Investigating the Impact of UW Cold Storage on Human Islet Basement Membrane Integrity Following Islet Isolation



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Abstract

The islet basement membrane (BM) is a specialised sheet of extracellular matrix comprised primarily of laminins, collagen IV and perlecan. It is essential for islet function and survival. During the enzymatic digestion phase of islet isolation, the islet BM is significantly disrupted. Further loss of the islet BM occurs during the mandatory culture period, when islets are placed in CMRL media at 37°C. Previous studies have demonstrated the superiority of UW cold storage solution for maintaining islets during the post-isolation culture period. However, the impact of UW storage on BM integrity during culture has not been explored. The hypothesis is that the increased viability and recovery of islets following culture in UW, may result from the preserved integrity of the islet BM.

Human islet cells collected from 4 human islet isolations were divided into two groups and cultured for up to 72 hours in either: CMRL media at 37°C or UW at 4°C. Islet samples were collected every 24 hours from each condition, which included collection of a post-isolation sample. Samples were assessed for purity, viability, and islet yield. Subsequently, human islet BM integrity was determined histologically via insulin, collagen IV, and laminin labelling. Total protein concentration was determined by ELISA. Results from the cultured samples were normalised to the post-isolation sample. Statistical analysis was by way of ANOVA.

The number of islets, IEQ, and islet viability significantly declined following culture in CMRL up to 72 hours compared to fresh samples collected at the conclusion of the isolation procedure. This same loss was not seen in islets stored in UW, for up to 72 hours when compared to the fresh islets. The islet BM (measured via laminin, collagen IV, laminin-α5, and perlecan) is significantly more stable in UW storage solution, compared to CMRL.

The findings in this thesis provide novel insights into the maintenance of the human islet BM during the required culture period. The increased survival of key islet BM proteins in UW provides important molecular evidence, which could underpin the reasons for the enhanced islet survival seen in UW culture systems. Further evaluation of the presence of the human islet BM following storage in UW will aid in optimising current isolation practices, and lead to greater transplantation outcomes.

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Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
BCA	Bicinchoninic acid
BM	Basement membrane
BMI	Body mass index
Ca^{2+}	Calcium
CIT	Cold ischemia time
CMRL	Connaught's Medical Research Laboratory
CSII	Continuous intra-cutaneous injection of insulin
CVD	Cardiovascular disease
DBD	Donation after Brainstem Death
DCCT	Diabetes Control and Complications Trial
DCD	Donation after Circulatory Death
DKA	Diabetic ketoacidosis
DNA	Deoxyribonucleic acid
DRWF	Diabetes Research and Wellness Foundation
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
EtOH	Ethanol alcohol
FDA	Fluorescein diacetate

FGF	Fibroblast growth factor
GLUT	Glucose transporter
GMP	Good manufacturing practice
GSIS	Glucose stimulated insulin secretion
HbA1c	Haemoglobin A1c
HBSS	Hank's balanced buffer solution
HGF	Hepatocyte growth factor
HSPG	Heparan sulfate proteoglycan
HTA	Human Tissue Authority
IEQ	Islet equivalent
IRT	Insulin replacement therapy
K_{ATP} Channel	Adenosine triphosphate-sensitive potassium channel
KRB	Krebs Ringer Buffer
NaOH	Sodium hydroxide
NP	Neutral protease
OCDEM	Oxford Centre for Diabetes, Endocrinology, and Metabolism
OCT	Optimal cutting temperature compound
OGTT	Oral Glucose Intolerance Test
OXCIT	Oxford Consortium for Islet Transplantation
PFA	Paraformaldehyde
PIC	Protein inhibitor cocktail
PP	Polypeptide-producing

QOL	Quality of life
SD	Standard deviation
SGLT	Sodium glucose cotransporter
SOP	Standard operating procedure
T1DM	Type 1 Diabetes Mellitus
TBS	Tris-buffered saline
TCA	Tricarboxylic
UK	UK Islet Transplant Consortium
UK	United Kingdom
UW	University of Wisconsin solution
VEGF	Vascular endothelial growth factor
VGCC	Voltage-gated calcium channel
α	Alpha
β	Beta

Chapter 1

General Introduction

1.1 Type 1 Diabetes Mellitus

Type 1 Diabetes Mellitus (T1DM) is understood to be an autoimmune disease leading to the destruction of insulin-producing beta-cells (β-cells) within the islets of Langerhans ("islets") in the pancreas [1]. Normally, insulin, a peptide hormone, works to refine the metabolism of carbohydrates, fats, and proteins leading to the uptake of glucose from the blood into fat, liver, and skeletal muscle cells [2] and allows glucose to enter cells and produce energy. In patients with T1DM, β-cell destruction results in failure to produce insulin with consequent hyperglycaemia and ultimately the development of diabetic ketoacidosis (DKA). People with T1DM require daily exogenous insulin and regular glucose monitoring.

Although Type 2 Diabetes accounts for the majority of global cases of diabetes mellitus, there has been a steady increase in the presence of T1DM [3] and in 2021 there were an estimated 8.4 million individuals with T1DM globally [4]. Although T1DM can occur at any age, the onset of T1DM is usually in the first two decades of life (most commonly presenting between the ages of 4 to 6 and 10 to 14), and T1DM is one of the most frequent chronic diseases in children and adolescents [1]. Additionally, there are reported geographic and ethnic variations in the incidence of T1DM worldwide [4, 5].

Whilst the exact aetiology of T1DM is still unknown, researchers suggest there is a genetic predisposition strongly linked to specific HLA (DR and DQ, and some DP) alleles [6]. Considering the increased prevalence of cases, however, T1DM cannot be solely due to genetic predisposition. The pathogenesis of T1DM suggests that there is a complex interaction between

β-cells and the innate and adaptive immune systems [3]. T1DM develops in 3 stages: In the first stage, affected individuals present as asymptomatic with normal fasting glucose and glucose intolerance, but demonstrate two or more pancreatic autoantibodies. Individuals will remain asymptomatic in stage 2 but will start to demonstrate abnormalities in blood sugar stability. This will manifest as impaired fasting glucose (100 to 125 mg/dL), impaired glucose tolerance (2-hour post-load glucose level of 140 to 199 mg/dL), or a haemoglobin A1c (HbA1c) between 5.7% to 6.4%. In stage 3, individuals develop clinical symptoms secondary to hyperglycaemia [7]. Individuals with T1DM may experience the onset of symptoms suddenly or over an extended period. The symptoms commonly seen in patients with T1DM include excessive thirst, frequent urination, excessive hunger, unexplained and persistent weight loss, extreme fatigue, blurry vision, slow healing of cuts and bruises [8]. The most common symptoms of hyperglycaemia are polydipsia, polyuria, and polyphagia [1]. A diagnosis of T1DM can be confirmed by HbA1c (fasting blood sugar test), oral glucose tolerance test (OGTT), or random blood sugar test measure considering the respective criteria (Table 1), referenced from the American Diabetes Association.

