & Section 4.2.4) and this assumption agrees with previous studies (110). The finding that somatostatin, a modulator of acinar secretions, was found bound to acinar cells was an interesting and may to represent a protective reaction to the acinar autolysis following pancreas digestion (Figure 4.2.7). However the mechanism by which somatostatin acts is likely to be inefficient at inhibiting acinar secretions from lethally damaged cells, although it may modulate enzyme secretion from healthy acini and this forms an interesting avenue of future study.

In summary, the study presented in Chapter 4 showed that co-culture of islets with non-islet pancreatic tissue supported the maintenance of intracellular insulin stores whilst highly purified islets showed a significant decrease over the 8-day culture period. This finding suggests that non-islet tissue may play an important role in maintaining islet function either through cell to cell contact or through soluble factors. To compliment this work, the study presented in Chapter 5 investigated the impact of islet-cell precursors and non-islet hormone positive cells on long-term autograft outcomes, based on the hypothesis that they have the capacity to contribute to the beta-cell mass of the graft post-transplant.

6.2.3 The impact of non-islet endocrine cells, pre-endocrine and ductal cells on islet graft outcomes.

Over the past decade an increasing number of research groups have shown that islet precursor cells and adult stem cells of the pancreas can be expanded clonally and, after manipulation, can be induced to express low levels of insulin and other pancreatic markers (44,150,159). These findings are provocative and have triggered the hypothesis that islet precursors have the capacity to convert into a beta cell phenotype post-transplant (112,248). Indeed, the study by Street and

colleagues in 2004 provided evidence that allo transplantation of large numbers of ductal epithelial cells was associated with superior graft function.

It has been shown that chronic pancreatitis induces a marked increase in tissue regeneration and remodelling, causing a distinct increase in the prevalence of pancreatic precursors including: ductal epithelial cells and non-islet endocrine cells (162,255,262). Thus, it can be assumed that correlations between the histological profile of source pancreata and clinical outcomes would be more apparent in the auto rather than allo transplantation setting.

Preliminary analysis showed that levels of ductal cells, PDX-1 positive cells and non-islet insulin positive cells were indeed higher in CP pancreata compared with normal pancreata. Correlations between potential pancreas precursors and islet auto transplantation outcomes showed that high levels of ductal epithelial cells, PDX-1 positive progenitors and glucagon positive NIECs, correlated significantly with higher C-peptide secretion, lower glucose levels and lower HbA1c%. Furthermore these results were observable despite the fact that the results should have been completely masked by 'active' part of the islet graft i.e. the islets. The results presented here, combined with the seminal paper published by Street *et al* in 2004 (112) therefore provide good evidence that complete purification of islets may not be best practice. Although, the benefits of including ductal cells in islet transplants has previously been explored (112,202,249) the finding that non-islet PDX-1 cells also correlate with improved outcomes presents a novel finding. However, the pattern of PDX-1 staining observed was often ductal in nature (both in CP and normal pancreata), and may support the relatively new concept that non-islet PDX-1 positive cells represent acinar cells transdifferentiating into a ductal cell phenotype (268) and an investigation of a larger patient cohort may prove a significant relationship between these two cell types. Although the metaplastic

change of acinar tissue into ductal tissue is well recognised in chronic pancreatitis pathology (262) it is not commonly believed to occur in the normal pancreas. Therefore the observation that approximately 10% of non-islet cells within the control group (normal pancreata) were PDX-1 positive was surprising and may be due to improvements in standard immunohistochemistry techniques and available antibodies.. The results presented here potentially suggest that strategies to conserve, harvest and co-transplant PDX-1 positive cells with the main islet graft may significantly improve islet outcomes. However due to the fact that the existence of non-islet PDX-1 postive cells is in question further definitive characterisation of PDX-1 positive cells, possibly using in-situ hybrisation, is necessary.

Finally, the occurrence of rare precursors cells e.g. glucagon positive NIECs and glucagon positive ductal cells, were also correlated with enhanced graft function. To account for the potency and these relatively rare cells, it could be speculated that they undergo rapid proliferation post transplant; however another explanation may be that these cells act as a marker of pancreas plasticity with highly regenerative pancreata displaying an up-regulation of NIECs, PDX-1 positive cells and ductal epithelia. In line with this theory, glucagon positive ductal cells were shown to strongly correlate with levels of CK19 positive cells.

The results presented in Chapter 5 suggest that ductal cells, non-islet PDX-1 positive cells and glucagon positive NIECs are associated with improved long-term islet incomes, although analysis of a larger cohort of patients would be more informative. Combined with the findings of Chapters 4 & 5 suggest that non-purified islet transplants are associated with more stable islet function than purified islets

6.3 Future work

Taken together the studies presented suggest that transplantation of non-purified islets is potentially associated with enhanced graft function. Although the transplantation of non-purified islets is standard protocol in the field of islet auto transplantation, it is possible that islet allo transplantation protocols may also benefit from this approach. However, it is likely that the potential risk of thrombosis associated with allo transplantation of non-purified will remain a major focus of concern, particularly in light of the fact that volumes of non-purified islets procured from healthy pancreata for islet allo transplantation are likely be much larger than volumes procured from chronic pancreatitic pancreata, which have been shown to display marked levels of acinar atrophy (84). As such, assuming that ductal cells and islet precursors do improve islet graft function, the following strategies may prove useful:

1) Due to the fact that islet transplantation is associated with transient rises in hepatic portal pressure (Table 3.1), sub-therapeutic amounts of non-purified islets could be transplanted over a prolonged period. This strategy has been associated with superior graft outcomes in animals transplanted over a 14 day (144) and may further increase the safety of the procedure.
2) The culture of impure islet fractions following purification potentially provides an additional step to the COBE process. Based on the data presented Section 4.2.2.3, approximately 60% of the islets found within the impure gradient fractions could be recovered whilst retaining non-islet tissue which may confer continued bio-trophic support to the islets post transplant.
3) Although previous clinical and experimental studies (112,248) and the present study suggest that the transplantation of various non-islets pancreatic cells can significantly enhance islet graft outcomes, further work in this area may change current isolation protocols to include the isolation ductal cells, islets precursors and other cells that may improve long-term graft function.

Conclusion 6.4

While the long-term future of islet transplantation may involve the use of haemopoetic and embryonic stem cells, a shorter-term and much needed target is to maximise the recovery of insulin secreting cells from the digested pancreas. This study suggests that purification of islets may not be optimal for long-term graft function and that the inclusion non-islet pancreatic cells may have more profound benefits post-transplant than has been shown to date. Indeed additional research in this area, particularly further studies comparing non-islet cells transplanted with clinical graft outcomes, could significantly change current clinical islet transplantation practices. Although the safety of infusing larger amounts of tissue into the liver needs to be addressed, islet isolation procedures that allow the successful harvest of islets as well non-islet cells particularly ductal epithelia tissue (including glucagon positive ductal cells) and PDX-1 positive acinar cells, may greatly improve the outcome of islet allo transplantation and make single donor transplants for the treatment of type 1 diabetes, a reality.

A comparison of islet auto transplantation data of the 3 largest centres worldwide

	Number of Procedures	**Death rate at year 1**	**Death rate at year 10**	**Insulin independence**	**C-peptide positive patients**	**Operation length (hr)**	**Estimated Blood loss (ml)**	***Hospital stay (days)***
Minnesota series	136	*2%*	*27%*	33%	<100%	10+/-1.7	1500ml (50ml – 30L)	*22 days (1-89)*
Cincinnati series	50	6%	ND	40%	ND	ND	ND	*ND*
Leicester series	*50*	*2%*	*16%*	*26%*	*100%*	*7 (5.5-11)*	*700 (200-2000)*	*21.5 (8-52)*

Table i. The Islet auto transplant data of the 3 major centres in the world. *All data is correct as at December 2006, excluding Cincinnati whose data is correct up to December 2004. Data for the Minnesota and Cincinnati series were obtained from a articles provided by Blondet and colleagues 2007* (85,98)

From this data presented in table I it is clear that in terms of mortality and peri-operative data (operation length, blood loss length of total hospital stay) Leicester compares favorably with the Minnesota group who hold the largest islet auto islet series in the world. Additionally C-peptide maintenance rates within the Leicester remain at 100% whilst Minnesota quote that one 3rd of patients shown no or little C-peptide secretion (98). Conversely, insulin independence rates within the Leicester group as at the December 2006 were shown to be poorer than those reported in both the Minnesota and Cincinnati series, however it must be taken into account that up until December 2006 the majority of patients within the Leicester series presented with primary diagnosis of ethanol abuse (272), whereas the Minnesota and Cincinnati groups transplanted larger numbers of idiopathic chronic pancreatitis patients. This factor is important based on observations that patients with an etiology of ethanol abuse tend to fare poorly due to destructive habits that negatively impact glucose control (272).

Statistical model developed to compare the clinical outcomes of patients either transplanted with purified or non-purified islets

The clinical graft outcomes (e.g. serum glucose, HbA1c and C-peptide) of patients transplanted with either purified or non-purified were compared over a 5 years period.

- The model used was a: repeated measures multiple regression analysis and was used to compare the purified and non-purified groups, over the 5 years, after taking into account the effect of year.
- Random intercepts and random slopes models were fitted to the data.
- This model took into account that clinical patient data can widely vary, therefore, instead of fitting a common slope and a common intercept as would be carried out in a normal regression model, the intercept and slope was allowed to vary across individuals, allowing each individual had their own intercept and slope.
- As well as fitting random intercepts and random slope models, random intercept only models were also fitted. Subsequently a comparison of the goodness of fit (using Akaike information criteria (AIC)) was made and the model was selected (either random intercepts and random slopes or random intercepts only) based upon which had the lowest AIC.
- LS means (least squares means (mean score after adjusting for other effects in the model).
- All data was analysed using SAS version 9.1 using Proc Mixed and statistical analysis was carried out in association with Dr John Bankart, Senior Lecturer, Statistics, the University Hospitals of Leicester. P values of <0.05 were considered statistically significant.

Analysis	**Model Used**
Analysis 1a: GTT 0	Intercept and Slope
Analysis 1b: GTT 30	Intercept and Slope
Analysis 1c: GTT 120	Intercept
Analysis 2: HbA1c	Intercept and Slope
Analysis 3a: C-PEP 0	Intercept and Slope
Analysis 3b: C-PEP 30	Intercept
Analysis 3c: C-PEP 120	Intercept
Analysis 4a: Insulin requirement	Intercept

Analysis 1a: GTT 0, Random Intercepts and Random Slopes Model, N subjects = 31

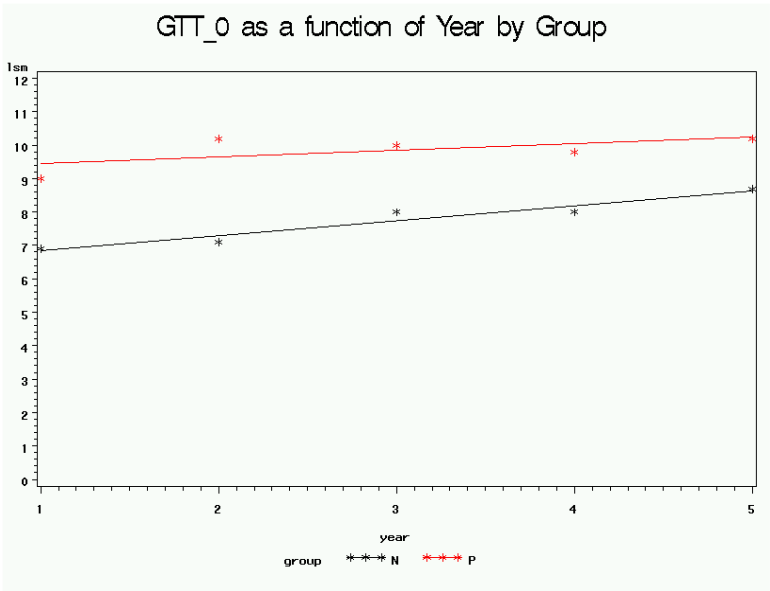
Effect	F	P
Group	4.67	0.0282 *
Year	4.33	0.0947
	LS Mean	Std Error
Group N	7.59	0.64
Group P	9.68	0.74

Where:

F=	F-test
P=	p value
Group N =	Non-purified Group
Group P =	Purified Group
LS Means =	Least squares means

- **Group P have a significantly higher GTT0 score than Group N.**
- Slope for Year = 0.43. Non-significant.
- There is no significant interaction between Group and Year.

Year	Mean predicted value for GTT score	
	P	N
1	9.0	6.9
2	10.4	7.0
3	9.8	8.0
4	9.7	8.1
5	10.4	8.7



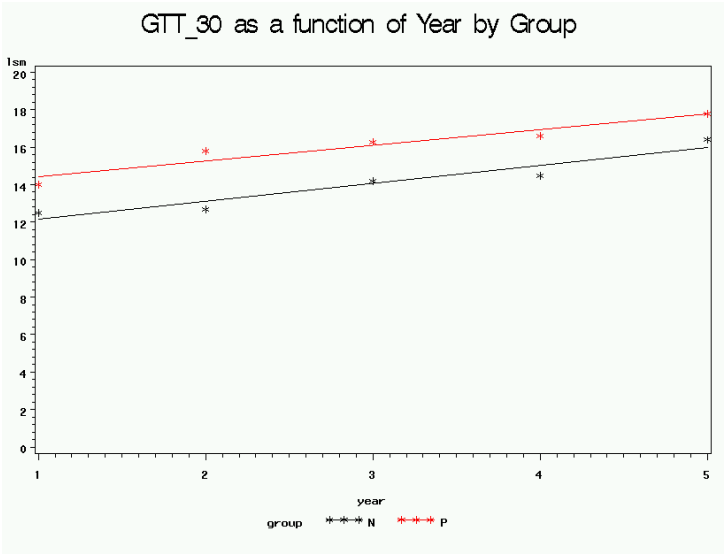
Analysis 1b: GTT30, Random Intercepts and Random Slopes Model,

N subjects = 31

Effect	F	P
Group	1.01	0.3210
Year	9.04	0.0063
	LS Mean	Std Error
Group N	14.2	0.92
Group P	15.5	1.09

- No significant difference in mean GTT30 score between the groups.
- **Significant slope for Year = 1.0. For each extra year we get an increase of 1 in GTT30.**
- There is no significant interaction between Group and Time.
- There is a significant linear effect of Year. With each year we get on average an increase in mean GTT score of 1.0.