Result*	A1C Test	Fasting Blood Sugar Test	Glucose Tolerance Test	Random Blood Sugar Test
Diabetes	6.5% or above	126 mg/dL or above	200 mg/dL or above	200 mg/dL or above
Prediabetes	5.7 – 6.4%	100 – 125 mg/dL	140 – 199 mg/dL	N/A
Normal	Below 5.7%	99 mg/dL or below	140 mg/dL or below	N/A

Table 1: Testing for confirmation of T1DM diagnosis
Sourced from the American Diabetes Association (2023)

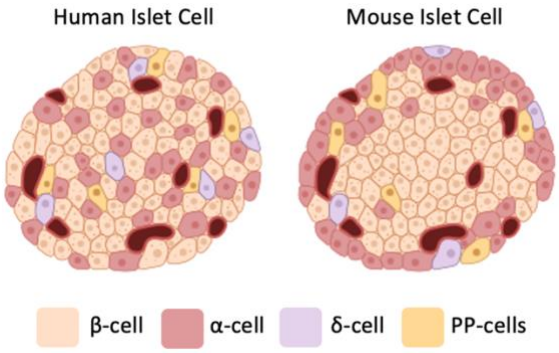
In addition to the acute presentation of T1DM, people with T1DM are at risk of developing debilitating secondary complications (Table 2) of the disease that result from longstanding hyperglycaemia. These arise from micro-vascular, macro-vascular, and neurological damage, and the most common clinical manifestations are renal failure (nephropathy), blindness (retinopathy), changes in sensation (peripheral neuropathy), lower limb ischaemia leading to amputation, erectile dysfunction, gastroparesis, and cardiovascular disease [9]. Secondary complications not only have a significant impact on the individual's quality of life (QOL) but also place a substantial economic impact on health care providers.

<table>
<tr><th colspan="2">Complications of Type 1 Diabetes</th></tr>
<tr><th>Microvascular</th><th>Macrovascular</th></tr>
<tr><td>Retinopathy: Damage to blood vessels and nerve tissue in the retina leading to vision loss, and in some cases blindness.</td><td>Cardiovascular disease (CVD): Impact to the heart and circulation throughout the body, most commonly displaying as coronary heart disease in individuals with diabetes.</td></tr>
<tr><td>Nephropathy: Deterioration of globular filtration rate (GFR) leading to loss of kidney function.</td><td>Ischemic Stroke: Narrowing or blockage of cerebral blood vessels leading to lack of blood supply to the brain.</td></tr>
<tr><td rowspan="2">Neuropathy: Damage to nerves throughout the body that can occur within both the peripheral and autonomic nervous systems.</td><td>Peripheral vascular disease: Narrowing or blockages of blood vessels carrying blood to multiple systems and the extremities.</td></tr>
<tr><td>Diabetic foot infection: Skin, soft tissue or bone infections below the ankle caused by bacterial pathogens, in severe cases requiring amputation.</td></tr>
</table>

Table 2: Complications of T1DM
Secondary complications seen in patients with T1DM without therapy to achieve glycaemic control.

1.2 The Pancreatic Islet

A detailed understanding of the structure and function of the pancreatic Islets of Langerhans is crucial for developing therapies for glucose homeostasis. Pancreatic islets are clusters of endocrine cells dispersed throughout the pancreas. The average adult human pancreas contains over a million islets, each containing several thousand endocrine cells [10]. Individual islet size is highly variable and can range from <50 µm to 500 µm in diameter (average diameter 150 µm) [11-13]. Islets contain at least five different secretory cell types: Alpha (α) cells producing glucagon (~20% of total islet cells); β-cells producing insulin and amylin (~70% of total islet cells); PP cells (gamma or F cells) producing pancreatic polypeptide; Delta cells producing somatostatin; and the more recently recognised Epsilon cells producing ghrelin (<1% of total islet cells) [14, 15]. Islet composition is species dependent [16]. In humans, β-cells are scattered within the whole islet structure, but in mice, β-cells are clustered together at the centre of the islet (Figure 1).



Within human islets, β-cells are the most prevalent, making up around 70% of the total cell volume [17].

Figure 1: Human Islet vs. Mouse Islet
The human islet shows the dispersion of β-cells among the entire islet, whilst the mouse islet shows the centralisation of β-cells to the middle of the islet.

In terms of T1DM, β-cells are the most important, and most studied, cell type of those within the Islets of Langerhans. The ultrastructure of the β-cell contains a continuous surrounding plasma membrane, and cytoplasmic organelles including the ergastoplasm, mitochondria, secretory granules, Golgi complex, as well as a nucleus. β-cell granules are dispersed throughout the cytoplasm in smooth membranous sacs [18]. These β-cells synthesise, store, and secrete insulin stored within the beta granules. Insulin is an anti-hyperglycaemic hormone that antagonises other hormones such as glucagon, growth hormone, glucocorticoids, epinephrine, and others that may shift glucose concentrations outside of normal physiologic range [19]. This shift occurs in a normal healthy pancreas as a response to electrical signals that are initiated upon glucose detection.

1.3 Beta-cell Insulin Release Mechanism

Pancreatic β-cells are electrically excitable cells regulated by various components. Both facilitated diffusion glucose transporters (GLUTs), and sodium-glucose cotransporters (SGLTs) play roles in glucose uptake and glucose-stimulated hormone release [20]. Upon absorption of glucose by GLUTs, glucose undergoes phosphorylation by glucokinase and is metabolised to pyruvate through glycolysis. GLUT presentation varies among different species [21]. In humans, GLUT1 and GLUT3 are understood to be the primary GLUTs involved in the transportation of glucose molecules upon detection on the cell surface [21, 22]. After being produced through glycolysis, pyruvate undergoes mitochondrial oxidation in the tricarboxylic (TCA) cycle leading to a build-up in intracellular adenosine triphosphate (ATP). The increased ATP inhibits the ATP-

sensitive potassium (K_{ATP}) channel activity, triggering membrane depolarisation. Changes in the β-cell membrane potential elicit an electrical response to regulate voltage-dependent calcium (Ca^{2+}) channels. When voltage gated Ca^{2+} channels (VGCCs) open there is an influx of Ca^{2+} released into the cytoplasm. Increased cytoplasmic Ca^{2+} induces insulin exocytosis, emphasising the connection between insulin release and glucose dependency, as well as the frequency of action potentials fired by the cell [20, 23]. See Figure 2 below.

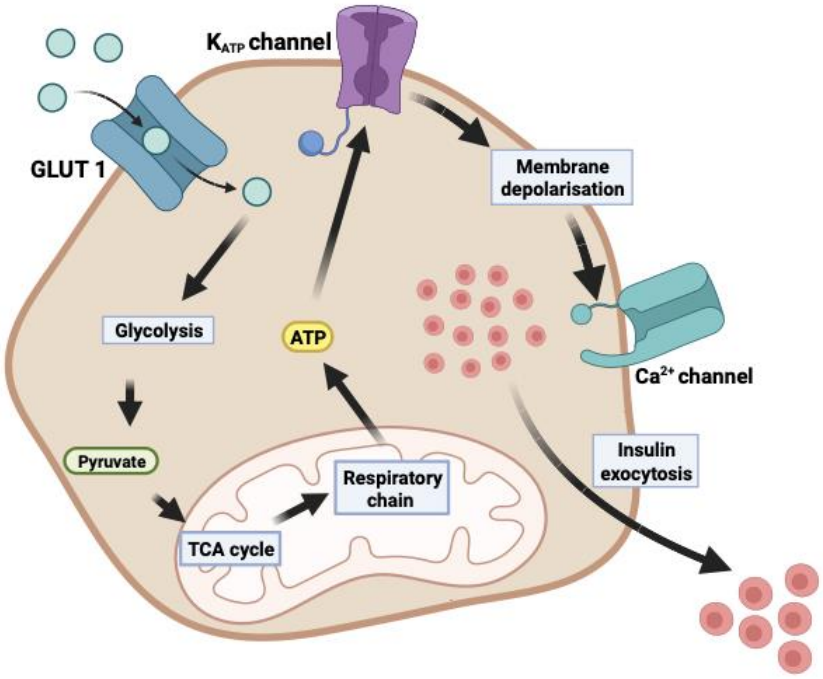


Figure 2: β-cell Insulin Secretion
Insulin secretion begins with glucose transportation via GLUT1 into the cell where glycolysis occurs producing pyruvate. Pyruvate then undergoes the TCA cycle and respiratory chain, here the level of ATP increases within the cell triggering the opening of K_{ATP} channels. The membrane is then depolarised and there is an influx of Ca^{2+} cells, triggering exocytosis of insulin-containing granules.