Year	Mean predicted value for GTT 30 score	
	P	N
1	14.0	12.5
2	15.8	12.7
3	16.3	14.2
4	16.6	14.5
5	17.8	16.4

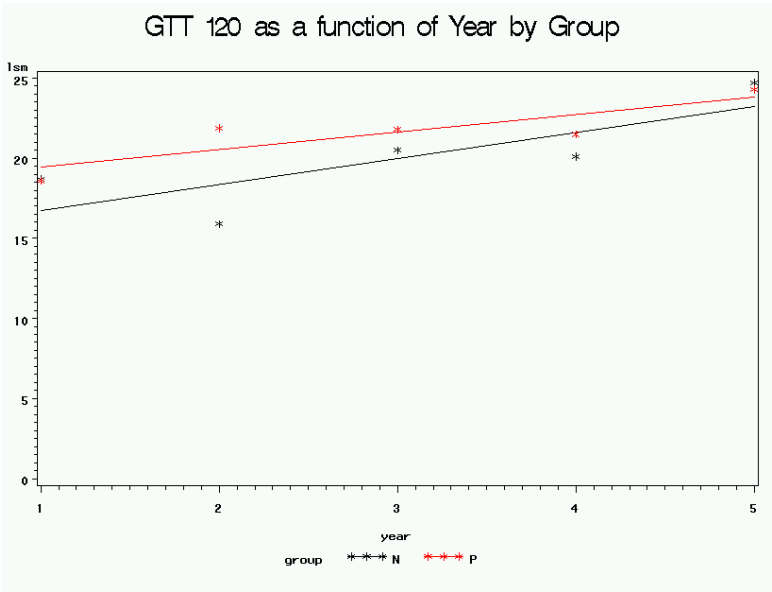


Analysis 1c: GTT120, Random Intercepts only Model, N subjects = 31

Effect	F	P
Group	0.07	0.7890
Year	10.48	0.0020*
	LS Mean	Std Error
Group N	19.4	1.6
Group P	20.1	1.9

- No significant difference in mean GTT120 score between the groups
- **Significant slope for Year = 1.01. For every extra year the score increases by 1.01.**
- There is no significant interaction between Group and Year.

Year	Mean predicted value for GTT 120 score	
	P	N
1	18.6	18.7
2	21.9	15.9
3	21.8	20.5
4	21.5	20.1
5	23.4	24.3

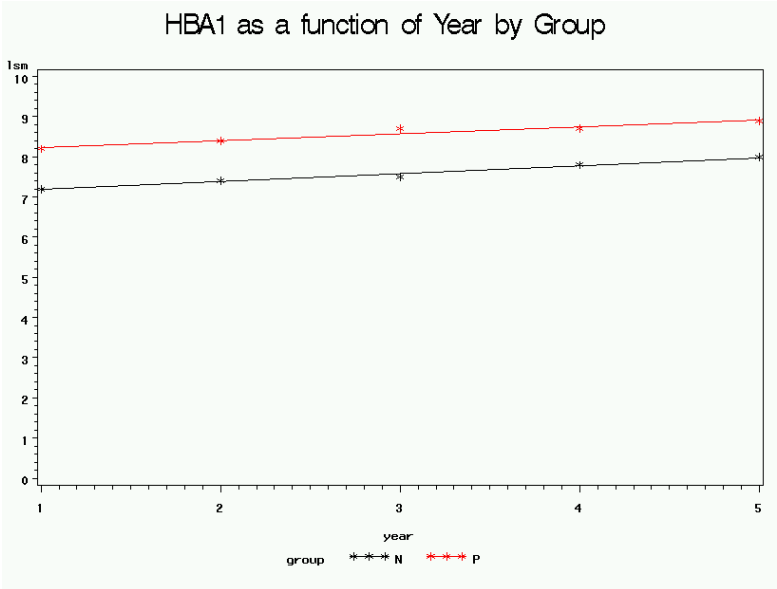


Analysis 2: HbA1c, Random Intercepts and Random Slopes Model, N subjects = 31

Effect	F	P
Group	5.99	0.019 *
Year	1.41	0.245
	LS Mean	Std Error
Group N	8.7	0.39
Group P	7.5	0.31

- **Group P have a significantly higher HbA1c score than Group N.**
- Slope for Year = 0.18 (not significant). Scores appear to increase with time but not significantly.
- No significant interaction between Group and Time (INT tested but not included in final model).

Year	Mean predicted value for HBA score	
	P	N
1	8.2	7.2
2	8.4	7.4
3	8.7	7.5
4	8.7	7.8
5	9.0	8.0

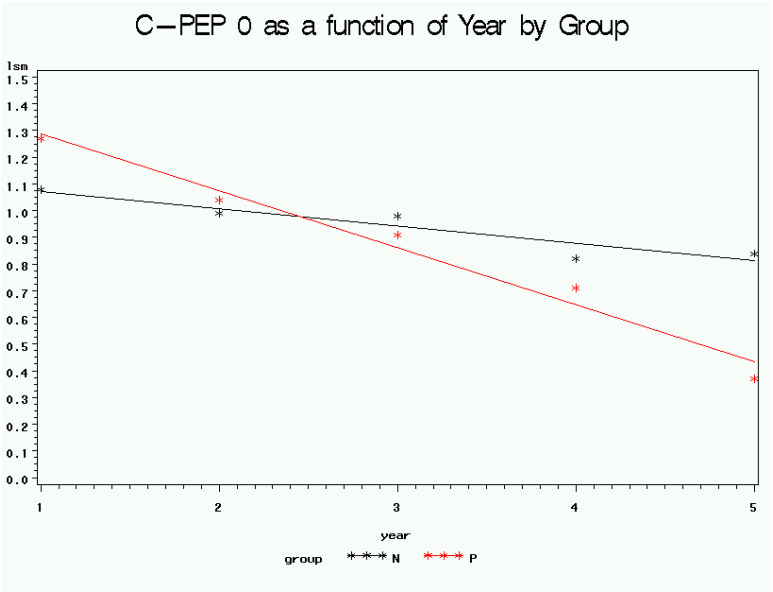


Analysis 3a: C-peptide 0, Random Intercepts and Random Slopes Model, N subjects = 30.

Effect	F	P
Group	0.83	0.3708
Year	4.67	0.0419*
LS Means		
	LS Mean	Std Error
Group N	0.85	0.092
Group P	0.96	0.1

- Group N and Group P do not differ significantly.
- **Significant slope for Year = -0.15. For every extra year scores decrease by 0.15.**
- No significant interaction between Group and Year

Year	Mean predicted value for C-PEP0 score	
	N	P
1	1.08	1.27
2	0.99	1.04
3	0.98	0.91
4	0.82	0.71
5	0.84	0.37

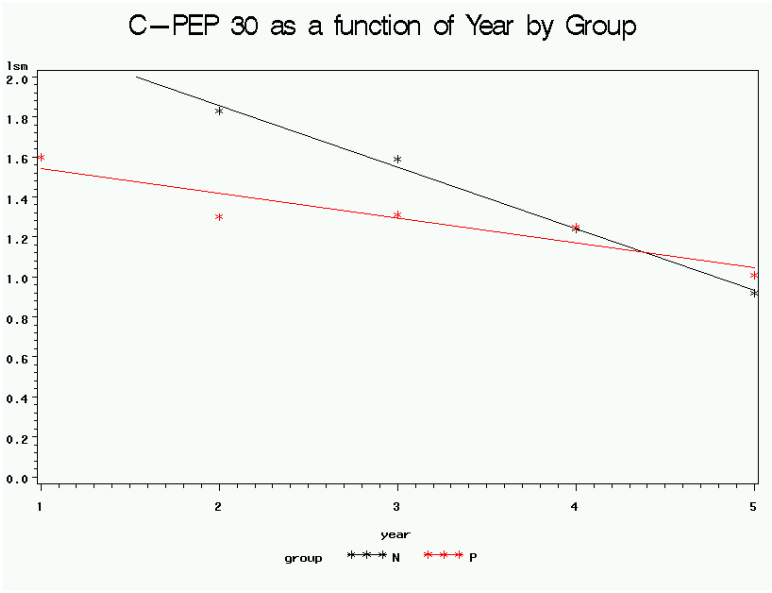


Analysis 3b: C-peptide 30, Random Intercepts only Model, N subjects = 30

Effect	F	P
Group	0.42	0.5220
Year	3.55	0.0664
LS Means		
	LS Mean	Std Error
Group N	1.62	0.28
Group P	1.32	0.36

- Group N and Group P do not differ significantly.
- Slope for Year = -0.21 (not significant).
- No significant interaction between Group and Year

Year	Mean predicted value for C-PEP 30 score	
	N	P
1	2.04	1.6
2	1.83	1.3
3	1.59	1.31
4	1.24	1.25
5	0.92	1.01

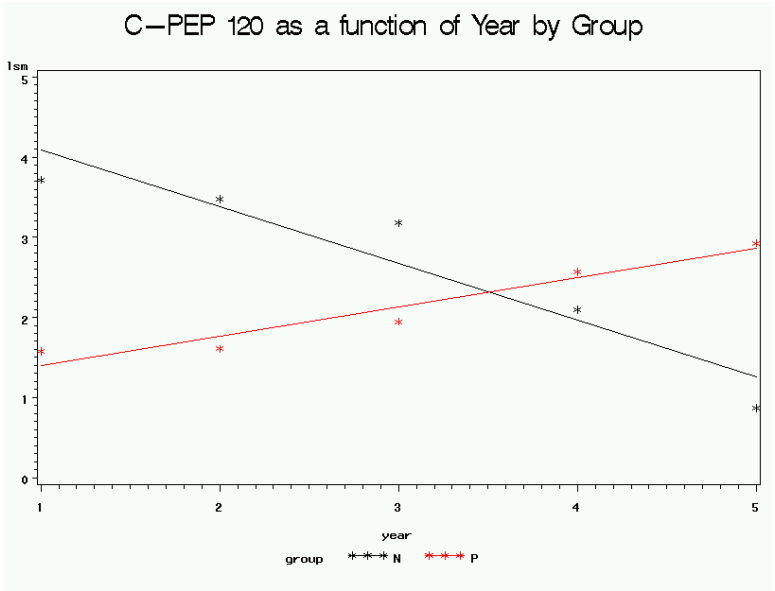


Analysis 3c: C-peptide, Random Intercepts only Model, N subjects = 30

Effect	F	P
Group	7.71	0.0081*
Year	0.02	0.8789
Group*Year	6.53	0.0142
LS Means		
	LS Mean	Std Error
Group N	2.94	0.49
Group P	1.82	0.63

- **Group P have a significantly higher C-peptide 120 score than Group N (p=0.0081)**
- The interaction between group and year is significant (0.0142). Group N rises whilst group P falls

Year	Mean predicted value For C-PEP 120 score	
	P	N
1	1.58	3.72
2	1.61	3.48
3	1.95	3.18
4	2.57	2.10
5	2.93	0.87

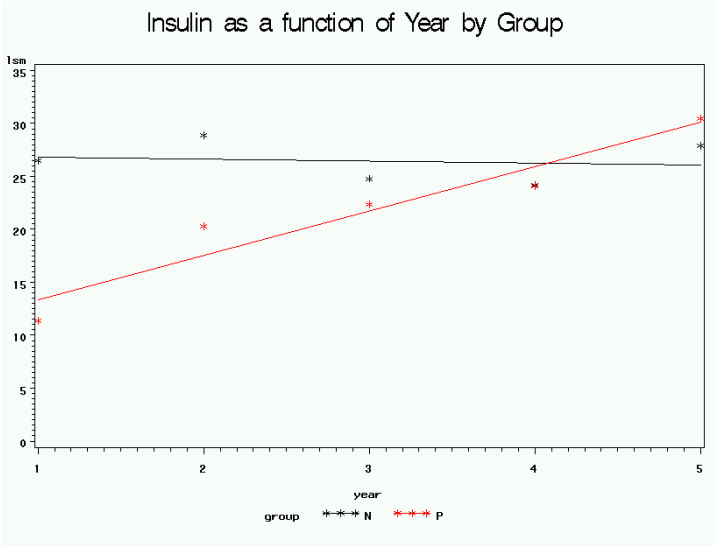


Analysis 4a: Insulin requirement, Random Intercepts only Model, N subjects = 31

Effect	F	P
Group	5.42	0.0229
Year	5.67	0.0201
Group*Year	8.6	0.0046*
	LS Mean	Std Error
Group N	25.6	4.1
Group P	20.3	5.4

- **Group P have a significantly higher C-peptide 120 score than Group N (p=0.0229)**
- The interaction between group and year is significant (0.0046). Group P rises whilst group N remains stable.

Year	Mean predicted value for Insulin score	
	N	P
1	26.5	11.4
2	28.9	20.3
3	24.8	22.4
4	24.2	24.1
5	27.9	30.5



This shows the pattern of results as a function of year to be different for the two groups. Group P shows a gradual linear increase, whereas group N is roughly flat until year 4 and then decreases.

Supplementary data relating to the culture of non-purified islets

Culture of non-purified islets, the experimental design and results reported by Weber and colleagues, 1977.

	Experimental design and observations Weber et al (206)
Pre-processing data	*Warm ischemia <10min in all cases*
Tissue digestion	*Minced pancreatic tissue was digested with collagenase (type IV at 1666U/ ml), trypsin (0.2mg/ml) and EDTA (0.2mg/ml) at 37ºC with agitation. Digested tissue was then passed through a pipette (40-60 repeats)*
Culture vessel	*Polystyrene, petri dish*
Culture Media	*M199 media & 10% FCS, Penicillin (400U/ml), D-glucose (11mM)*
Culture vessel and conditions	*Petri dish (Falcon), regulated air-Co2 (95-5%) incubator (37 C) humidification 98%*
Culture protocol	*Culture feed every 48hours, experiment terminated at 8 days*
Observations at day 4	*90% tissue non-viable, tissue adherent to petri dish, viable clumps with ovoid nuclei and pale cytoplasm characteristic of islet tissue.*

Table i The experimental design and observations of Weber et al 1977 (206)*, n=6.*

Total Units/48hr	**Control Media**	**0 to 2**	**2 to 4**	**4 to 6**	**6 to 8**
Insulin	0.2+01 (6)	334+/-128 (6)	183+/-114 (6)	124+/-83 (5)	67+/-31 (4)
Amylase	0 (6)	41,100+/-72,420 (6)	5100+/-2640 (6)	0 (2)	0 (6)

Table ii The results of experiments carried out by Weber et al 1977 (206)*. The content of insulin and amylase of media during tissue culture of human pancreas digestate. The number of replicates are in parenthesis.*

Experiments assessing the suitability of using islets procured from market weight pigs (6 months old) based islet response to a glucose challange

Experiment ID	Stimulation Index
MAW3	0.116
MAW4	0.022
MAW7	0.681
MAW8	1.061
MAW10	0.804
MAW11	0.659

Table iii Stimulation indices of non-purified porcine islets directly following pancreas digestion.

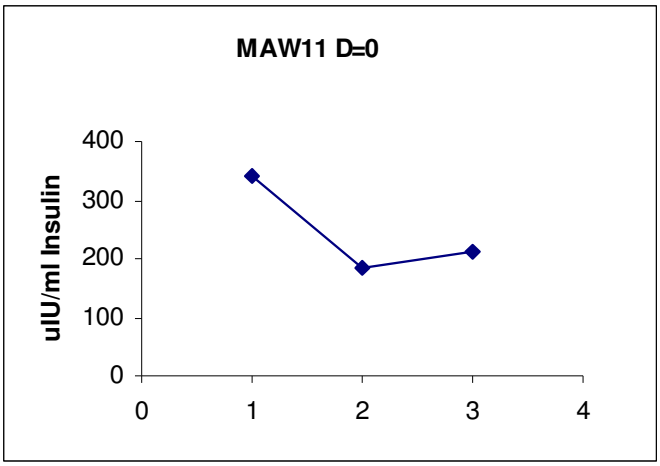


Figure i. The typical stimulation profile of porcine islets directly post digestion.

Raw data relating to stimulation indexes of rodent non-purified islets at Day 0 and Day 8

	Day 0		Day 8	
Experiment ID	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**
MAW26	1.84	1.00	1.00	1.00
MAW27	1.45	1.00	1.00	1.00
MAW28	1.20	1.42	2.01	1.00
MAW29	6.60	2.45	1.02	1.82
MAW31	1.07	1.68	3.67	3.90
MAW33	1.00	1.00	1.30	1.83
MAW34	1.00	5.55	1.00	1.56

Table iv Stimulation indices of non-purified at Day 0 and Day 8 of culture. The stimulation index was measured as the insulin released in response to high glucose (25mM) divided by insulin released in response to low glucose (1.8mM), Section 2.3.2. For each experiment 2 replicate samples of non-purified islets were tested, however, SI varied widely.

Schema and results of non-purified islets cultured with RPMI media (Table 4.1.2) or Memphis media.

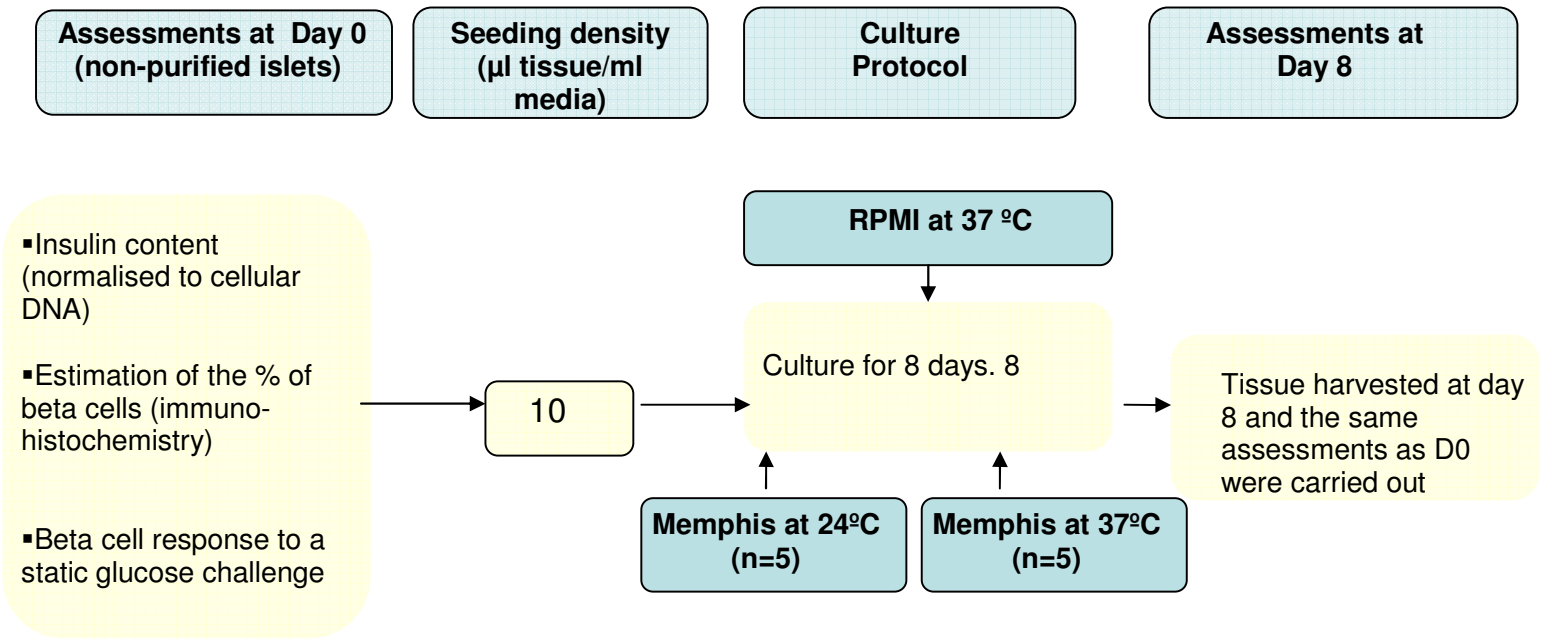


Figure ii. Schema of experiments carried out to compare the use of standard media with Memphis media. *Following the optimization of culture conditions for the successful culture of non-purified islets, attempts were made to further improve islet viability and function through the use of Memphis media, a serum free media specifically developed for islet culture. Manufactures recommend that Memphis media is used at 24ºC.*

	Day 0	RPMI media at 37º C	Memphis Media 24ºC	Memphis Media 37ºC
Insulin content ng/µg DNA	4.2 (1.15-18.7)	30.08 (5.65-152)	ND	ND
Insulin positive cells	1.5 (0.4-2.12)	7.56 (3.94-11.45)	ND	ND
Stimulation index to a glucose challenge	1.56(1-8.5)	1.78 (1-3.6)	ND	ND

Table v: The results of non-purified islets cultured in Standard RPMI media and Memphis media. Within days of culture non-purified islets cultured within Memphis media showed widespread tissue death. By day 8 of culture the tissue debris recovered contained no internal insulin or insulin positive cells, and did not secrete insulin in response to glucose.

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Apoptosis studies cell death immediately following pancreas digestion

Preliminary terminal deoxynucleotide transferase dUTP Nick End Labelling (TUNEL) staining of samples of non-purified islets at Day 0 was positive in 18.6% (5.4-24.9) of cells. However, this mode of staining was unable to between apoptosis, autolytic cell death or necrosis due to the fact that all 3 modes of death result in the DNA strand breaks which represent a target for Tdt-mediated dUTP labeling and this finding has already been discussed (184). However it is well accepted that cell death is largely autolytic or instigated by protease activity as such no further cell death studies were carried out on cultured tissue.

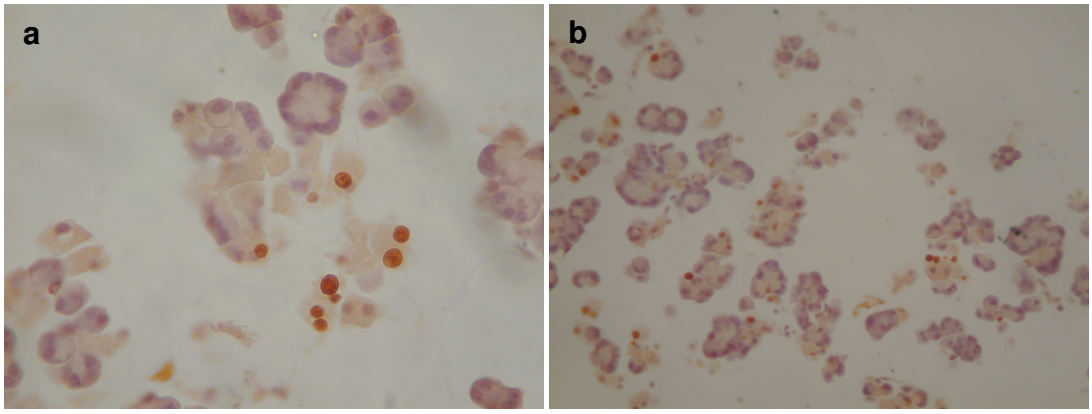

Figure iii Tunnel staining directly after pancreas digestion. 18.6% (5.4-24.9) of cells stained positive with tunnel staining although the mode of cell death could not be discriminated. Figures a & b photographed at magnifications of x 400 and 100 respectively.

Estimation of intracellular insulin per beta cell

To investigate whether culture had a detrimental affect on intracellular insulin stores, the insulin content was normalised against the percentage of insulin positive cells that had been characterised using immunohistochemistry, thus allowing an estimate of ng of intracellular insulin per µg of beta cell DNA.

a

	Non-purified islets D0			Non-purified islets D8		
Experiment ID	**% Insulin positive cells**	**Ng Insulin/µg DNA**	**Ng insulin/µg beta cell DNA**	**% Insulin positive cells**	**Ng Insulin/µg DNA**	**Ng insulin/µg beta cell DNA**
MAW26	0.766	5.9	770	8.691	16.25	187
MAW27	1.715	32	1866	5.63	167.3	2972
MAW28	0.97	7.18	740	4.02	8.67	216
MAW29	1.622	10.91	674	2.87	61.7	215
MAW31	0.615	22.65	3683	7.793	383	491
MAW109	0.4489	0.1	22	5.318	31.43	591

b

	Purified islets Day 0			Purified islets Day 8		
Experiment ID	**% Insulin positive cells**	**Ng Insulin/µg DNA**	**Ng insulin/µg beta cell DNA**	**% Insulin positive cells**	**Ng Insulin/µg DNA**	**Ng insulin/µg beta cell DNA**
MAW26	59.50	156.83	264	66.8	2.36	3.5
MAW27	62.30	166.30	267	68.4	20.72	30
MAW28	55.40	2083.03	3760	73.1	25.63	35
MAW29	54.80	578.12	1055	70.6	539.4	764
MAW31	65.10	53.38	82	75.4	24.88	33
MAW109	58.60	724.90	1237	68.9	711.73	1033

Tables vi a & b. Raw data relating to the calculation of insulin (ng) / µg of beta cell DNA. Method for the calculation of ng insulin/ µg of beta cell DNA can be found in Section 2.4.7

An assay of connexion 32 expression between endocrine and non-endocrine tissue following 8 days of culture

Following the culture of non-purified islets for 8 days, endocrine tissue and non-endocrine tissue frequently formed complexes. Therefore, experiments were carried out to explore whether connexin 32, an acinar-derived gap junction protein was involved in the attachment of these 2 tissue populations.

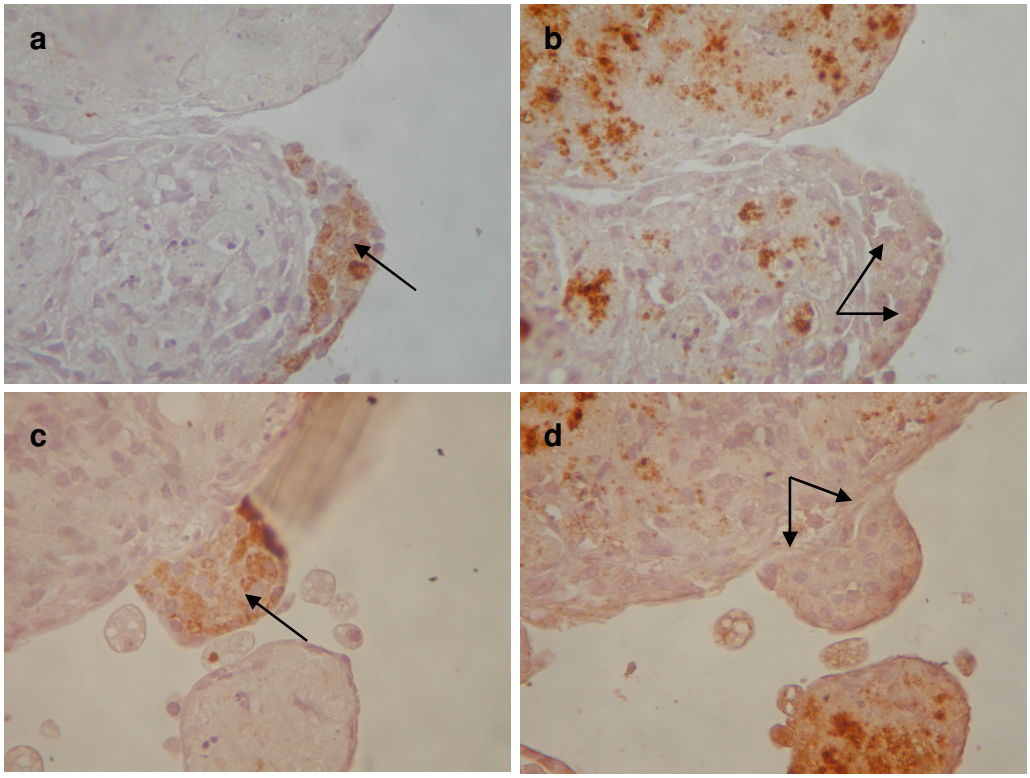

Figure iv. Assay of connexion 32 expression in the junction between islet and non-islet tissue. *Two examples of consecutive sections were stained for insulin (A&C) and Connexin 32 (B&D). In the areas where islet tissue and non-islet tissue meet were negative for the acinar derived factor Connexin 32 (gap junction protein) whilst adjoining non-islet tissue (previously shown to be chiefly ductal tissue and enzyme depleted acinar tissue, refer to section 4.2.2.7) were rich in Connexin 32 expression. Photographs taken under a magnification of X400*

Assay of central necrosis

Experiment ID	Attached islets	Free islets
26	100	50
27	100	53
28	*100*	*62.5*
29	*100*	*67*
31	*100*	*53*
109	*100*	*50*

Table vii, Levels of central necrosis within the non-purified islet culture. *Following 8 days of culture, islets attached to acinar tissue showed no central necrosis whilst, approximately half of free islets within the non-purified culture showed evidence of central necrosis (Section 2.4.3 for identification of central necrosis).*

Estimation of the proportions of alpha beta and delta cells in non-purified islets at Day 0 and Day 8

	Non-purified islets Day 0			Non-purified Day 8		
Experiment ID	**Alpha**	**Beta**	**Delta**	**Alpha**	**Beta**	**Delta**
MAW26	8.58 (5.689-14.625)	75.05 (45.80-82.41)	7.06 (3.78-36.79)	19.59 (6.54-37.037)	65.75 (54.22 - 78.27)	13.4 (3.70-15.49)
MAW27	12.07 (4.95-15.46)	70.48 (56.84-78.11)	9.62 (6.45-20.14)	10.97 (1.89-48.94)	69.34 (44.68-83.46)	12.19(1.55-24.7)
MAW28	7.85 (1.870-16.325)	74.22 (47.58-87.98)	8.85 (2.56-26.58)	16.82 (7.69-24.14)	75.25 (65.16-92.31)	0.60 (0-8.124)
MAW29	15.31 (6.254-42.016)	73.71 (68.54-87.56)	5.43 (0.50-9.00)	0.73 (0-12.46)	85.92 (60-100)	7.02 (0-40)
MAW31	12.53 (1.456-20.56)	78.93 (48.85-87.6)	10.39 (2.63-20.49)	6.78 (0-14.58)	76.57 (62.26-100)	5.55 (0-18.65)
MAW109	12.67 (2.74-20.45)	68.05 (36.45-87.60)	9.48 (0.55-14.33)	19.44 (0-35.46)	69.60 (58.48-92.50	1.252 (0-13.65)

Table viii Data showing the proportions of alpha, beta and delta cells in non-purified islets at Day 0 and Day 8.