1.4 Current Treatment Options for T1DM

1.4.1 Available Therapies for T1DM

Until the commercial availability of exogenous insulin in 1923, T1DM was an untreatable condition. The pioneering work by Banting and Best changed this [2]. The current standard of care for T1DM is exogenous insulin replacement therapy (IRT) [3]. Traditionally IRT was administered via regular insulin injections. However, over the last couple of decades, insulin pumps have been used widely. It has been demonstrated that an individual with T1DM will measure their blood glucose levels around 80,000 times and will have around 65,000 insulin injections throughout their lifetime [24]. At the heart of most current clinical management regimens for T1DM, is the understanding that the secondary complications of T1DM can be prevented/reduced by maintaining blood glucose levels within the normal range for as much time as possible ('time in range'). Although this might appear intuitive, the correlation between tightness of control of glycaemia and the development of T1DM secondary complications was only proven in the early 1990s with the Diabetes Control and Complications Trial (DCCT). This landmark trial compared intensive insulin treatment in T1DM with regular insulin treatment. The findings of this trial reflected the importance of intensive insulin therapies. Participants of the study were screened for the development or progression of secondary complications. When evaluated in a follow-up, researchers found that intensive insulin therapy during the DCCT minimised later development and progression of retinopathy, nephropathy, and neuropathy [25-27]. One of the problems identified in the DCCT however, was that there was a two- to three-fold increase in the prevalence of severe hypoglycaemic events in the patient population using intensive insulin therapies [28]. A more recent follow-up study completed by the DCCT group also

reported a threefold increase in severe hypoglycaemic events [26], indicating the maintained need for alternative therapeutic options.

Insulin pumps combined with glucose sensors (closed-loop systems), supply continuous insulin injection in response to glucose presence as a physiologically healthy pancreas would. This refined therapy still relies on exogenous insulin and mechanical devices but has been demonstrated to result in superior glycaemic control compared to daily insulin injections [7, 29]. Other treatment strategies attempt to arrest and/or reverse the autoimmune destructive process within the β-cells. One study found the β-cell function to persist despite the presence of T1DM through the use of serum c-peptide at a concentration of >0.03 nmol/L [30]. Immunotherapies and c-peptide hormone-based therapies [3, 31], have both been shown to produce benefits, but have yet to demonstrate long-lasting stabilisation of glycaemic control for T1DM patients. Immunotherapies work to reprogramme the immune system, stopping this system's attacks on β-cells [32]. As of 2022, the United States Federal Drug Association (FDA) has approved an immunotherapy drug, teplizumab, which can delay the onset of T1DM in high-risk individuals [33]. While teplizumab is not available in the UK, there are clinical trials underway for other immunotherapy options [32].

An alternative approach is to replace the insulin-producing β-cells. This can either be in the form of a whole pancreas transplant or as a cellular islet transplant. These therapies are deliberated further in the following section.

1.5 Beta-cell Replacement

1.5.1 Whole pancreas transplantation

Whole pancreas transplantation is available to patients with T1DM who also require a kidney transplant or who experience recurrent episodes of severe hypoglycaemia despite efforts of other medical therapy. However, the procedure carries risks associated with major surgery, making this treatment option less optimal for all eligible patients [34]. Complications may require reoperations due to haemorrhage, sepsis, pancreatitis, and thrombosis. The complexity of the pancreas transplantation procedure may result in significant mortality and morbidity [35]. The complications associated with whole pancreas transplantation surgery make islet transplantation a more attractive therapy for some patients eligible to receive this therapy.

1.5.2 Islet Isolation and Transplantation

Compared with whole pancreas transplantation, islet transplantation is a minimally invasive procedure which involves the transplantation of the endocrine component of the pancreas only. As a cell-based therapy, islet transplantation also has the potential for the islets to be manipulated to obviate the need for immunosuppression, thereby making it an exciting possibility for children.

1.5.2.1 History of Islet Isolation and Transplantation

Before the discovery of insulin, in 1892, Oscar Minkowski and Joseph von Mering subcutaneously transplanted autologous pancreatic fragments into a diabetic dog yielding transient

improvement of glycosuria [36]. In 1893, Patrick Watson-Williams performed a xenotransplant on a 15-year-old patient with diabetes using fragments of pancreatic sheep tissue [37]. Other individuals followed this lead, but for some, the discovery of insulin decreased interest in the use of pancreatic fragments for treatment. The discovery furthered researchers' understanding of the pancreas and the organs' insulin-secreting characteristics. In 1972, Walter Ballinger and Paul Lacy used islet isografts from normal rodents in streptozotocin-induced diabetic rodents, which influenced future research [38]. The first successful islet allotransplant was performed using the rodent model in 1972 and was applied to humans in 1980 [39]. Following the human application in 1980, over 600 islet transplants were performed during the 1990s. However, whilst many of these grafts were functional, very few recipients became insulin independent. In 2000, Dr. James Shapiro and colleagues in Edmonton, Canada, developed a revised islet isolation and transplantation protocol that led to insulin independence in seven consecutive T1DM patients [40]. The 'Edmonton Protocol' involved transplanting pancreatic islets into people with T1DM with life-threatening hypoglycaemia unawareness and used a steroid-free immunosuppressive regimen. Patients received two to three islet grafts in quick succession (within a few months of each other) and the resulting islet mass led to 'game-changing' outcomes with 85% of patients achieving insulin independence for at least a year following transplantation. This transformed the field of islet transplantation, and soon after Shapiro's landmark publication, nearly 50 centres across the world started using the Edmonton Protocol [40, 41].

1.5.2.2 Islet Isolation Protocol Overview

One of the greatest challenges of islet transplantation is the islet isolation procedure. The protocol for islet isolation (Figure 3) is briefly described below.

The goal of clinical islet isolation is to produce viable intact pancreatic islets for transplantation. The procedure is highly regulated to maintain the best possible viability and purity of the pancreatic islets being collected. Islet isolation occurs in various steps: pancreas preparation, pancreas perfusion and digestion, tissue collection, and purification [42]. To ensure the safety of the final product, islets that are intended for human application are produced in line with Good Manufacturing Practice (GMP) standards. With appropriate informed consent, pancreases are retrieved from donors via one of two donation pathways: Donation after Brainstem Death (DBD) or Donation after Circulatory Death (DCD). Organs are transited from the donor hospital to the islet isolation centre using static cold storage in University of Wisconsin (UW) solution [40, 43].