An assay of VEGF expression in purified islets and non-purified islets following 8-days of culture

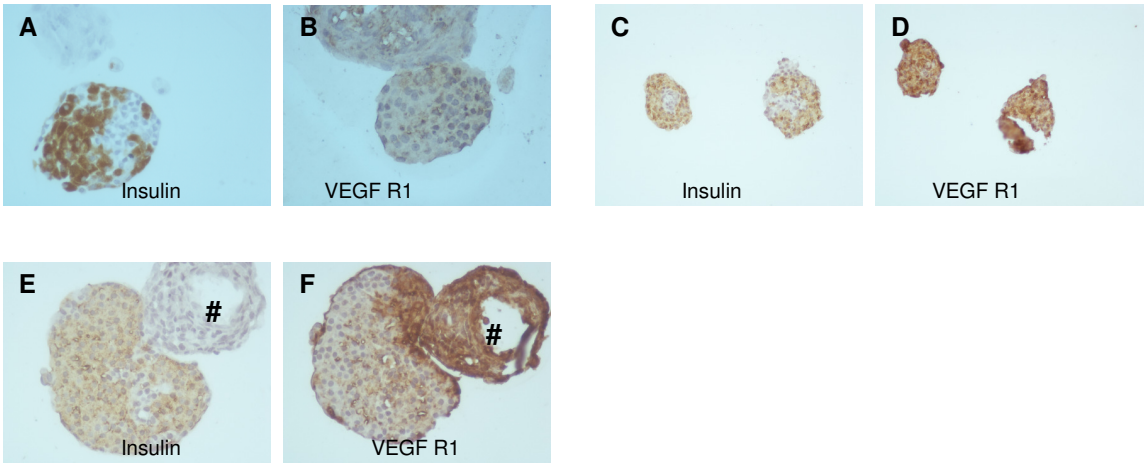

Figure v. Assay of VEGF R1 expression in non-purified and purified islets. *Non-purified islets attached to non-islet tissue (a&b) tended to express less VEGF R1 than purified islets (c&d) and free islets within non-purified islet preparations, however this finding did not reach significance (26.4 (1.5-68.9) vs. 48.7(1.2-71.6) vs. 35.2 (5.8-74.2), respectively, p=0.654). However it must be noted that VEGR R1 appeared to be a relatively crude assessment of central necrosis within this study. Mild central damage (as characterised by lack of hormone staining) did not consistently coincide with heightened levels of VEGF R1 (refer to Figures c&d). Whilst overtly damaged islets (islet* ***#*** *in Figure e&f shows no hormone staining and central tissue disintegration) exhibited intense VEGF R1 levels (a neighbouring healthy islets expressed relatively low VEGF R1 levels).*

Correlations tables comparing the prevalence of potential islet precursor cells (and islet IEQ) with islet graft outcomes between 6 months and 5 years post-transplant.

6 month		IEQ Transplanted	Insulin IC	PDX-1	CK-7	CK-19	Glucagon EIC	Glucagon +ve ducts
GTT0	R P N	-0.3091 0.2441 15	-0.2929 0.710 15	-0.556 0.013 15	-0.2634 0.3242 15	0.04415 0.8710 15	-0.4536 0.0776 15	-0.4829 0.0582 15
GTT30	R P N	-0.3250 0.2372 14	-0.3000 0.2773 14	-0.4393 0.1014 14	-0.475 0.074 14	-0.1143 0.6851 14	-0.486 0.078 14	-0.540 0.046 14
GTT120	R P N	-0.2412 0.3682 15	-0.1059 0.6963 15	-0.1676 0.5349 15	-0.3676 0.1612 15	0.04118 0.8797 15	-0.1442 0.5941 15	-0.2734 0.3506 15
HbA1c	R P N	-0.5469 0.123 17	0.008589 0.9739 17	-0.2138 0.4100 17	-0.004908 0.9851 17	0.08216 0.7539 17	0.04172 0.8737 17	-0.03859 0.8831 17
C-peptide 0	R P N	-0.3091 0.3560 9	-0.4182 0.2325 9	0.006061 >0.9999 9	-0.1394 0.7072 9	0.552 0.093 9	-0.2273 0.5034 9	0.01835 0.9573 9
C-peptide 30	R P N	-0.3217 0.3085 9	-0.3000 0.2773 9	-0.1818 0.5950 9	0.1091 0.7545 9	0.4091 0.2141 9	-0.02797 0.9312 9	0.09524 0.7684 9
C-peptide 120	R P N	0.0009091 0.9895 9	0.008398 0.9817 9	0.1515 0.6821 9	0.05455 0.8916 9	0.4667 0.1786 9	0.2727 0.4171 9	0.3357 0.3129 9
Stimulation Index 30 min	R P N	0.4273 0.1928 9	0.3000 0.3713 9	0.3636 0.2731 9	0.08182 0.8179 9	-0.1727 0.6147 9	0.648 0.043 9	0.3303 0.3132 9
Stimulation Index 120 min	R P N	0.5364 0.0939 9	0.3364 0.3132 9	0.5182 0.1072 9	0.1636 0.6337 9	0.04545 0.9033 9	0.633 0.067 9	0.729 0.026 9

Table i. Non-parametric 2-tailed correlations of antigen positivity versus patient outcomes at 6 months post-transplant.
Significant correlations highlighted.

1 year		IEQ Transplanted	Insulin IC	PDX-1	CK-7	CK-19	Glucagon EIC	Glucagon +ve ducts
GTT0	R P N	-0.2471 0.3746 15	0.05645 0.8416 15	-0.3611 0.1860 15	0.05909 0.8343 15	0.2650 0.3398 15	0.3004 0.2767 15	-0.09482 0.7368 15
GTT30	R P N	-0.1233 0.6615 15	-0.1047 0.7105 15	-0.3390 0.2164 15	-01412 0.6157 15	-0.07685 0.7854 15	0.1176 0.6764 15	-0.2949 0.2860 15
GTT120	R P N	-0.1321 0.6387 15	-0.1394 0.6202 15	-0.1769 0.5281 15	-0.2500 0.3688 15	-0.06786 0.8101 15	0.1901 0.4973 15	-0.02910 0.9180 15
HbA1c	R P N	-0.07858 0.7644 16	-0.3575 0.1589 16	-0.2138 0.4100 16	-0.559 0.025 16	-0.544 0.029 16	-0.2799 0.2766 16	-0.493 0.062 16
C-peptide 0	R P N	0.2821 0.3083 15	0.02860 0.9227 15	-0.2728 0.3453 14	0.04176 0.8873 14	0.635 0.015 14	0.07735 0.7927 14	0.1686 0.5645 14
C-peptide 30	R P N	0.5321 0.0412 15	-0.2354 0.4178 15	-0.4213 0.1237 15	0.1648 0.5733 15	0.512 0.061 15	0.01989 0.9462 15	0.3124 0.2768 15
C-peptide 120	R P N	0.3321 0.2265 15	-0.1474 0.6510 15	0.1515 0.6821 15	0.1868 0.5225 15	0.3931 0.1644 15	0.006409 0.8277 15	0.2225 0.4445 15
Stimulation Index 30 min	R P N	0.4357 0.1045 15	-0.2842 0.3046 15	0.1019 0.7179 15	0.04545 0.9033 15	-0.2071 0.4588 15	-0.2547 0.3596 15	0.09456 0.7375 15
stimulation index 120min	R P N	-0.351 0.1913 15	0.03217 0.9094 15	0.2002 0.4744 15	0.08571 0.7613 15	-0.3964 0.1435 15	-0.07713 0.7847 15	8.044E-19 >0.9999 15

Table ii. Non-parametric 2-tailed correlations of antigen positivity versus patient outcomes at 1 years post-transplant.
Significant correlations highlighted.

2 years		IEQ Transplanted	Insulin IC	PDX-1	CK-7	CK-19	Glucagon EIC	Glucagon +ve ducts
GTT0	R P N	0.02198 0.9432 12	-0.03026 0.9218 12	-0.650 0.022 12	-0.1816 0.5527 12	-0.0548 0.8588 12	-0.02762 0.9286 12	-0.2207 0.4686 12
GTT30	R P N	-0.02747 0.9290 12	0.09629 0.7543 12	-0.4380 0.1344 12	-0.0575 0.8520 12	-0.2584 0.3939 12	-0.1492 0.6267 12	-0.712 0.009 12
GTT120	R P N	-0.2473 0.4154 12	-0.1871 0.5406 12	-0.587 0.045 12	-0.557 0.06 12	-0.1736 0.5705 12	-0.2210 0.4681 12	-0.4472 0.1255 12
HbA1c	R P N	-0.2594 0.3505 14	-0.2596 0.501 14	-0.2016 0.4713 14	-0.4375 0.1030 14	-0.4158 0.1232 14	-0.1240 0.6598 14	-0.671 0.009 14
C -peptide 0	R P N	0.08333 0.8432 10	0.565 0.089 10	0.1944 0.6163 10	0.0743 0.8492 10	-0.4884 0.1822 10	0.1513 0.7081 10	0.1187 0.7756 10
C-peptide 30	R P N	-5.204E-19 >0.9999 10	0.553 0.097 10	0.4065 0.2776 10	0.3165 0.4066 10	-0.1282 0.7424 10	-0.08368 0.8432 10	0.1356 0.7435 10
C-peptide 120	R P N	0.1879 0.6073 10	0.1459 0.6821 10	0.0076 0.9834 10	0.5984 0.0676 10	-0.3201 0.3673 10	0.01829 0.9730 10	0.3689 0.2957 10
Stimulation Index 30 min	R P N	-0.1833 0.6436 9	0.1198 0.7930 9	0.04762 0.9349 9	0.2857 0.5008 9	0.3571 0.3894 9	-0.1317 0.7520 9	0.3593 0.3894 9
Stimulation Index 120 min	R P N	-0.1333 0.7435 9	-0.1925 0.6134 9	-0.01667 0.9816 9	0.2500 0.5206 9	0.600 0.0968 9	-0.1345 0.7435 9	0.3899 0.2912 9

Table iii. Non-parametric 2-tailed correlations of antigen positivity versus patient outcomes at 2 years post-transplant.
Significant correlations highlighted

3 years		IEQ Transplanted	Insulin IC	PDX-1	CK-7	CK-19	Glucagon EIC	Glucagon +ve ducts
GTT0	R P N	0.3091 0.3869 10	-0.4303 0.2182 10	-0.5648 0.0889 10	-0.2504 0.4853 10	-0.0475 0.8964 10	0.01220 0.9730 10	-0.3754 0.2788 10
GTT30	R P N	0.3091 0.3869 10	-0.2121 0.5603 10	-0.6168 0.0575 10	-0.2230 0.5358 10	-0.0258 0.9436 10	-0.1220 0.7730 10	-0.2339 0.5135 10
GTT120	R P N	0.3333 0.3487 10	-0.4303 0.2182 10	-0.5686 0.0863 10	-0.3463 0.3269 10	-0.1711 0.6365 10	0.07317 0.8382 10	-0.1477 0.6821 10
HbA1c	R P N	0.1961 0.5431 12	-0.4904 0.1055 12	-0.3590 0.2157 12	-0.571 0.084 12	0.5207 0.0826 12	-0.3796 0.2236 12	-0.5554 0.0628 12
C -peptide 0	R P N	0.4500 0.2298 9	0.0500 0.9116 9	-0.1267 0.7606 9	0.0852 0.8275 9	0.0437 0.9110 9	0.5967 0.0968 9	0.0509 0.9116 9
C-peptide 30	R P N	0.4833 0.1938 9	0.1833 0.6436 9	0.3201 0.3749 9	0.3728 0.3230 9	0.1329 0.7333 9	-0.5120 0.1475 9	-0.0339 0.9484 9
C-peptide 120	R P N	0.3167 0.4101 9	0.2333 0.5517 9	0.4860 0.1846 9	0.4090 0.2744 9	0.1139 0.7705 9	0.4958 0.1777 9	0.2543 0.4933 9
Stimulation Index 30 min	R P N	-0.1833 0.6436 9	0.6000 0.0968 9	0.3333 0.3853 9	0.4167 0.2696 9	0.2506 0.4596 9	-0.3025 0.4366 9	-0.3899 0.2912 9
Stimulation Index 120 min	R P N	-0.1333 0.7435 9	0.4500 0.2298 9	0.3000 0.4366 9	0.4000 0.2912 9	-0.1167 0.7756 9	-0.1765 0.6436 9	0.2204 0.5809 9

Table iv. Non-parametric 2-tailed correlations of antigen positivity versus patient outcomes at 3 years post-transplant.

4 years		IEQ Transplanted	Insulin IC	PDX-1	CK-7	CK-19	Glucagon EIC	Glucagon +ve ducts
GTT0	R P N	-0.1216 0.7730 10	0.1702 0.6321 10	-0.5978 0.0680 10	-0.1314 0.7175 10	-0.6293 0.0512 10	-0.009174 0.9730 10	-0.4369 0.2044 10
GTT30	R P N	0.3455 0.3304 10	-0.1818 0.9730 10	-0.4480 0.1524 10	0.01401	-0.0980 0.7876 10	0.1646 0.6567 10	0.04295 0.9184 10
GTT120	R P N	-0.3939 0.2632 10	0.4424 02044 10	-0.2654 0.4586 10	-0.3679 0.2956 10	-0.1946 0.5901 10	0.3110 0.3869 10	-0.2270 0.5367 10
HbA1c	R P N	0.004400 0.9881 13	0.1606 0.5833 13	0.0246 0.9336 13	-0.2537 0.3815 13	-0.492 0.087 13	-0.05298 0.8572 13	-0.3398 0.2346 13
C -peptide 0	R P N	-0.1667 0.7033 8	0.04762 0.9349 8	0.1492 0.7243 8	-0.6529 0.0792 8	0.0574 0.8927 8	0.2048 0.6191 8	0.1952 0.6646 8
C-peptide 30	R P N	0.02381 0.9768 8	-0.02381 0.9768 8	0.4724 0.2372 8	-0.4241 0.2950 8	0.1983 0.6379 8	-0.01205 0.9768 8	0.2928 0.4618 8
C-peptide 120	R P N	0.02381 0.9768 8	-0.09524 0.8401 8	0.4624 0.2468 8	-0.3620 0.3782 8	0.1625 0.7005 8	-0.2530 0.5364 8	0.2440 0.5821 8
Stimulation Index 30 min	R P N	-0.09524 0.8401 8	-0.4524 0.2675 8	0.3571 0.3894 8	-0.09524 0.8401 8		-0.6145 0.1150 8	-0.2440 0.5821 9
Stimulation Index 120 min	R P N	0.02381 0.9768 8	-0.5238 0.1966 8	0.3571 0.3894 8	-0.4286 0.2992 8	-0.09524 0.8401 8	-0.5302 0.1710 8	-0.1952 0.6646 8

Table v. Non-parametric 2-tailed correlations of antigen positivity versus patient outcomes at 4 years post-transplant.