The isolation procedure begins with the inspection of the donor paperwork and a visual examination of the donor organ to check for any organ damage or gross ischaemia. The pancreas may be found to have factors contraindicated for islet transplantation including a positive serology after acceptance, an HbA1c level in the diabetic range, signs of acute or chronic pancreatitis, fatty infiltration of the pancreas, or significant damage to the organ during procurement and/or preservation [44]. If present, these factors can negatively impact islet isolation outcomes. Once the isolation team have approved the pancreas, any accessory organs that are still attached to the pancreas are dissected, such as the spleen and the bowel. In younger

donors, the bowel may be more closely fixed to the pancreas requiring precise dissection to preserve pancreatic tissue whilst also ensuring no perforation of the bowel occurs, as the contents can contaminate the organ and impact transplantation. The pancreas is then cannulated via the pancreatic duct, where a commercially available enzymatic blend consisting of collagenase and neutral protease (NP) is delivered. When the enzyme blend is delivered through the cannulated duct, distension of the organ should occur [45]. In some instances where portions of the organ are not distending, the organ will be sectioned and the pancreatic duct cannulated in each section, then the same process to deliver the enzymes is completed. The distended organ is sectioned into smaller fragments (around 1-2 inch(s)) and placed in the Ricordi chamber, where enzymatic and mechanical digestion occur, using a sterile closed-loop digestion circuit. Once the islets are sufficiently free from the exocrine tissue, the pancreatic tissue digest is processed for purification. Within the pelleted tissue sample, islets are separated from the exocrine tissue due to their differing densities using density gradient centrifugation methods [46]. Islets are then cultured in Connaught's Medical Research Laboratory (CMRL) culture media with supplementation for a minimum of 24 hours, prior to reassessment for transplantation. If the product of the islet isolation meets the number and viability needed for transplantation after the necessary culture period, the islets acquired from the isolation procedure are then transplanted into the recipient's liver via the portal vein in interventional radiology.

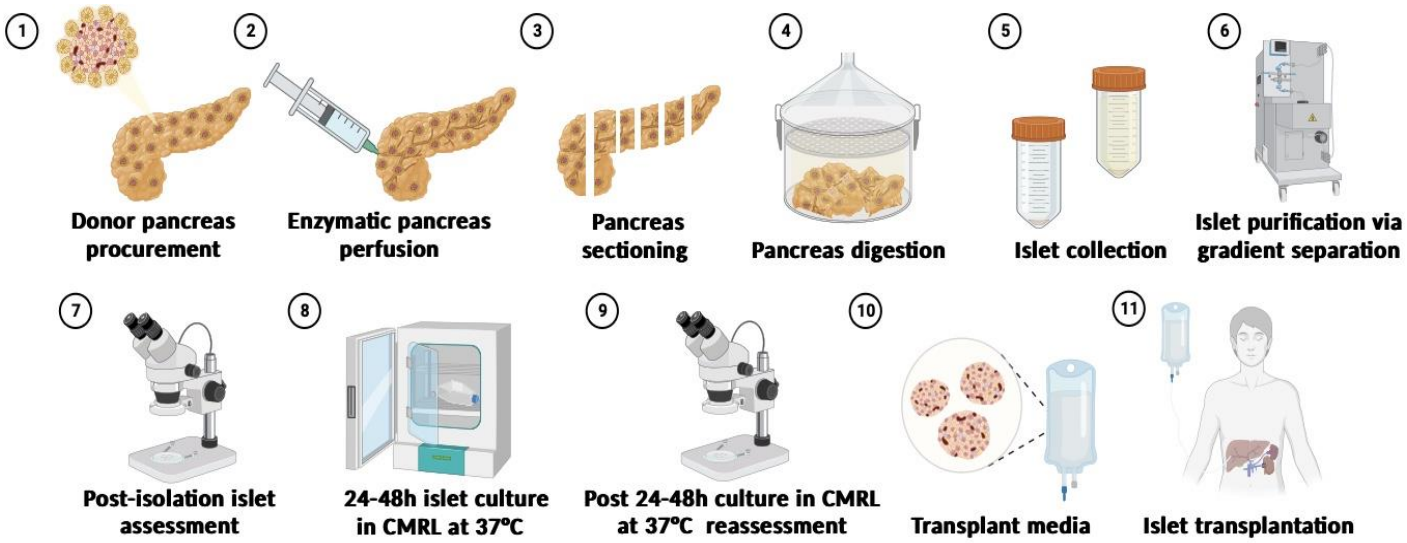


Figure 3: Pancreatic Islet Isolation Protocol Overview
① Donor pancreas arrives at the facility for isolation in static cold storage (UW solution). ② Accessory organs and exogenous fat are removed, and the pancreas is cannulated at the pancreatic duct and perfused using a commercially available enzymatic blend of collagenase and neutral protease. ③ The pancreas is sectioned to increase surface area for digestion. ④ Sectioned pieces are placed within the Ricordi chamber and are digested via enzymatic and mechanical digestion at around 35°C. ⑤ Islet pooling and collection begins when islets are visually free (determined via multiple dithizone stained biopsies throughout the digestion phase). ⑥ Islet purification is completed via continuous density gradient for optimal separation, using a Cobe 2991 cell processor. ⑦ Islet isolation product is assessed for respective parameters (see Chapter 2, Table 3). ⑧ If suitable for transplant, islets are cultured for 24-48 hours in supplemented CMRL culture media. ⑨ Islets are reassessed to confirm the product is still viable and maintains each respective parameter necessary. ⑩ Islets are resuspended into transplant media. ⑪ Islets are transplanted into the recipient's liver via the portal vein in interventional radiology.

1.5.2.3 Donor Organ Criteria

For pancreatic islet transplantation, donor pancreases are retrieved from deceased donors as part of the multi-organ retrieval process. The acceptance criteria of a donor pancreas for islet isolation and transplantation varies slightly worldwide. For this thesis, the donor inclusion and exclusion criteria referred to will be those of the UK Islet Transplant Consortium (UKITC) and the

Diabetes Research and Wellness Foundation (DRWF) Oxford Human Islet Isolation Facility at the Churchill Hospital, Oxford (see appendix, Table A1: Donor Pancreas Acceptance Criteria).

The success of human islet isolation is impacted by multiple factors which don't always correlate with optimal islet function. These include donor age, body mass index (BMI), cause of death, and cold ischemia time (CIT). Research has demonstrated that a lower donor age has a beneficial effect on islet function after transplantation [47]. However, isolating islets from younger donors remains a technical challenge compared with the functionally less favourable older donor. Islets isolated from younger donors often remain partially embedded in the surrounding exocrine tissue following pancreas digestion, which prevents them from being purified [44, 48]. Numerous reports express that a higher BMI is commonly associated with significantly higher islet yields and viability, compared with donors with a lower BMI [49-51].

Other factors that impact the success of islet isolation and transplantation is the selected donation pathway (i.e., the donor type) and the CIT. Donation can proceed via one of two pathways: DBD or DCD. Most donor organs are procured from DBD donors. However, in the UK, there has been an increase in the number of DCD donors due to limited DBD donor availability [52]. Nonetheless, the occurrence of DCD donors resulting in islet transplantation remains less than that of DBD donors, as reflected in Figure 4, from the UK National Health Service (NHS) Annual Report on Pancreas and Islet Transplantation for the year 2022/2023 [53]. Findings vary as to whether DCD donors produce the same level of success as DBD donors during islet isolation, with some studies demonstrating similar outcomes from the two types, and others expressing

differences in islet isolation outcomes, islet secretory function, and clinical transplantation outcomes [43, 54, 55]. Although study outcomes proved that DCD donor islet function both in vivo and in vitro was comparable to that of DBD donors, the main difference reported was the islet yield recovered from the islet isolation, with DCD donors producing a significantly lower yield [43, 55]. Research suggests that reduced CIT is associated with better isolation outcomes. Moreover a CIT beyond 8 hours results in reductions in islet yield and quality [56]. Other criteria that are evaluated are substance usage including drug and alcohol abuse. Significant alcohol abuse in the donor's history may impact islet isolation outcomes due to a lack of accessibility to desired cells. Donor history of drug use is permitted; however, it is important to note that due to the potential increased risks of transmissible disease, donors with a history or evidence of intravenous drug use are not acceptable candidates for organ donation for clinical islet isolation and transplantation.