5 years		IEQ Transplanted	Insulin IC	PDX-1	CK-7	CK-19	Glucagon EIC	Glucagon +ve ducts
GTT0	R P N	0.2571 0.6583 6	-0.2571 0.6583 6	-0.3861 0.4497 6	-0.0567 0.9151 6	-0.2130 0.6854 6	-0.02899 >0.9999 6	-0.2899 0.5639 6
GTT30	R P N	0.2571 0.6583 6	-02571 0.6583 6	-0.3620 0.4807 6	0.0852 0.8725 6	-0.1995 0.7048 6	-0.02899 >0.9999 6	-2899 0.5639 6
GTT120	R P N	-0.2571 0.6583 6	0.3143 0.5639 6	-0.0863 0.8708 6	-0.2039 0.6984 6	-0.3609 0.4822 6	0.2029 0.7139 6	0.1160 0.8028 6
HbA1c	R P N	-0.09524 0.8401 8	-0.1190 0.7930 8	0.0408 0.9236 8	-0.2320 0.5804 8	-0.0701 0.8690 8	-0.05988 0.8820 8	-0.1708 0.7033 8
C -peptide 0	R P N	0.4000 0.5167 5	1.735E-19 >0.9999 5	0.1465 0.8141 5	0.5464 0.3406 5	0.1689 0.7860 5	0.4617 0.4500 5	0.6156 0.3500 5
C-peptide 30	R P N	0.3000 0.6833 5	-0.1000 0.9500 5	0.1791 0.7731 5	0.3980 0.5070 5	0.3926 0.5133 5	0.2052 0.7833 5	0.3591 0.5167 5
C-peptide 120	R P N	0.1000 0.950 5	-0.3000 0.6833 5	0.0019 0.9975 5	0.2717 0.6583 5	0.2813 0.6466 5	0.05130 0.9500 5	0.3591 0.5167 5
Stimulation Index 30 min	R P N	0.1000 0.950 5	-0.3000 0.6833 5	-0.3000 0.6833 5	-0.6000 0.3500 5	0.1091 0.6786	0.05130 0.9500 5	0.3591 0.5167 5
Stimulation Index 120 min	R P N	0.1000 0.950 5	-0.5000 0.4500 5	-0.3000 0.6833 5	-0.1000 0.9500 -0.1000	0.5000 0.4500 5	-0.3078 0.6833 5	0.3044 0.5656 5

Table vi. Non-parametric 2-tailed correlations of antigen positivity versus patient outcomes at 5 years post-transplant.

Correlation table comparing patient demographic data and non-islet glucagon positive cells

Identifier	**Smoker**	**Age (yrs)**	**Gender**	**BMI**	**Days on ventilator**	**Cold ischaemia pancreas**	**Medical Conditions**	**Cause of Death**	***Glucagon positive NIECs***
HP696	Yes	67	F	28.58	3	7hr	Hypertension, previous breast cancer	Subarachnoid haemmorhage	*0.5*
HP698	Yes	59	M	ND	7	15hr	Unremarkable	Subarachnoid haemmorhage	*1.4*
HP699	No	54	F	ND	2	10hr20	Unremarkable	Intracranial bleed	*1.333333*
HP701	No	42	F	29.3	2	10hr20	Unremarkable	Intracranial bleed secondary to septacemia	*3.1*
HP702	Unknown	44	F	28.58	0	12hr	Unremarkable	Hypoxic brain injury	*1.7*
HP705	Unknown	17	M	23.63	0	12hr	Unremarkable	Hypoxic brain damage	*2.7*
HP706	*Ex*	*65*	*F*	*ND*	*12*	*6hr 50*	*TB, Hyperthroadism, giant cell vasculitis of lung*	*Stroke, respiratory failure*	*0.68*

Table vii. Patient demographic data vs. the prevalence of glucagon positive NIECs. *The above demographic data was compared with the prevalence of potential precursor cells (CK7, CK19, PDX-1, insulin NIECs, glucagon NIECs and glucagon positive cells associated with the duct). The prevalence of glucagon positive NIECs was negatively correlated with donor patient age ($p=0.0067$, $r=-0.9286$, $n=7$). Of the 8 normal pancreas samples available for analysis demographic data was available for 7 patients.*

Bibliography

(1) Roglic G, Unwin N, Bennett PH, Mathers C, Tuomilehto J, Nag S, et al. The burden of mortality attributable to diabetes: realistic estimates for the year 2000. Diabetes Care 2005 Sep;28(9):2130-2135.

(2) World Health Organisation. World Health Organisation Fact Sheet 312 November 2009 Diabetes. 2009 November 2009.

(3) Collaborative Islet Transplant Registry. Collaborative Islet Transplant Registry 6th Annual Report. 2009 November 1, 2009;6:1.

(4) Goldfine ID, Abraira C, Gruenewald D, Goldstein MS. Plasma insulin levels during imaginary food ingestion under hypnosis. Proc Soc Exp Biol Med 1970 Jan;133(1):274-276.

(5) Parra-Covarrubias A, Rivera-Rodriguez I, Almaraz-Ugalde A. Cephalic phase of insulin secretion in obese adolescents. Diabetes 1971 Dec;20(12):800-802.

(6) Thomson G, Robinson WP, Kuhner MK, Joe S, Klitz W. HLA and insulin gene associations with IDDM. Genet Epidemiol 1989;6(1):155-160.

(7) Richer MJ, Horwitz MS. Viral infections in the pathogenesis of autoimmune diseases: focus on type 1 diabetes. Front Biosci 2008 May 1;13:4241-4257.

(8) Lorenzi M, Cagliero E, Schmidt NJ. Racial differences in incidence of juvenile-onset type 1 diabetes: epidemiologic studies in southern California. Diabetologia 1985 Oct;28(10):734-738.

(9) Hemminki K, Li X, Sundquist J, Sundquist K. Familial association between type 1 diabetes and other autoimmune and related diseases. Diabetologia 2009 Sep;52(9):1820-1828.

(10) Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2001 Jul 21;358(9277):221-229.

(11) Norris JM. Infant and Childhood Diet and Type 1 Diabetes Risk: Recent Advances and Prospects. Curr Diab Rep 2010 Jul 20.

(12) Serreze DV, Ottendorfer EW, Ellis TM, Gauntt CJ, Atkinson MA. Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets. Diabetes 2000 May;49(5):708-711.

(13) American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care 2008 Jan;31 Suppl 1:S55-60.

(14) NEEL JV. Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? Am J Hum Genet 1962 Dec;14:353-362.

(15) Mitrakou A, Kelley D, Veneman T, Jenssen T, Pangburn T, Reilly J, et al. Contribution of abnormal muscle and liver glucose metabolism to postprandial hyperglycemia in NIDDM. Diabetes 1990 Nov;39(11):1381-1390.

(16) DeFronzo RA, Bonadonna RC, Ferrannini E. Pathogenesis of NIDDM. A balanced overview. Diabetes Care 1992 Mar;15(3):318-368.

(17) Kitzmiller JL, Buchanan TA, Kjos S, Combs CA, Ratner RE. Pre-conception care of diabetes, congenital malformations, and spontaneous abortions. Diabetes Care 1996 May;19(5):514-541.

(18) Girach A, Vignati L. Diabetic microvascular complications--can the presence of one predict the development of another? J Diabetes Complications 2006 Jul-Aug;20(4):228-237.

(19) Koya D, King GL. Protein kinase C activation and the development of diabetic complications. Diabetes 1998 Jun;47(6):859-866.

(20) Brownlee M. Advanced protein glycosylation in diabetes and aging. Annu Rev Med 1995;46:223-234.

(21) Lee AY, Chung SK, Chung SS. Demonstration that polyol accumulation is responsible for diabetic cataract by the use of transgenic mice expressing the aldose reductase gene in the lens. Proc Natl Acad Sci U S A 1995 Mar 28;92(7):2780-2784.

(22) Lemons JA, Vargas P, Delaney JJ. Infant of the diabetic mother: review of 225 cases. Obstet Gynecol 1981 Feb;57(2):187-192.

(23) Key TC, Giuffrida R, Moore TR. Predictive value of early pregnancy glycohemoglobin in the insulin-treated diabetic patient. Am J Obstet Gynecol 1987 May;156(5):1096-1100.

(24) National Diabetes Support Team. Diabetes in the NHS: commissioning and providing specialist services. 2007; . Accessed September.

(25) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993 Sep 30;329(14):977-986.

(26) Nathan DM, Cleary PA, Backlund JY, Genuth SM, Lachin JM, Orchard TJ, et al. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. N Engl J Med 2005 Dec 22;353(25):2643-2653.

(27) Cryer PE. The barrier of hypoglycemia in diabetes. Diabetes 2008 Dec;57(12):3169-3176.

(28) Workgroup on Hypoglycemia, American Diabetes Association. Defining and reporting hypoglycemia in diabetes: a report from the American Diabetes Association Workgroup on Hypoglycemia. Diabetes Care 2005 May;28(5):1245-1249.

(29) Mohamadi A, Cooke DW. Type 2 diabetes mellitus in children and adolescents. Adolesc Med State Art Rev 2010 Apr;21(1):103-19, x.

(30) Ekberg K, Brismar T, Johansson BL, Jonsson B, Lindstrom P, Wahren J. Amelioration of sensory nerve dysfunction by C-Peptide in patients with type 1 diabetes. Diabetes 2003 Feb;52(2):536-541.

(31) Wahren J, Ekberg K, Johansson J, Henriksson M, Pramanik A, Johansson BL, et al. Role of C-peptide in human physiology. Am J Physiol Endocrinol Metab 2000 May;278(5):E759-68.

(32) Forst T, Kunt T. Effects of C-peptide on microvascular blood flow and blood hemorheology. Exp Diabesity Res 2004 Jan-Mar;5(1):51-64.

(33) Bertelli E, Bendayan M. Association between endocrine pancreas and ductal system. More than an epiphenomenon of endocrine differentiation and development? J Histochem Cytochem 2005 Sep;53(9):1071-1086.

(34) Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000 Jul 27;343(4):230-238.

(35) Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K. Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. Diabetes 1989 Jan;38 Suppl 1:199-201.

(36) Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW. Automated method for isolation of human pancreatic islets. Diabetes 1988 Apr;37(4):413-420.

(37) Gruessner AC, Sutherland DE. Pancreas transplant outcomes for United States (US) and non-US cases as reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR) as of June 2004. Clin Transplant 2005 Aug;19(4):433-455.

(38) Zeng Y, Ricordi C, Lendoire J, Carroll PB, Alejandro R, Bereiter DR, et al. The effect of prednisone on pancreatic islet autografts in dogs. Surgery 1993 Jan;113(1):98-102.

(39) Egidi FM. Management of hyperglycaemia after pancreas transplantation: are new immunosuppressants the answer? Drugs 2005;65(2):153-166.

(40) Venstrom JM, McBride MA, Rother KI, Hirshberg B, Orchard TJ, Harlan DM. Survival after pancreas transplantation in patients with diabetes and preserved kidney function. JAMA 2003 Dec 3;290(21):2817-2823.

(41) Soria B. In-vitro differentiation of pancreatic beta-cells. Differentiation 2001 Oct;68(4-5):205-219.

(42) Baeyens L, De Breuck S, Lardon J, Mfopou JK, Rooman I, Bouwens L. In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells. Diabetologia 2005 Jan;48(1):49-57.

(43) Gmyr V, Kerr-Conte J, Vandewalle B, Proye C, Lefebvre J, Pattou F. Human pancreatic ductal cells: large-scale isolation and expansion. Cell Transplant 2001 Jan-Feb;10(1):109-121.

(44) Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz K, Song KH, Sharma A, et al. In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci U S A 2000 Jul 5;97(14):7999-8004.

(45) Li WC, Horb ME, Tosh D, Slack JM. In vitro transdifferentiation of hepatoma cells into functional pancreatic cells. Mech Dev 2005 Jun;122(6):835-847.

(46) Rossi JM, Dunn NR, Hogan BL, Zaret KS. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev 2001 Aug 1;15(15):1998-2009.

(47) Jung J, Zheng M, Goldfarb M, Zaret KS. Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science 1999 Jun 18;284(5422):1998-2003.

(48) Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am J Pathol 2005 Jun;166(6):1781-1791.

(49) Lindeborg E, Kumagai-Braesch M, Tibell A, Christensson B, Moller E. Biological activity of pig islet-cell reactive IgG antibodies in xenotransplanted diabetic patients. Xenotransplantation 2004 Sep;11(5):457-470.

(50) Clemenceau B, Jegou D, Martignat L, Sai P. Microchimerism and transmission of porcine endogenous retrovirus from a pig cell line or specific pathogen-free pig islets to mouse tissues and human cells during xenografts in nude mice. Diabetologia 2002 Jun;45(6):914-923.

(51) Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, et al. Five-year follow-up after clinical islet transplantation. Diabetes 2005 Jul;54(7):2060-2069.

(52) Brandhorst H, Brandhorst D, Brendel MD, Hering BJ, Bretzel RG. Assessment of intracellular insulin content during all steps of human islet isolation procedure. Cell Transplant 1998 Sep-Oct;7(5):489-495.

(53) Balamurugan AN, He J, Guo F, Stolz DB, Bertera S, Geng X, et al. Harmful delayed effects of exogenous isolation enzymes on isolated human islets: relevance to clinical transplantation. Am J Transplant 2005 Nov;5(11):2671-2681.

(54) Hughes SJ, Clark A, McShane P, Contractor HH, Gray DW, Johnson PR. Characterisation of collagen VI within the islet-exocrine interface of the human pancreas: implications for clinical islet isolation? Transplantation 2006 Feb 15;81(3):423-426.

(55) Matsumoto S, Noguichi H, Shimoda M, Ikemoto T, Naziruddin B, Jackson A, et al. Seven consecutive successful clinical islet isolations with pancreatic ductal injection. Cell Transplant 2010;19(3):291-297.

(56) Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006 Sep 28;355(13):1318-1330.

(57) Ichii H, Pileggi A, Molano RD, Baidal DA, Khan A, Kuroda Y, et al. Rescue purification maximizes the use of human islet preparations for transplantation. Am J Transplant 2005 Jan;5(1):21-30.

(58) Gores PF, Sutherland DE. Pancreatic islet transplantation: is purification necessary? Am J Surg 1993 Nov;166(5):538-542.

(59) Lloveras J, Farney AC, Sutherland DE, Wahoff D, Field J, Gores PF. Significance of contaminated islet preparations in clinical islet transplantation. Transplant Proc 1994 Apr;26(2):579-580.

(60) Johansson H, Lukinius A, Moberg L, Lundgren T, Berne C, Foss A, et al. Tissue factor produced by the endocrine cells of the islets of Langerhans is associated with a negative outcome of clinical islet transplantation. Diabetes 2005 Jun;54(6):1755-1762.

(61) Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. Diabetes 2002 Jun;51(6):1779-1784.

(62) Carlsson PO, Palm F, Mattsson G. Low revascularization of experimentally transplanted human pancreatic islets. J Clin Endocrinol Metab 2002 Dec;87(12):5418-5423.

(63) Mattsson G, Jansson L, Carlsson PO. Decreased vascular density in mouse pancreatic islets after transplantation. Diabetes 2002 May;51(5):1362-1366.

(64) Zhang N, Richter A, Suriawinata J, Harbaran S, Altomonte J, Cong L, et al. Elevated vascular endothelial growth factor production in islets improves islet graft vascularization. Diabetes 2004 Apr;53(4):963-970.

(65) Ryan EA, Paty BW, Senior PA, Shapiro AM. Risks and side effects of islet transplantation. Curr Diab Rep 2004 Aug;4(4):304-309.

(66) Zhang N, Su D, Qu S, Tse T, Bottino R, Balamurugan AN, et al. Sirolimus is associated with reduced islet engraftment and impaired beta-cell function. Diabetes 2006 Sep;55(9):2429-2436.

(67) Chatenoud L. Chemical immunosuppression in islet transplantation--friend or foe? N Engl J Med 2008 Mar 13;358(11):1192-1193.

(68) Vincenti F. The role of newer monoclonal antibodies in renal transplantation. Transplant Proc 2001 Feb-Mar;33(1-2):1000-1001.

(69) Mineo D, Ricordi C, Xu X, Pileggi A, Garcia-Morales R, Khan A, et al. Combined islet and hematopoietic stem cell allotransplantation: a clinical pilot trial to induce chimerism and graft tolerance. Am J Transplant 2008 Jun;8(6):1262-1274.

(70) Monti P, Scirpoli M, Maffi P, Ghidoli N, De Taddeo F, Bertuzzi F, et al. Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells. J Clin Invest 2008 May;118(5):1806-1814.

(71) Froud T, Yrizarry JM, Alejandro R, Ricordi C. Use of D-STAT to prevent bleeding following percutaneous transhepatic intraportal islet transplantation. Cell Transplant 2004;13(1):55-59.

(72) Gray DW. The role of exocrine tissue in pancreatic islet transplantation. Transpl Int 1989 Apr;2(1):41-45.

(73) Kin T, Mirbolooki M, Salehi P, Tsukada M, O'Gorman D, Imes S, et al. Islet isolation and transplantation outcomes of pancreas preserved with University of Wisconsin solution versus two-layer method using preoxygenated perfluorocarbon. Transplantation 2006 Nov 27;82(10):1286-1290.

(74) Collaborative Islet Transplant Registry. Collaborative Islet Transplant Registry Fifth Annual Report. 2008 September 15th 2008;5.

(75) Webb MA, Illouz SC, Pollard CA, Gregory R, Mayberry JF, Tordoff SG, et al. Islet auto transplantation following total pancreatectomy: a long-term assessment of graft function. Pancreas 2008 Oct;37(3):282-287.

(76) Robertson RP, Lanz KJ, Sutherland DE, Kendall DM. Prevention of diabetes for up to 13 years by autoislet transplantation after pancreatectomy for chronic pancreatitis. Diabetes 2001 Jan;50(1):47-50.

(77) Sutherland DE, Gruessner AC, Carlson AM, Blondet JJ, Balamurugan AN, Reigstad KF, et al. Islet autotransplant outcomes after total pancreatectomy: a contrast to islet allograft outcomes. Transplantation 2008 Dec 27;86(12):1799-1802.

(78) Levy P, Barthet M, Mollard BR, Amouretti M, Marion-Audibert AM, Dyard F. Estimation of the prevalence and incidence of chronic pancreatitis and its complications. Gastroenterol Clin Biol 2006 Jun-Jul;30(6-7):838-844.

(79) Warshaw AL, Banks PA, Fernandez-Del Castillo C. AGA technical review: treatment of pain in chronic pancreatitis. Gastroenterology 1998 Sep;115(3):765-776.

(80) Layer P, Yamamoto H, Kalthoff L, Clain JE, Bakken LJ, DiMagno EP. The different courses of early- and late-onset idiopathic and alcoholic chronic pancreatitis. Gastroenterology 1994 Nov;107(5):1481-1487.

(81) Steer ML, Waxman I, Freedman S. Chronic pancreatitis. N Engl J Med 1995 Jun 1;332(22):1482-1490.

(82) Wang LW, Li ZS, Li SD, Jin ZD, Zou DW, Chen F. Prevalence and clinical features of chronic pancreatitis in China: a retrospective multicenter analysis over 10 years. Pancreas 2009 Apr;38(3):248-254.

(83) Howard TJ, Jones JW, Sherman S, Fogel E, Lehman GA. Impact of pancreatic head resection on direct medical costs in patients with chronic pancreatitis. Ann Surg 2001 Nov;234(5):661-667.

(84) Kloppel G. Chronic Pancreatitis: The initial damage and resulting fibrosis patterns. In: Pour PM, editor. Toxicology of the Pancreas: Taylor & Francis Group; 2006. p. 329.

(85) Ahmed SA, Wray C, Rilo HL, Choe KA, Gelrud A, Howington JA, et al. Chronic pancreatitis: recent advances and ongoing challenges. Curr Probl Surg 2006 Mar;43(3):127-238.

(86) Evans JD, Wilson PG, Carver C, Bramhall SR, Buckels JA, Mayer AD, et al. Outcome of surgery for chronic pancreatitis. Br J Surg 1997 May;84(5):624-629.

(87) Alexakis N, Ghaneh P, Connor S, Raraty M, Sutton R, Neoptolemos JP. Duodenum- and spleen-preserving total pancreatectomy for end-stage chronic pancreatitis. Br J Surg 2003 Nov;90(11):1401-1408.

(88) Garcea G, Weaver J, Phillips J, Pollard CA, Ilouz SC, Webb MA, et al. Total Pancreatectomy With and Without Islet Cell Transplantation for Chronic Pancreatitis: A Series of 85 Consecutive Patients. Pancreas 2008 Jul 24.

(89) Linehan IP, Lambert MA, Brown DC, Kurtz AB, Cotton PB, Russell RC. Total pancreatectomy for chronic pancreatitis. Gut 1988 Mar;29(3):358-365.

(90) Ammann RW, Heitz PU, Kloppel G. Course of alcoholic chronic pancreatitis: a prospective clinicomorphological long-term study. Gastroenterology 1996 Jul;111(1):224-231.

(91) Brunicardi FC, Chaiken RL, Ryan AS, Seymour NE, Hoffmann JA, Lebovitz HE, et al. Pancreatic polypeptide administration improves abnormal glucose metabolism in patients with chronic pancreatitis. J Clin Endocrinol Metab 1996 Oct;81(10):3566-3572.

(92) Schrader H, Menge BA, Schneider S, Belyaev O, Tannapfel A, Uhl W, et al. Reduced pancreatic volume and beta-cell area in patients with chronic pancreatitis. Gastroenterology 2009 Feb;136(2):513-522.

(93) Kobayashi T, Manivel JC, Bellin MD, Carlson AM, Moran A, Freeman ML, et al. Correlation of pancreatic histopathologic findings and islet yield in children with chronic pancreatitis undergoing total pancreatectomy and islet autotransplantation. Pancreas 2010 Jan;39(1):57-63.

(94) Takita M, Naziruddin B, Matsumoto S, Noguchi H, Shimoda M, Chujo D, et al. Implication of Pancreatic Image Findings in Total Pancreatectomy With Islet Autotransplantation for Chronic Pancreatitis. Pancreas 2010 Sep 28.

(95) Robertson GS, Chadwick DR, Contractor H, James RF, Bell PR, London NJ. The use of continuous density gradients for the assessment of islet and exocrine tissue densities and islet purification. Acta Diabetol 1993;30(3):175-180.

(96) Sever CE, Demetris AJ, Zeng J, Carroll P, Tzakis A, Fung JJ, et al. Composition of human islet cell preparations for transplantation. Acta Diabetol 1992;28(3-4):233-238.

(97) Gray DW, Sutton R, McShane P, Peters M, Morris PJ. Exocrine contamination impairs implantation of pancreatic islets transplanted beneath the kidney capsule. J Surg Res 1988 Nov;45(5):432-442.

(98) Blondet JJ, Carlson AM, Kobayashi T, Jie T, Bellin M, Hering BJ, et al. The role of total pancreatectomy and islet autotransplantation for chronic pancreatitis. Surg Clin North Am 2007 Dec;87(6):1477-501, x.

(99) Lucas-Clerc C, Massart C, Campion JP, Launois B, Nicol M. Long-term culture of human pancreatic islets in an extracellular matrix: morphological and metabolic effects. Mol Cell Endocrinol 1993 Jul;94(1):9-20.

(100) Pyzdrowski KL, Kendall DM, Halter JB, Nakhleh RE, Sutherland DE, Robertson RP. Preserved insulin secretion and insulin independence in recipients of islet autografts. N Engl J Med 1992 Jul 23;327(4):220-226.

(101) Wahoff DC, Sutherland DE, Hower CD, Lloveras JK, Gores PF. Free intraperitoneal islet autografts in pancreatectomized dogs--impact of islet purity and posttransplantation exogenous insulin. Surgery 1994 Oct;116(4):742-8; discussion 748-50.

(102) Kaufman DB, Morel P, Field MJ, Munn SR, Sutherland DE. Purified canine islet autografts. Functional outcome as influenced by islet number and implantation site. Transplantation 1990 Sep;50(3):385-391.

(103) Brendel MD, Hering BJ, Schulz A, Bretzel RG. Internation Islet Transplant Registry report. Giessen 1999. 1999:1.

(104) Wahoff DC, Nelson LA, Papalois BE, Curtis DA, Gores PF, Sutherland DE. Decreased chronic failure of intraperitoneal impure islets versus intrahepatic pure islets in a canine autograft model. Transplant Proc 1995 Dec;27(6):3186.

(105) Beuneu C, Vosters O, Movahedi B, Remmelink M, Salmon I, Pipeleers D, et al. Human pancreatic duct cells exert tissue factor-dependent procoagulant activity: relevance to islet transplantation. Diabetes 2004 Jun;53(6):1407-1411.

(106) Bertelli E, Regoli M, Bastianini A. Endocrine tissue associated with the pancreatic ductal system: a light and electron microscopic study of the adult rat pancreas with special reference to a new endocrine arrangement. Anat Rec 1994 Aug;239(4):371-378.

(107) Bouwens L, Pipeleers DG. Extra-insular beta cells associated with ductules are frequent in adult human pancreas. Diabetologia 1998 Jun;41(6):629-633.

(108) Wang RN, Kloppel G, Bouwens L. Duct- to islet-cell differentiation and islet growth in the pancreas of duct-ligated adult rats. Diabetologia 1995 Dec;38(12):1405-1411.

(109) Zhou J, Pineyro MA, Wang X, Doyle ME, Egan JM. Exendin-4 differentiation of a human pancreatic duct cell line into endocrine cells: involvement of PDX-1 and HNF3beta transcription factors. J Cell Physiol 2002 Sep;192(3):304-314.

(110) Ilieva A, Yuan S, Wang RN, Agapitos D, Hill DJ, Rosenberg L. Pancreatic islet cell survival following islet isolation: the role of cellular interactions in the pancreas. J Endocrinol 1999 Jun;161(3):357-364.

(111) Murray HE, Paget MB, Bailey CJ, Downing R. Sustained insulin secretory response in human islets co-cultured with pancreatic duct-derived epithelial cells within a rotational cell culture system. Diabetologia 2009 Mar;52(3):477-485.

(112) Street CN, Lakey JR, Shapiro AM, Imes S, Rajotte RV, Ryan EA, et al. Islet graft assessment in the Edmonton Protocol: implications for predicting long-term clinical outcome. Diabetes 2004 Dec;53(12):3107-3114.

(113) Hayashi KY, Tamaki H, Handa K, Takahashi T, Kakita A, Yamashina S. Differentiation and proliferation of endocrine cells in the regenerating rat pancreas after 90% pancreatectomy. Arch Histol Cytol 2003 May;66(2):163-174.

(114) De Haro-Hernandez R, Cabrera-Munoz L, Mendez JD. Regeneration of beta-cells and neogenesis from small ducts or acinar cells promote recovery of endocrine pancreatic function in alloxan-treated rats. Arch Med Res 2004 Mar-Apr;35(2):114-120.

(115) Liu T, Wang CY, Gou SM, Wu HS, Xiong JX, Zhou J. PDX-1 expression and proliferation of duct epithelial cells after partial pancreatectomy in rats. Hepatobiliary Pancreat Dis Int 2007 Aug;6(4):424-429.

(116) Movahedi B, Gysemans C, Jacobs-Tulleneers-Thevissen D, Mathieu C, Pipeleers D. Pancreatic duct cells in human islet cell preparations are a source of angiogenic cytokines interleukin-8 and vascular endothelial growth factor. Diabetes 2008 Aug;57(8):2128-2136.

(117) Wu T, Levay-Young B, Heuss N, Sozen H, Kirchhof N, Sutherland DE, et al. Inducing tolerance to MHC-matched allogeneic islet grafts in diabetic NOD mice by simultaneous islet and bone marrow transplantation under nonirradiative and nonmyeloablative conditioning therapy. Transplantation 2002 Jul 15;74(1):22-27.

(118) Gotoh M, Maki T, Satomi S, Porter J, Monaco AP. Immunological characteristics of purified pancreatic islet grafts. Transplantation 1986 Oct;42(4):387-390.

(119) Bartlett ST, Chin T, Dirden B, Quereshi A, Hadley G. Inclusion of peripancreatic lymph node cells prevents recurrent autoimmune destruction of islet transplants: evidence of donor chimerism. Surgery 1995 Aug;118(2):392-7; discussion 397-8.

(120) Johansson U, Elgue G, Nilsson B, Korsgren O. Composite islet-endothelial cell grafts: a novel approach to counteract innate immunity in islet transplantation. Am J Transplant 2005 Nov;5(11):2632-2639.

(121) Fandrich F, Lin X, Chai GX, Schulze M, Ganten D, Bader M, et al. Preimplantation-stage stem cells induce long-term allogeneic graft acceptance without supplementary host conditioning. Nat Med 2002 Feb;8(2):171-178.

(122) Pearson KW, Scott D, Torrance B. Effects of partial surgical pancreatectomy in rats. I. Pancreatic regeneration. Gastroenterology 1977 Mar;72(3):469-473.

(123) Kodama S, Kuhtreiber W, Fujimura S, Dale EA, Faustman DL. Islet regeneration during the reversal of autoimmune diabetes in NOD mice. Science 2003 Nov 14;302(5648):1223-1227.

(124) el-Naggar MM, Elayat AA, Ardawi MS, Tahir M. Isolated pancreatic islets of the rat: an immunohistochemical and morphometric study. Anat Rec 1993 Dec;237(4):489-497.

(125) Wang RN, Rosenberg L. Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. J Endocrinol 1999 Nov;163(2):181-190.

(126) Paraskevas S, Maysinger D, Wang R, Duguid TP, Rosenberg L. Cell loss in isolated human islets occurs by apoptosis. Pancreas 2000 Apr;20(3):270-276.

(127) Rosenberg L, Wang R, Paraskevas S, Maysinger D. Structural and functional changes resulting from islet isolation lead to islet cell death. Surgery 1999 Aug;126(2):393-398.

(128) Toso C, Oberholzer J, Ris F, Triponez F, Bucher P, Demirag A, et al. Factors affecting human islet of Langerhans isolation yields. Transplant Proc 2002 May;34(3):826-827.

(129) Fernandez LA, Turgeon NA, Odorico JS, Leverson G, Pirsch JD, Becker BN, et al. Superior long-term results of simultaneous pancreas-kidney transplantation from pediatric donors. Am J Transplant 2004 Dec;4(12):2093-2101.

(130) Balamurugan AN, Chang Y, Bertera S, Sands A, Shankar V, Trucco M, et al. Suitability of human juvenile pancreatic islets for clinical use. Diabetologia 2006 Aug;49(8):1845-1854.

(131) Ihm SH, Moon HJ, Kang JG, Park CY, Oh KW, Jeong IK, et al. Effect of aging on insulin secretory function and expression of beta cell function-related genes of islets. Diabetes Res Clin Pract 2007 Sep;77 Suppl 1:S150-4.

(132) Krishnamurthy J, Ramsey MR, Ligon KL, Torrice C, Koh A, Bonner-Weir S, et al. p16INK4a induces an age-dependent decline in islet regenerative potential. Nature 2006 Sep 28;443(7110):453-457.

(133) Maedler K, Schumann DM, Schulthess F, Oberholzer J, Bosco D, Berney T, et al. Aging correlates with decreased beta-cell proliferative capacity and enhanced sensitivity to apoptosis: a potential role for Fas and pancreatic duodenal homeobox-1. Diabetes 2006 Sep;55(9):2455-2462.