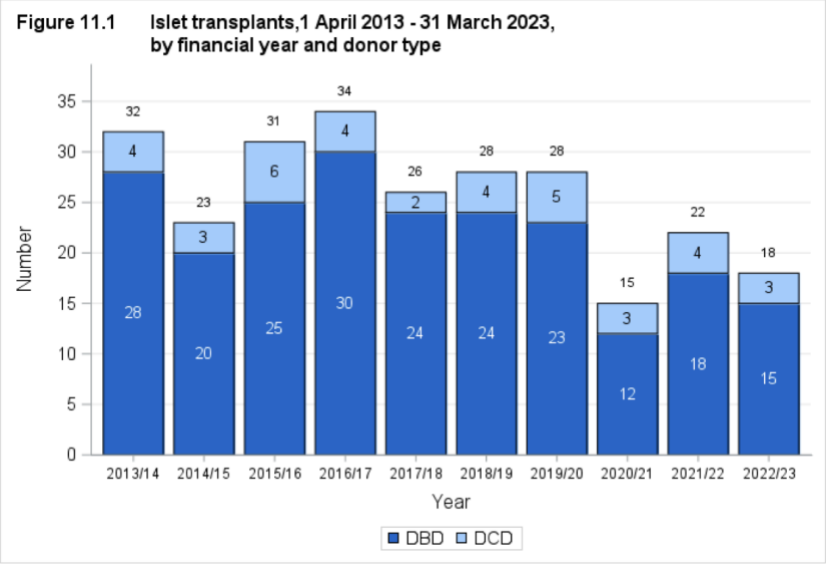


Figure 4: Islet transplantation record in the UK from April 2013 to March 2023
A comparison of the frequency of DBD: DCD donors across all UK centres (Cambridge, Cardiff, Edinburgh, Guy's, Manchester, Newcastle, Oxford, and WLRTC). Figure adapted from NHS Annual Report on Pancreas and Islet Transplantation, Report For 2022/2023 (1 April 2013 - 31 March 2023).

1.5.2.4 Recipient Criteria

The islet recipient criteria has been established and followed throughout the United Kingdom (UK) (see appendix, Table A2, for recipient criteria), but the criteria may vary between countries [57]. However, research shares that the ideal candidate for islet transplantation includes an insulin-sensitive individual with T1DM. Within the UK, an individual must report at least 2 severe hypoglycaemic episodes within the last 2 years, defined by the need for a third-party intervention to be eligible for islet transplantation.

In the UK islet transplantation success has been proven via reports on islet graft survival, annual rate of severe hypoglycaemic events, HbA1c, and insulin requirements between April 2018 and March 2022 [53]. Graft survival one year following routine islet transplant was 83%. A decrease in the median annual rate of severe hypoglycaemic events for islet-only transplants, from 10 events to 0 events, was seen from the time of registration, compared to 1 year following the islet transplant. There was a reduction in median HbA1c from 65 mmol/mol before islet transplantation to 58 mmol/mol one-year post-transplant. Lastly, the median insulin dose per kilogram of recipient body weight at the time of transplant compared to 6 months after transplantation declined from 0.42 units/kg to 0.20 units/kg, respectively. Insulin dose remained relatively stable after 1 year following the transplant with the median dose at 0.23 units/kg. 6 of the 16 patients reporting insulin dosing also achieved insulin independence at some point during their first-year post-transplantation. As reflected in this report, islet transplantation can be a lifesaving procedure for individuals with severe hypoglycaemic unawareness [58]. Optimisation

of the islet isolation process is crucial to increase the availability of this intervention within the T1DM patient population.

1.6 Current Limitations in Islet Isolation

1.6.1 Donor Pancreas Availability

One of the greatest limitations to whole pancreas and islet transplantation is the worldwide scarce availability of organ donors [34]. With the strict donor organ acceptance criteria, various complications can lead to the decline of an organ. In addition, recipients require two islet transplants to obtain maximum benefit from the procedure. Currently, the recipient will receive a minimum dose of 5,000 IEQ/kg of body weight for their first islet transplant, and within 6 months of the first transplant, the patients will receive a top-up graft with an aim to achieve 8,000 IEQ/kg overall. Although there have been developments, such as accepting DCD organs, there is still a significant shortage of donor organs that are found to be of optimal condition for pancreatic islet isolation and transplantation.

1.6.2 Long-Term Immunosuppression Medications

To prevent immune-mediated rejection, recipients of pancreatic islet transplants must be maintained on immunosuppressive drug therapy. For each potential recipient, it is important to weigh up the risks of immunosuppression with the benefits of the islet transplant. The most common immunosuppression regimens used for islet transplantation begin with the

administration of etanercept and alemtuzumab induction one hour before the patient's first islet transplant, with mycophenolate mofetil and tacrolimus immunosuppressants for maintenance following the procedure [52]. Unfortunately, severe side effects have been related to long-term use of these drugs, making these regimens a barrier to accessible islet transplantation for some, particularly, paediatric patients who may benefit greatly from an islet transplant prior to the development of any diabetes-related secondary complications [59]. Although immunosuppressive regimes impact the availability of transplantation for some subsets of patients, immunomodulation and immune-isolation techniques may help mitigate these barriers. Immunomodulation in islet transplantation involves the local release of immunomodulatory molecules that suppress local immunity. Autologous regulatory T cells (Tregs) are being explored as a potential therapy for immunomodulation which will reduce the need for immunosuppression with transplantation [60]. The goal of Tregs-mediated therapies is to increase immune tolerance via the expansion of the lymphocyte subset prior to islet transplantation [61]. Some researchers have also explored coating islets with ex-vivo expanded Tregs for local immunoprotection leading to prolonged islet survival [62, 63]. One study conveys another method with the success of synthetic biomaterials for control over material properties, molecule presentation, and therapeutic release in the facilitation of islet transplantation [64]. Evidence has shown success in biomaterial-based delivery of anti-inflammatory molecules in conjunction with islets for the prevention of immune targeting of islet allografts [64]. Immuno-isolation involves harbouring the transplanted islets from the recipient’s immune system by either using macro-encapsulation or micro-encapsulation of the islets. Such capsules are composed of hydrogels or synthetic devices which are often mimetic of the pancreas to aid

engraftment and islet survival [65]. These approaches remain experimental now, but they offer enormous potential to eliminate the need for lifelong immunosuppression, which would enable the goal of transplanting children soon after diagnosis to be reached.