(134) Matsumoto S, Tanaka K, Strong DM, Reems JA. Efficacy of human islet isolation from the tail section of the pancreas for the possibility of living donor islet transplantation. Transplantation 2004 Sep 27;78(6):839-843.

(135) Bluth M, Mueller CM, Pierre J, Callender G, Kandil E, Viterbo D, et al. Pancreatic regenerating protein I in chronic pancreatitis and aging: implications for new therapeutic approaches to diabetes. Pancreas 2008 Nov;37(4):386-395.

(136) Downing R, Morrissey S, Kiske D, Scharp DW. Does the purity of intraportal islet isografts affect their endocrine function? J Surg Res 1986 Jul;41(1):41-46.

(137) Hegre OD, Ketchum RJ, Popiela H, Eide CR, Meloche RM, Serie JR, et al. Allotransplantation of culture-isolated neonatal rat islet tissue. Absence of MHC class II positive antigen-presenting cells in nonimmunogenic islets. Diabetes 1989 Feb;38(2):146-151.

(138) Heuser M, Wolf B, Vollmar B, Menger MD. Exocrine contamination of isolated islets of Langerhans deteriorates the process of revascularization after free transplantation. Transplantation 2000 Mar 15;69(5):756-761.

(139) Beuneu C, Vosters O, Ling Z, Pipeleers D, Pradier O, Goldman M, et al. N-Acetylcysteine derivative inhibits procoagulant activity of human islet cells. Diabetologia 2007 Feb;50(2):343-347.

(140) Pavlovic D, Chen MC, Bouwens L, Eizirik DL, Pipeleers D. Contribution of ductal cells to cytokine responses by human pancreatic islets. Diabetes 1999 Jan;48(1):29-33.

(141) Sever CE, Demetris AJ, Zeng Y, Carroll P, Tzakis A, Starzl TE, et al. Cellular composition of islet cell suspensions for transplantation. Transplant Proc 1992 Jun;24(3):979.

(142) Froberg MK, Leone JP, Jessurun J, Sutherland DE. Fatal disseminated intravascular coagulation after autologous islet transplantation. Hum Pathol 1997 Nov;28(11):1295-1298.

(143) White SA, Robertson GS, Davies JE, Rees Y, London NJ, Dennison AR. Splenic infarction after total pancreatectomy and autologous islet transplantation into the spleen. Pancreas 1999 May;18(4):419-421.

(144) Morsiani E, Rozga J, Dellagiacoma G, Demetriou AA. Repeated intraportal injections of subtherapeutic islet cell isografts restore normoglycemia in streptozotocin-diabetic rats. Cell Transplant 1997 Jan-Feb;6(1):17-22.

(145) Lack EE. Embryology, Anatomy and Function of the Exocrine and Endocrine Pancreas. Pathology of the Pancreas, Gallbladder, Extrahepatic Biliary Tracy and Ampullary Region. 198 Maddison Avenue, New York, 10016: Oxford University Press; 2003. p. 3.

(146) Butler AE, Cao-Minh L, Galasso R, Rizza RA, Corradin A, Cobelli C, et al. Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. Diabetologia 2010 Jun 5.

(147) Pour PM. Pancreatic centroacinar cells. The regulator of both exocrine and endocrine function. Int J Pancreatol 1994 Feb;15(1):51-64.

(148) Suzuki T, Kadoya Y, Sato Y, Handa K, Takahashi T, Kakita A, et al. The expression of pancreatic endocrine markers in centroacinar cells of the normal and regenerating rat pancreas: their possible transformation to endocrine cells. Arch Histol Cytol 2003 Oct;66(4):347-358.

(149) Lipsett M, Finegood DT. Beta-Cell Neogenesis during Prolonged Hyperglycemia in Rats. Diabetes 2002 Jun;51(6):1834-1841.

(150) Bonner-Weir S, Inada A, Yatoh S, Li WC, Aye T, Toschi E, et al. Transdifferentiation of pancreatic ductal cells to endocrine beta-cells. Biochem Soc Trans 2008 Jun;36(Pt 3):353-356.

(151) Potten CS, Morris RJ. Epithelial stem cells in vivo. J Cell Sci Suppl 1988;10:45-62.

(152) Paku S, Dezso K, Kopper L, Nagy P. Immunohistochemical analysis of cytokeratin 7 expression in resting and proliferating biliary structures of rat liver. Hepatology 2005 Oct;42(4):863-870.

(153) Theise ND, Saxena R, Portmann BC, Thung SN, Yee H, Chiriboga L, et al. The canals of Hering and hepatic stem cells in humans. Hepatology 1999 Dec;30(6):1425-1433.

(154) Githens S, Schexnayder JA, Moses RL, Denning GM, Smith JJ, Frazier ML. Mouse pancreatic acinar/ductular tissue gives rise to epithelial cultures that are morphologically, biochemically, and functionally indistinguishable from interlobular duct cell cultures. In Vitro Cell Dev Biol Anim 1994 Sep;30A(9):622-635.

(155) Rooman I, Heremans Y, Heimberg H, Bouwens L. Modulation of rat pancreatic acinoductal transdifferentiation and expression of PDX-1 in vitro. Diabetologia 2000 Jul;43(7):907-914.

(156) Sphyris N, Logsdon CD, Harrison DJ. Improved retention of zymogen granules in cultured murine pancreatic acinar cells and induction of acinar-ductal transdifferentiation in vitro. Pancreas 2005 Mar;30(2):148-157.

(157) Kurup S, Bhonde RR. Analysis and optimization of nutritional set-up for murine pancreatic acinar cells. JOP 2002 Jan;3(1):8-15.

(158) Means AL, Meszoely IM, Suzuki K, Miyamoto Y, Rustgi AK, Coffey RJ,Jr, et al. Pancreatic epithelial plasticity mediated by acinar cell transdifferentiation and generation of nestin-positive intermediates. Development 2005 Aug;132(16):3767-3776.

(159) Mashima H, Ohnishi H, Wakabayashi K, Mine T, Miyagawa J, Hanafusa T, et al. Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. J Clin Invest 1996 Apr 1;97(7):1647-1654.

(160) Mashima H, Shibata H, Mine T, Kojima I. Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. Endocrinology 1996 Sep;137(9):3969-3976.

(161) Melmed RN, Benitez CJ, Holt SJ. Intermediate cells of the pancreas. I. Ultrastructural characterization. J Cell Sci 1972 Sep;11(2):449-475.

(162) Szynaka B, Szynaka P, Andrzejewska A, Puchalski Z. Intermediate cells in chronic pancreatitis--ultrastructural observations. Rocz Akad Med Bialymst 1997;42 Suppl 2:26-33.

(163) Bertelli E, Bendayan M. Intermediate endocrine-acinar pancreatic cells in duct ligation conditions. Am J Physiol 1997 Nov;273(5 Pt 1):C1641-9.

(164) Korc M, Friess H, Yamanaka Y, Kobrin MS, Buchler M, Beger HG. Chronic pancreatitis is associated with increased concentrations of epidermal growth factor receptor, transforming growth factor alpha, and phospholipase C gamma. Gut 1994 Oct;35(10):1468-1473.

(165) Friess H, Yamanaka Y, Buchler M, Beger HG, Do DA, Kobrin MS, et al. Increased expression of acidic and basic fibroblast growth factors in chronic pancreatitis. Am J Pathol 1994 Jan;144(1):117-128.

(166) Cras-Meneur C, Elghazi L, Czernichow P, Scharfmann R. Epidermal growth factor increases undifferentiated pancreatic embryonic cells in vitro: a balance between proliferation and differentiation. Diabetes 2001 Jul;50(7):1571-1579.

(167) Bouckenooghe T, Vandewalle B, Lukowiak B, Kerr-Conte J, Belaich S, Gmyr V, et al. Modulation of specific beta cell gene (re)expression during in vitro expansion of human pancreatic islet cells. Cell Transplant 2003;12(7):799-807.

(168) Szynaka B, Zimnoch L, Puchalski Z, Szynaka P. Endocrine and intermediate cells in pancreatic ductal carcinoma and in chronic pancreatitis. Rocz Akad Med Bialymst 2002;47:21-30.

(169) Szynaka B, Andrzejewska A. Intermediate cells in the rat pancreas after chronic exposure to lead. Rocz Akad Med Bialymst 1997;42 Suppl 2:222-230.

(170) Bonner-Weir S, Sharma A. Pancreatic stem cells. J Pathol 2002 Jul;197(4):519-526.

(171) Yalniz M, Pour PM. Are there any stem cells in the pancreas? Pancreas 2005 Aug;31(2):108-118.

(172) Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, et al. Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes 2001 Mar;50(3):521-533.

(173) Lardon J, Rooman I, Bouwens L. Nestin expression in pancreatic stellate cells and angiogenic endothelial cells. Histochem Cell Biol 2002 Jun;117(6):535-540.

(174) Bouwens L, De Blay E. Islet morphogenesis and stem cell markers in rat pancreas. J Histochem Cytochem 1996 Sep;44(9):947-951.

(175) Yoshitomi H, Zaret KS. Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a. Development 2004 Feb;131(4):807-817.

(176) Noguchi H, Kaneto H, Weir GC, Bonner-Weir S. PDX-1 protein containing its own antennapedia-like protein transduction domain can transduce pancreatic duct and islet cells. Diabetes 2003 Jul;52(7):1732-1737.

(177) Gao R, Ustinov J, Pulkkinen MA, Lundin K, Korsgren O, Otonkoski T. Characterization of endocrine progenitor cells and critical factors for their differentiation in human adult pancreatic cell culture. Diabetes 2003 Aug;52(8):2007-2015.

(178) Hunziker E, Stein M. Nestin-expressing cells in the pancreatic islets of Langerhans. Biochem Biophys Res Commun 2000 Apr 29;271(1):116-119.

(179) Zimmermann A, Gloor B, Kappeler A, Uhl W, Friess H, Buchler MW. Pancreatic stellate cells contribute to regeneration early after acute necrotising pancreatitis in humans. Gut 2002 Oct;51(4):574-578.

(180) Swift SM, White SA, Clayton HA, Kimber D, London NJ, Dennison AR. Intraportal human islet autotransplantation: an immunohistochemical study of islets in situ. Transplant Proc 1998 Mar;30(2):417.

(181) Huang GC, Zhao M, Jones P, Persaud S, Ramracheya R, Lobner K, et al. The development of new density gradient media for purifying human islets and islet-quality assessments. Transplantation 2004 Jan 15;77(1):143-145.

(182) Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967 Jan;16(1):35-39.

(183) O'Neil JJ, Stegemann JP, Nicholson DT, Gagnon KA, Solomon BA, Mullon CJ. The isolation and function of porcine islets from market weight pigs. Cell Transplant 2001;10(3):235-246.

(184) Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 1995 May;21(5):1465-1468.

(185) Gruessner RW, Sutherland DE, Dunn DL, Najarian JS, Jie T, Hering BJ, et al. Transplant options for patients undergoing total pancreatectomy for chronic pancreatitis. J Am Coll Surg 2004 Apr;198(4):559-67; discussion 568-9.

(186) Webb MA, Illouz SC, Pollard CA, Musto PP, Berry D, Dennison AR. Long-term maintenance of graft function after islet autotransplantation of less than 1000 IEQ/kg. Pancreas 2006 Nov;33(4):433-434.

(187) Illouz S, Webb M, Pollard C, Musto P, O'Reilly K, Berry D, et al. Islet autotransplantation restores normal glucose tolerance in a patient with chronic pancreatitis. Diabetes Care 2007 Dec;30(12):e130.

(188) White SA, Davies JE, Pollard C, Swift SM, Clayton HA, Sutton CD, et al. Pancreas resection and islet autotransplantation for end-stage chronic pancreatitis. Ann Surg 2001 Mar;233(3):423-431.

(189) Kloppel G, Bommer G, Commandeur G, Heitz P. The endocrine pancreas in chronic pancreatitis. Immunocytochemical and ultrastructural studies. Virchows Arch A Pathol Anat Histol 1978 Feb 10;377(2):157-174.

(190) Larsen S. Diabetes mellitus secondary to chronic pancreatitis. Dan Med Bull 1993 Apr;40(2):153-162.

(191) Jaffer AK, Barsoum WK, Krebs V, Hurbanek JG, Morra N, Brotman DJ. Duration of anesthesia and venous thromboembolism after hip and knee arthroplasty. Mayo Clin Proc 2005 Jun;80(6):732-738.

(192) Comfere TB, Sprung J, Case KA, Dye PT, Johnson JL, Hall BA, et al. Predictors of mortality following symptomatic pulmonary embolism in patients undergoing noncardiac surgery. Can J Anaesth 2007 Aug;54(8):634-641.

(193) Barbich M, Hyon SH, Vieiro Laucirica M, Mullen J, Sorroche P, Furzi A, et al. Effect of embolization of impure islets on liver histology and biochemical variables. Transplant Proc 1998 Mar;30(2):415-416.

(194) Barshes NR, Lee TC, Goodpastor SE, Balkrishnan R, Schock AP, Mote A, et al. Transaminitis after pancreatic islet transplantation. J Am Coll Surg 2005 Mar;200(3):353-361.

(195) Rafael E, Ryan EA, Paty BW, Oberholzer J, Imes S, Senior P, et al. Changes in liver enzymes after clinical islet transplantation. Transplantation 2003 Nov 15;76(9):1280-1284.

(196) Shaw S, Korts D, Stimmel B. Abnormal liver function tests as biological markers for alcoholism in narcotic addicts. Am J Drug Alcohol Abuse 1982 - 1983;9(3):345-354.

(197) Steffensen FH, Sorensen HT, Brock A, Vilstrup H, Lauritzen T. Alcohol consumption and serum liver-derived enzymes in a Danish population aged 30-50 years. Int J Epidemiol 1997 Feb;26(1):92-99.

(198) Hirshberg B, Rother KI, Digon BJ,3rd, Lee J, Gaglia JL, Hines K, et al. Benefits and risks of solitary islet transplantation for type 1 diabetes using steroid-sparing immunosuppression: the National Institutes of Health experience. Diabetes Care 2003 Dec;26(12):3288-3295.

(199) Peavy DE, Taylor JM, Jefferson LS. Time course of changes in albumin synthesis and mRNA in diabetic and insulin-treated diabetic rats. Am J Physiol 1985 Jun;248(6 Pt 1):E656-63.

(200) Wanke IE, Wong NC. Diabetes mellitus decreases the activity of the albumin promoter in vitro. J Biol Chem 1991 Apr 5;266(10):6068-6072.

(201) Jefferson LS, Liao WS, Peavy DE, Miller TB, Appel MC, Taylor JM. Diabetes-induced alterations in liver protein synthesis. Changes in the relative abundance of mRNAs for albumin and other plasma proteins. J Biol Chem 1983 Jan 25;258(2):1369-1375.

(202) Ichii H, Miki A, Yamamoto T, Molano RD, Barker S, Mita A, et al. Characterization of pancreatic ductal cells in human islet preparations. Lab Invest 2008 Nov;88(11):1167-1177.

(203) Walsh TJ, Eggleston JC, Cameron JL. Portal hypertension, hepatic infarction, and liver failure complicating pancreatic islet autotransplantation. Surgery 1982 Apr;91(4):485-487.