1.6.3 Suboptimal Islet Survival Post-isolation

Islets undergo considerable stress throughout the isolation process, sometimes leading to partial or full cell death. Isolations can result in substantial damage to islets, which can be visualised morphologically by assessing the shape, fragmentation/disintegration, non-rounded or irregular surface of islets, or islet clumping because of deoxyribonucleic acid (DNA) released from damaged cells [66]. After isolation, the islets undergo a culture period. This allows the islets to recover from the islet isolation procedure and enables a more detailed assessment of the islets before being implanted. It also benefits the logistics of patient admission and transplant timing. The standard culture media used for this post-isolation culture period is CMRL with supplementation, however, reports share that there is a decrease in the number of IEQs, insulin secretory function, and insulin content during culture under these current conditions [43, 67]. Culture conditions including media compositions and temperature modifications have been scrutinised in hopes of enhancing the outcomes of islet culture. The addition of growth factors and compounds, such as, glutamine compounds, human albumin, insulin, and sericin has been explored as well [68]. Modifications to the temperature at which islets are cultured in CMRL have also been explored in multiple studies, with findings suggesting advantages to islet culture at 4°C compared to the standardly used culture at 37°C [66, 69]. However, further evaluation proved islet preservation at 4°C in CMRL after 48h to be more detrimental to islet survival when

compared to standard conditions currently utilised (islet culture in CMRL at 37°C) [66]. In summary, current conditions of islet culture are still sub-optimal resulting in a significant loss of islets compared to pre-culture assessment. Moreover, apoptosis (cell death) is commonly seen in islets suspended in CMRL through evident central necrosis detected during viability staining.

1.7 Future Endeavours for Pancreatic Islet Isolation and Transplantation

The future of islet isolation and transplantation for the treatment of T1DM will include efforts to optimise isolation and transplantation techniques. With the shortage of available donor organs, isolation outcomes from lower BMI donors and younger donors must be improved. Exploration and development of techniques to achieve higher islet yield and viability from unlikely donors can help mitigate the impact of donor shortages [46, 51].

Potential aspects of islet isolation that may be evaluated for improvement include the realisation of alternative cell sources such as xenogeneic and stem cell-derived β-cells. Additionally, concerning the transplantation procedure, the use of an alternative transplant site may yield to better overall islet engraftment [70]. Some sites that have been explored for islet engraftment include the pancreas, omentum, kidney capsule, peritoneum, gastric submucosa, testis, and thymus [71]. However, throughout these explorations challenges have impacted the success of islet transplantation using sites other than the liver, hindering the discovery of a more optimal

alternative transplant site. With further research, an alternative transplantation site for pancreatic islets may increase the efficiency of this intervention.

1.8 Human Islet Recovery Following Isolation

Following the islet isolation procedure, human islets require time to recover due to the stressful conditions of the isolation process. This period is beneficial to clinical islet transplantation outcomes as it allows for islets to recover, reassessment of islet quality, recipient travel time to the transplant facility, and initiation of immunosuppressive drug therapy [72]. It is reported by various studies, however, that isolated islets deteriorate in this culture period [69, 72, 73]. The Oxford Group have previously shown that there is significant disruption to the human islet basement membrane (BM) during the post-isolation culture period when islets are cultured in CMRL-supplemented media. The key matrix proteins assessed in this study include collagen IV, pan-laminin (total laminin), perlecan, and laminin-α5. Findings expressed a significant loss of pan-laminin in islets after culture up to 72 hours, compared to islets pre-culture, and complete loss of laminin-α5 and perlecan islets after isolation, which was not re-established during culture [74]. Based on the conclusions drawn in this study, there is a need for further evaluation of post-isolation culture period conditions to optimise islet recovery and survival for transplant. Further evaluation of the human islet BM structure during this period is important for understanding the BM's impact on islet survival in culture.

1.9 Extracellular Matrix (ECM) in the Human Pancreas

1.9.1 Human Pancreatic ECM Overview

The pancreatic islet BM in humans is comprised of various ECM proteins. This structure is integral in pancreatic islet function and survival in vivo. Removal of the islet BM can result in anoikis, a type of cell death characterised by matrix detachment. Key ECM proteins found in the pancreatic islet BM include laminins, collagen IV, and heparan sulfate proteoglycans (HSPGs). A distinguishing component of human pancreatic islets is the duplex BM structure containing the peri-islet BM and the vascular BM.

1.9.2 Peri-islet BM

Extracellular matrices, commonly referred to as the BM, can be detected on some, but not all cell surfaces, surrounding muscle, fat, epithelial and endothelial cells, and Schwann cells. These matrices assemble themselves from various components via binding interactions between proteins including collagen IV, laminins, nidogens, and HSPGs, such as perlecan. The BM is an extension of the plasma membrane that acts to protect tissues and cells from external disruption and serves as an interface for cell signalling throughout the body [75, 76]. The islet BM has been well studied for pancreatic islet isolation and transplantation in rodent models because of genetic similarities between the two species and the limited availability of human islets. However, the islet BM in mice differs substantially from the human BM. The pancreatic islet BM in mice is understood to exist as a single-layer BM, but in humans' islet BMs have been described with a duplex BM including the peri-islet BM surrounding the perivascular BM [76-78]. This human islet

structure suggests endocrine islets are surrounded by a BM separate from the endothelia [77]. Due to stress induced upon islets during enzymatic digestion in isolation a considerable portion of the peripheral ECM surrounding islets are effaced. This damage to the BM increases cell anoikis and immunogenicity, enhancing risks of graft rejection during transplantation [76, 79, 80].

1.9.3 Vascular BM

Pancreatic islets are highly vascularized with dense vascular capillary networks. These networks are primarily formed from vascular endothelial growth factor (VEGF) R2-expressing endothelial cells that respond to elevated levels of the vascular endothelial growth factor VEGF-A. The importance of the dense vascular network lays in its contribution to endocrine function and islet size. Although islets contribute only 1-2% of the pancreas, these cells receive up to 20% of total blood flow to the organ [81, 82]. Adequate vascularization of islets is necessary for insulin release kinetics, as well as the removal of metabolic waste [83]. Due to the harsh enzymatic digestion procedure during islet isolation, islet endothelial cells may be removed or damaged, contributing to transplant failure [84]. Further damage to this highly innervated vasculature during the post-isolation culture period may induce cell apoptosis due to hypoxia and impair islet engraftment post-transplantation [68].

1.9.3.1 Collagen IV

Collagen IV is one of the major glycoprotein components within the vascular BM [84], which is suggested to define the BM scaffolding through the integration of other components [85]. Each

collagen molecule is made up of a triple helix structure of three polypeptide-producing (PP) chains. The structure of this fibrous protein provides scaffolding for structural stability and assembly with other proteins (Figure 5) [86]. A key collagen IV isoform found within BMs possesses PP chains α1.α1.α2 (IV), which can lead to defects in BM stability if both α-chains are deleted. All collagen IV isoforms have more structural flexibility than fibrillar collagens, allowing for interaction via uncleaved terminal domains that guide the assembly of a multi-layered network [87]. As collagen IV works within the pancreas as a scaffold for the basement membrane, it functions to regulate insulin secretion [88]. The integrity of collagen IV is disrupted in islet isolation due to the use of digestive enzymes. Collagenases, used for enzymatic digestion in isolation, are proteins that disrupt native collagens in vivo and in vitro [89]. NPs, which also degrade collagens, are used in conjunction with collagenases to further ECM digestion. With the use of this enzymatic blend, there is increased accessibility to collagen. The presence of NPs damage proteoglycans that surround collagen structures, exposing the collagen molecule to be cleaved [90]. As collagen IV provides a self-assembling scaffolding for nidogen and perlecan crosslinking [75], the degradation of this protein may impact the survival and function of the islets upon and following isolation from the pancreas.