(204) Bucher P, Mathe Z, Bosco D, Becker C, Kessler L, Greget M, et al. Morbidity associated with intraportal islet transplantation. Transplant Proc 2004 May;36(4):1119-1120.

(205) Matas AJ, Sutherland DE, Kretschmer G, Steffes MW, Najarian JS. Pancreatic tissue culture: depletion of exocrine enzymes and purification of islets for transplantation. Transplant Proc 1977 Mar;9(1):337-339.

(206) Weber CJ, Hardy MA, Lerner RL, Reemtsma K. Tissue culture isolation and preservation of human cadaveric pancreatic islets. Surgery 1977 Mar;81(3):270-273.

(207) Resau JH, Hudson EA, Jones RT. Organ explant culture of adult Syrian golden hamster pancreas. In Vitro 1983 Apr;19(4):315-325.

(208) Matsumoto S, Lawrence O, Rigley TH, Lakey JR, Stevens RB, Strong DM. University of wisconsin solution with trypsin inhibitor pefabloc improves survival of viable human and primate impure islets during storage. Cell Tissue Bank 2001;2(1):15-21.

(209) Ikemoto T, Noguchi H, Fujita Y, Takita M, Shimoda M, Sugimoto K, et al. New stepwise cooling system for short-term porcine islet preservation. Pancreas 2010 Oct;39(7):960-963.

(210) Ilieva A, Yuan S, Wang R, Duguid WP, Rosenberg L. The structural integrity of the islet in vitro: the effect of incubation temperature. Pancreas 1999 Oct;19(3):297-303.

(211) Groebe K, Mueller-Klieser W. Distributions of oxygen, nutrient, and metabolic waste concentrations in multicellular spheroids and their dependence on spheroid parameters. Eur Biophys J 1991;19(4):169-181.

(212) Gaber AO, Fraga D. Advances in long-term islet culture: the memphis experience. Cell Biochem Biophys 2004;40(3 Suppl):49-54.

(213) Rush BT, Fraga DW, Kotb MY, Sabek OM, Lo A, Gaber LW, et al. Preservation of human pancreatic islet in vivo function after 6-month culture in serum-free media. Transplantation 2004 Apr 27;77(8):1147-1154.

(214) Moran A, Zhang HJ, Olson LK, Harmon JS, Poitout V, Robertson RP. Differentiation of glucose toxicity from beta cell exhaustion during the evolution of defective insulin gene expression in the pancreatic islet cell line, HIT-T15. J Clin Invest 1997 Feb 1;99(3):534-539.

(215) Jonkers FC, Jonas JC, Gilon P, Henquin JC. Influence of cell number on the characteristics and synchrony of Ca2+ oscillations in clusters of mouse pancreatic islet cells. J Physiol 1999 Nov 1;520 Pt 3:839-849.

(216) Hauge-Evans AC, Squires PE, Persaud SJ, Jones PM. Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca2+ and insulin secretory responses of MIN6 pseudoislets. Diabetes 1999 Jul;48(7):1402-1408.

(217) Resau JH, Marzella L, Trump BF, Jones RT. Degradation of zymogen granules by lysosomes in cultured pancreatic explants. Am J Pathol 1984 May;115(2):139-150.

(218) Dionne KE, Colton CK, Yarmush ML. Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. Diabetes 1993 Jan;42(1):12-21.

(219) Balamurugan AN, Ramakrishna B, Gunasekaran S. Optimization of culture conditions for the preservation of monkey pancreatic islets in culture. Pancreas 1999 Jul;19(1):83-88.

(220) Matsumoto S, Goel S, Qualley S, Strong DM, Reems JA. A comparative evaluation of culture conditions for short-term maintenance (<24 hr) of human islets isolated using the Edmonton protocol. Cell Tissue Bank 2003;4(2/4):85-93.

(221) Rutzky LP, Bilinski S, Kloc M, Phan T, Zhang H, Katz SM, et al. Microgravity culture condition reduces immunogenicity and improves function of pancreatic islets1. Transplantation 2002 Jul 15;74(1):13-21.

(222) Rose NL, Palcic MM, Shapiro AM, Lakey JR. An evaluation of endogenous pancreatic enzyme levels following enzymatic digestion. Transplant Proc 2003 Nov;35(7):2458-2460.

(223) Tang C, Zhao X, Han J. Primary culture of porcine pancreatic acinar cells. Pancreas 2002 Jul;25(1):68-70.

(224) Brunstedt J, Nielsen JH. Long-term effect of pH on B-cell function in isolated islets of Langerhans in tissue culture. Diabetologia 1978 Sep;15(3):181-185.

(225) Webb M, James RF. Growth restriction and exendin 4 promote endocrine expression in cultured islet cells derived from patients with persistent hyperinsulinemic hypoglycemia of infancy (PHHI). Endocr Res 2005;31(2):99-109.

(226) Webb MA, Platton SL, Dennison AR, James RF. Immunohistochemical evidence that culture in the high aspect rotating vessel can up-regulate hormone

expression in growth dedifferentiated PHHI-derived islet cells. In Vitro Cell Dev Biol Anim 2007 Jul-Aug;43(7):210-214.

(227) Holmes MA, Clayton HA, Chadwick DR, Bell PR, London NJ, James RF. Functional studies of rat, porcine, and human pancreatic islets cultured in ten commercially available media. Transplantation 1995 Oct 27;60(8):854-860.

(228) Bottino R, Balamurugan AN, Smetanka C, Bertera S, He J, Rood PP, et al. Isolation outcome and functional characteristics of young and adult pig pancreatic islets for transplantation studies. Xenotransplantation 2007 Jan;14(1):74-82.

(229) Beattie GM, Otonkoski T, Lopez AD, Hayek A. Functional beta-cell mass after transplantation of human fetal pancreatic cells: differentiation or proliferation? Diabetes 1997 Feb;46(2):244-248.

(230) Brendel MD, Kong SS, Alejandro R, Mintz DH. Improved functional survival of human islets of Langerhans in three-dimensional matrix culture. Cell Transplant 1994 Sep-Oct;3(5):427-435.

(231) Yoffe B, Darlington GJ, Soriano HE, Krishnan B, Risin D, Pellis NR, et al. Cultures of human liver cells in simulated microgravity environment. Adv Space Res 1999;24(6):829-836.

(232) Khaoustov VI, Darlington GJ, Soriano HE, Krishnan B, Risin D, Pellis NR, et al. Induction of three-dimensional assembly of human liver cells by simulated microgravity. In Vitro Cell Dev Biol Anim 1999 Oct;35(9):501-509.

(233) Matsumoto S, Goel S, Qualley S, Strong DM, Reems JA. A comparative evaluation of culture conditions for short-term maintenance (<24 hr) of human islets isolated using the Edmonton protocol. Cell Tissue Bank 2003;4(2/4):85-93.

(234) Arita S, Kasraie A, Une S, Ohtsuka S, Kawahara T, Smith CV, et al. Improved recovery of cryopreserved canine islets by use of beraprost sodium. Pancreas 1999 Oct;19(3):289-296.

(235) Vasir B, Jonas JC, Steil GM, Hollister-Lock J, Hasenkamp W, Sharma A, et al. Gene expression of VEGF and its receptors Flk-1/KDR and Flt-1 in cultured and transplanted rat islets. Transplantation 2001 Apr 15;71(7):924-935.

(236) Esteve JP, Susini C, Vaysse N, Antoniotti H, Wunsch E, Berthon G, et al. Binding of somatostatin to pancreatic acinar cells. Am J Physiol 1984 Jul;247(1 Pt 1):G62-9.

(237) Garry DJ, Garry MG, Williams JA, Mahoney WC, Sorenson RL. Effects of islet hormones on amylase secretion and localization of somatostatin binding sites. Am J Physiol 1989 May;256(5 Pt 1):G897-904.

(238) Sever CE, Demetris AJ, Zeng Y, Tzakis A, Fung JJ, Starzl TE, et al. Islet cell allotransplantation in diabetic patients. Histologic findings in four adults simultaneously receiving kidney or liver transplants. Am J Pathol 1992 May;140(5):1255-1260.

(239) Carter J, Karmiol S, Nagy M, McElreath R, Calloway C, Motley A, et al. Pretransplant islet culture: a comparison of four serum-free media using a murine model of islet transplantation. Transplant Proc 2005 Oct;37(8):3446-3449.

(240) Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. Diabetes 1989 Jan;38 Suppl 1:140-142.

(241) Gerling IC, Kotb M, Fraga D, Sabek O, Gaber AO. No correlation between in vitro and in vivo function of human islets. Transplant Proc 1998 Mar;30(2):587-588.

(242) Srikant CB, Patel YC. Somatostatin receptors on rat pancreatic acinar cells. Pharmacological and structural characterization and demonstration of down-regulation in streptozotocin diabetes. J Biol Chem 1986 Jun 15;261(17):7690-7696.

(243) Gullo L. Somatostatin analogues and exocrine pancreatic secretion. Digestion 1996;57 Suppl 1:93.

(244) Rossowski WJ, Gu ZF, Akarca US, Jensen RT, Coy DH. Characterization of somatostatin receptor subtypes controlling rat gastric acid and pancreatic amylase release. Peptides 1994;15(8):1421-1424.

(245) Evert M, Seiler C, Dombrowski F. Aberrant acinar cell CA 19-9 expression and peri-insular acinar cell alterations in an adult human pancreas. Virchows Arch 2005 Jan;446(1):68-72.

(246) Leeson TS, Leeson R. Close association of centroacinar/ductular and insular cells in the rat pancreas. Histol Histopathol 1986 Jan;1(1):33-42.

(247) National Institutes of Health Clinical Center (CC). Solitary Islet Transplantation for Type 1 Diabetes Mellitus Using Steroid Sparing Immunosuppression. May 12th, 2010; .

(248) Bogdani M, Lefebvre V, Buelens N, Bock T, Pipeleers-Marichal M, In't Veld P, et al. Formation of insulin-positive cells in implants of human pancreatic duct cell preparations from young donors. Diabetologia 2003 Jun;46(6):830-838.

(249) Muharram G, Beucher A, Moerman E, Belaich S, Gmyr V, Vandewalle B, et al. Endocrine pancreatic tissue plasticity in obese humans is associated with cytoplasmic expression of PBX-1 in pancreatic ductal cells. Biochem Biophys Res Commun 2005 Aug 12;333(4):1153-1159.

(250) Peters K, Panienka R, Li J, Kloppel G, Wang R. Expression of stem cell markers and transcription factors during the remodeling of the rat pancreas after duct ligation. Virchows Arch 2005 Jan;446(1):56-63.

(251) Bouwens L, Braet F, Heimberg H. Identification of rat pancreatic duct cells by their expression of cytokeratins 7, 19, and 20 in vivo and after isolation and culture. J Histochem Cytochem 1995 Mar;43(3):245-253.

(252) Ko SH, Suh SH, Kim BJ, Ahn YB, Song KH, Yoo SJ, et al. Expression of the intermediate filament vimentin in proliferating duct cells as a marker of pancreatic precursor cells. Pancreas 2004 Mar;28(2):121-128.

(253) Wu KL, Gannon M, Peshavaria M, Offield MF, Henderson E, Ray M, et al. Hepatocyte nuclear factor 3beta is involved in pancreatic beta-cell-specific transcription of the pdx-1 gene. Mol Cell Biol 1997 Oct;17(10):6002-6013.

(254) Stoffers DA, Heller RS, Miller CP, Habener JF. Developmental expression of the homeodomain protein IDX-1 in mice transgenic for an IDX-1 promoter/lacZ transcriptional reporter. Endocrinology 1999 Nov;140(11):5374-5381.

(255) Song SY, Gannon M, Washington MK, Scoggins CR, Meszoely IM, Goldenring JR, et al. Expansion of Pdx1-expressing pancreatic epithelium and islet neogenesis in transgenic mice overexpressing transforming growth factor alpha. Gastroenterology 1999 Dec;117(6):1416-1426.

(256) Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997 Dec 15;90(12):5002-5012.

(257) Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007 Jun;132(7):2542-2556.

(258) Immervoll H, Hoem D, Sakariassen PO, Steffensen OJ, Molven A. Expression of the "stem cell marker" CD133 in pancreas and pancreatic ductal adenocarcinomas. BMC Cancer 2008 Feb 8;8:48.

(259) Pang K, Mukonoweshuro C, Wong GG. Beta cells arise from glucose transporter type 2 (Glut2)-expressing epithelial cells of the developing rat pancreas. Proc Natl Acad Sci U S A 1994 Sep 27;91(20):9559-9563.

(260) Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 2002 May;129(10):2447-2457.

(261) Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 2002 Sep;32(1):128-134.

(262) Strobel O, Dor Y, Alsina J, Stirman A, Lauwers G, Trainor A, et al. In vivo lineage tracing defines the role of acinar-to-ductal transdifferentiation in inflammatory ductal metaplasia. Gastroenterology 2007 Dec;133(6):1999-2009.

(263) Ryan EA, Lakey JR, Rajotte RV, Korbutt GS, Kin T, Imes S, et al. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. Diabetes 2001 Apr;50(4):710-719.

(264) Moberg L. The role of the innate immunity in islet transplantation. Ups J Med Sci 2005;110(1):17-55.

(265) Giuliani M, Moritz W, Bodmer E, Dindo D, Kugelmeier P, Lehmann R, et al. Central necrosis in isolated hypoxic human pancreatic islets: evidence for postisolation ischemia. Cell Transplant 2005;14(1):67-76.

(266) Guz Y, Montminy MR, Stein R, Leonard J, Gamer LW, Wright CV, et al. Expression of murine STF-1, a putative insulin gene transcription factor, in beta cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. Development 1995 Jan;121(1):11-18.

(267) Fernandes A, King LC, Guz Y, Stein R, Wright CV, Teitelman G. Differentiation of new insulin-producing cells is induced by injury in adult pancreatic islets. Endocrinology 1997 Apr;138(4):1750-1762.

(268) Miyatsuka T, Kaneto H, Shiraiwa T, Matsuoka TA, Yamamoto K, Kato K, et al. Persistent expression of PDX-1 in the pancreas causes acinar-to-ductal metaplasia through Stat3 activation. Genes Dev 2006 Jun 1;20(11):1435-1440.

(269) Hayashi K, Takahashi T, Kakita A, Yamashina S. Regional differences in the cellular proliferation activity of the regenerating rat pancreas after partial pancreatectomy. Arch Histol Cytol 1999 Oct;62(4):337-346.

(270) Hirshberg B, Montgomery S, Wysoki MG, Xu H, Tadaki D, Lee J, et al. Pancreatic islet transplantation using the nonhuman primate (rhesus) model predicts that the portal vein is superior to the celiac artery as the islet infusion site. Diabetes 2002 Jul;51(7):2135-2140.

(271) Gores PF, Najarian JS, Stephanian E, Lloveras JJ, Kelley SL, Sutherland DE. Insulin independence in type I diabetes after transplantation of unpurified islets from single donor with 15-deoxyspergualin. Lancet 1993 Jan 2;341(8836):19-21.

(272) Clayton HA, Davies JE, Pollard CA, White SA, Musto PP, Dennison AR. Pancreatectomy with islet autotransplantation for the treatment of severe chronic pancreatitis: the first 40 patients at the leicester general hospital. Transplantation 2003 Jul 15;76(1):92-98.