1.9.3.2 Laminins

Laminins are classified as heterotrimeric glycoproteins that are made up of α-, β-, and γ-chain(s). Laminins are vital to the survival of an organism [88]. Like collagen IV, laminin is a self-assembling protein that supports binding interactions among other BM proteins. As nidogen and perlecan possess binding properties to both collagen IV and laminin, it is suspected that they connect the

collagen IV and laminin networks in the BM (Figure 5) [87]. When assessing the impact of BM laminin presence on insulin gene expression in mice, findings concluded that β-cells must interact with a significant density of laminin molecules to activate insulin gene expression [84]. In pancreatic islets pan-laminin and laminin-α5 assist in cell proliferation and insulin transcription [88]. Evaluation of laminin chains in the duplex structure of the human islet BM has been completed via antibody-specific markers for human laminin. Findings imply that laminin-α5, is detected in both layers of the duplex BM structure, unlike other proteins investigated. [77]. The existence of laminin-α5 in both layers of the human islet BM presents the opportunity for exploration into the depth of disruption seen in the islet BM following isolation, and culture.

1.9.3.3 Heparan Sulfate Proteoglycans (HSPG)

HSPGs are glycoproteins that possess multiple binding sites for other ECM components and receptors. One of the most referred to HSPGs that mediates both growth factors and adhesive cell molecules is perlecan. Growth factors mediated by this protein include fibroblast growth factors (FGFs), VEGFs, and hepatocyte growth factor (HGF). This intra-islet vascular BM component also interacts with laminins and collagen IV, contributing to the overall superstructure of the human islet BM. Perlecan may affect insulin transcription, secretion, and/or cell proliferation within pancreatic islets [88].

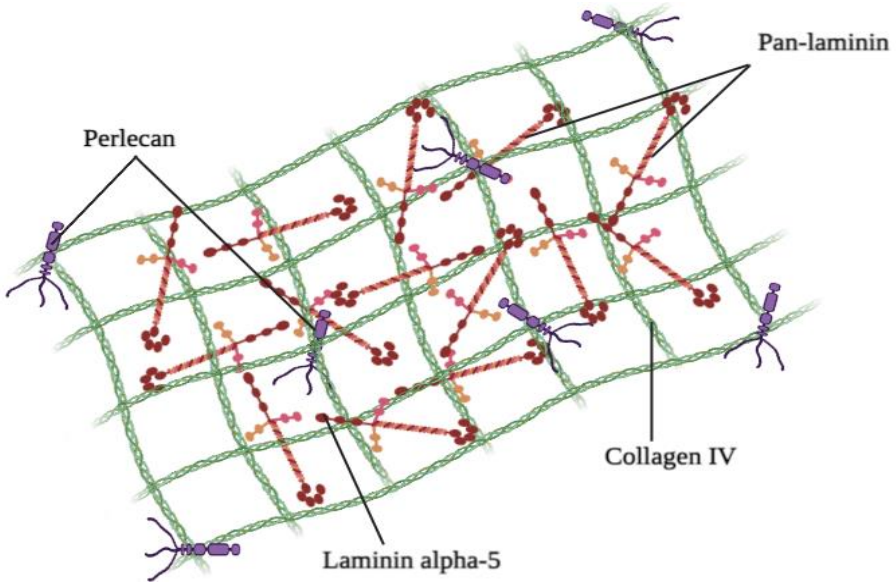


Figure 5: Basement Membrane (BM) Scaffolding Structure
Collagen IV protein scaffolding with binding interactions from laminins and perlecan.

1.10 Investigation of Post-Isolation Culture Conditions

1.10.1 Currently Practiced Post-Isolation Culture Conditions

Currently, the conditions of the post-isolation culture period for recovery of islets are suboptimal. As discussed above, during this time islets are placed in culture media (CMRL-1066 with supplementation) and incubated at 37°C for up to 48 hours. However, under these conditions there is a significant loss in the number and viability of islets, impacting transplant outcomes. Considering the effects of cold storage, researchers investigated the preservation of islets during this period under such conditions [91]. Some studies have explored the use of UW solution for the cold storage of animal islets following isolation [92, 93]. Investigations of the impact of cold storage using CMRL has also been completed. Some findings suggested that the initial success of islet culture in CMRL at lower temperatures is due to the decreased oxygen demand during

preservation. However, another study demonstrated that after 48 hours in CMRL at 4°C there was an abundance of fragmentation and a decline in overall IEQ within the islets suspended and stored under these conditions. These same outcomes were not seen in islets suspended in UW solution [66]. The composition of UW cold storage solution may help better preserve cells as the use of certain additives to prevent necrotic injury, including cell swelling and lack of osmotic support may contribute to the prolonged survival of islets in these conditions [94]. The direct mechanism of better islet survival in UW solution is not fully understood, however, the preservation and/or recovery of the islet basement membrane in UW cold storage may contribute to prolonged cell survival and function in vivo.

1.10.2 UW Cold Storage Solution

UW solution is used for the preservation of various transplant organs including the liver, kidney, and pancreas. This solution preserves organs by stopping the occurrence of osmotic imbalance caused by surrounding impermeable anions, and cell damage by external free radicals [94]. Used within the Oxford Consortium for Islet Transplantation (OXCIT), Bridge to Life, Belzer UW® Cold Storage Solution (UW Solution) is composed of various components (see appendix, Table A3, Bridge to Life, Belzer UW® Cold Storage Solution Components, for full solution composition) that work to preserve organs and cells. The impact of this media for islet storage has been investigated, and some findings suggest that the Glutathione component of the solution aids in maintaining membrane integrity [95].

1.10.3 CMRL-1066 Medium

CMRL is a nucleoside and vitamin-rich medium suitable for various human cells. This media was created initially as Medium 199 by Connaught Medical Research Laboratories. That media was amended to CMRL-1066, which is used today, to culture mouse L-cells in protein-free conditions. The modifications to the initial media included elevating the levels of reducing substances (cysteine, glutathione, and ascorbic acid), eliminating fat-soluble vitamins, variations to nucleic-acid precursors, and adding coenzymes [96]. Currently CMRL-1066 Medium (PAN-Biotech) with supplementation is the gold standard media used to house post-isolation islets during the initial 24-hour culture period and up to the point of infusion (see appendix, Table A4, for complete PAN-Biotech CMRL-1066 component composition). Currently, islets are preserved in cold CMRL media for autologous and allogeneic transplants whilst the patient is prepped for the infusion procedure. One study compares the survival of human islets cultured/preserved up to 48 hours in CMRL at 4°C and 37°C. Findings infer that islets stored in CMRL at 4°C showed increased fragmentation after 24 hours, lack of recovery after 48 hours, and significant loss in viability after 48 hours [66]. Thus, the composition of the media, when compared to other culture/preservation solutions, may be a large contributing factor to islet survival.

1.10.4 Differentiation Between CMRL and UW for Islet Culture/Preservation

When comparing the use of UW solution to CMRL for post-isolation islet culture and preservation, various aspects are evaluated. In previous studies, islet morphology, islet recovery, islet viability and function, presence of mRNA-375, and expression of inflammatory and hypoxia-

specific genes were evaluated for both medias [66]. One group has shared better-maintained islet morphology after preservation in UW cold storage solution at 4°C compared to storage in CMRL media at 22° and 37°C [97]. In another study, researchers evaluated the use of UW solution and CMRL media at these temperatures and reported islets suspended in UW to have displayed the highest recovery percentage among all groups at 24 and 48 hours [66]. A novel consideration is the behaviour of the islet BM during culture in both systems. Although islet culture and preservation have been experimented with under these proposed conditions, there is minimal understanding of the BM integrity before and after this culture period. As referenced above, previous work within the Oxford Group investigated the presence of key matrix proteins following islet culture in CMRL [74]. Further evaluation of the impact on these key matrix proteins of islets preserved in UW cold storage solution at 4°C is necessary, as the optimisation of islet BM preservation will lead to the improvement of islet survival and function.

Aims of Thesis

The research outlined in this thesis aims to compare the impact of UW cold storage solution for the maintenance of human pancreatic islets post-isolation to culturing of human pancreatic islets post-isolation in CMRL, the current gold standard culture media. Specifically, the hypothesis was that UW may better maintain the integrity of the islet BM which in turn, might help preserve islet viability and increase the number and quality of islets available for transplantation. To best understand the impact of each post-isolation culture/preservation condition, an extensive review

of the use of CMRL and UW solution for post-isolation, pre-transplantation storage of human pancreatic islets is investigated below.

Chapter 2

Pancreatic Islet Isolation, Collection, and Post-Isolation Assessment

2.1 Introduction

Isolated islets are assessed using various standard operating procedures (SOPs) for IEQ, islet count, islet viability, and sample purity. The findings of these required post-isolation assessments determine whether the isolation product will be suitable for transplantation. Current clinical release criteria for pancreatic islet transplantation products in the UK can be found below in Table 3. If the pancreatic isolation product meets the respective release criteria, islets are then placed into culture for the mandatory culture period. If the isolation product does not meet the required parameters the cells may be released for research, where there is appropriate consent for research purposes.

During the mandatory culture period, islets are placed into CMRL media supplemented with human albumin, HEPES, and glutamine. After a minimum of 24 hours in culture, islets are then re-assessed for the same parameters (IEQ, count, viability, and islet purity). If the assessments prove that the islets still meet the respective criteria, then the cells are prepared for transplant.

Although CMRL is considered the gold standard media for this culture period, it has been shown that there is a large loss of islets under these culture conditions [69]. Apoptosis is known to occur during the extended culture of islets in vitro, primarily impacting the insulin-producing β-cells [98]. Several factors are understood to cause apoptosis of the cells during this phase including hypoxia due to loss of vasculature, and cellular stresses leading to disruption in the cell-to-matrix relationship (resulting in anoikis) [99].

UK Release Criteria for Post-Isolation Islet Transplant Product	
Parameter	**Outcome**
IEQ*	5000 IEQ/kg of recipient body weight
Viability	> 70%
Purity	> 50%
Sterility	Negative gram stain

Table 3: UK Release Criteria for Post-Isolation Islet Transplant Product
*IEQ, Islet Equivalent.

2.2 Chapter Aims

The experiments outlined in this chapter compared the efficiency of CMRL medium and UW cold storage solution for maintaining pancreatic islets during the standard culture period. The protocols and procedures, beginning with donor organ procurement, up to post-isolation morphology assessments (islet counting, cell viability assessment, and purity assessments) are deliberated in depth to convey the importance and significance of islet survival in both media.

2.3 Methods

2.3.1 Donor Organ Procurement

Pancreases were offered as part of the UK NHS Islet Isolation and Transplantation Programme. The organs were transported to the DRWF Oxford Islet Isolation Facility under nationally agreed conditions within an NHSBT-certified organ transport box. Pancreases were perfused at the point of retrieval with UW solution. Following isolation, islets that did not go for transplantation

(mainly due to insufficient islet yield for named recipients') were made available for this study. For all included samples, appropriate written informed consent for research purposes was provided by the donor families and ethical approval for the study was in place. One pancreas used in this study was procured following decline for clinical use after retrieval. Although this organ was not accepted for transplantation purposes, the isolation procedure was performed to the same clinical standard as the other organs that had been accepted for transplantation.

2.3.2 Islet Collection and Purification Protocol

As per the process described in Chapter 1, islets were isolated using the well-established semi-automated approach.

2.3.3 Sample Plating and Collection

Following the isolation process islets were plated out as per the sample collection overview displayed below (Figure 6). 'Fresh' samples were collected immediately following isolation prior to any culture period. 1,000 fresh islets were collected and assessed for count, viability, purity, and GSIS. To compare the different culture conditions, islets were plated in 6-well plates. Each well contained 1,500 islets, suspended in 3 ml of the indicated media. One 6-well plate contained UW solution, supplemented with an additional 500 μl of human albumin, which was used to coat the plate before the addition of the islets, to prevent cell adhesion. The second 6-well plate contained islets suspended in CMRL. The UW solution plate was placed in a fridge at 4°C, whereas the CMRL islets were cultured in a tissue culture incubator (Binder) at 37°C containing 5% CO_2.

Sample collection was then completed every 24 hours. Samples were collected in the microbiology safety cabinet to prevent contamination to subsequent sample time points. For each time point, the islets were gently agitated and 100 μl was collected from each well for count and viability. For the GSIS assessment, approximately 300 islets were collected from each well at the 48-hour sample collection time point (See appendix Figures E1-E3 for detailed GSIS findings).

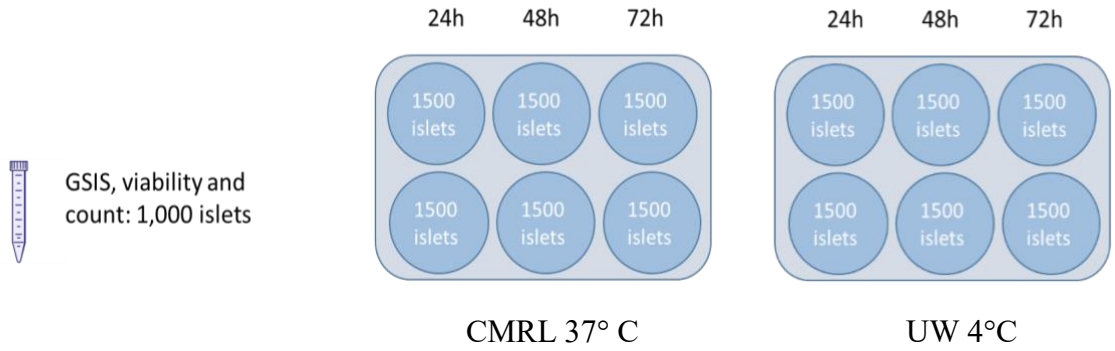


Figure 6: 6-well sample plate layout for UW and CMRL
1,000 islets were collected at each sample collection time point for count, viability, and GSIS assessment. 9,000 islets were plated out into respective CMRL or UW 6-well plates with 1,500 islets/well.

2.3.3.1 Islet Counting

At all collection time points the number of islets and islet equivalents were counted manually as per OXCIT SOP 5.6.5. 200 μl of prepared dithizone solution was added to 4 pre-labelled tubes. 100 μl of sample was collected from each well at each respective collection time point (2 counts per sample collection, per medium) was added to the corresponding pre-labelled tube and an additional 800 μl Hank's Balanced Salt Solution (HBSS) (Sigma) was added to each falcon tube. Using a surgical pipette, the contents from each falcon tube were pipetted onto either side of a 90 mm petri dish (Fisher Brand) linearly, creating "streaking" of the sample. Each well plate was counted using a stereomicroscope (Wild Heerbrugo) equipped with a visual islet size reference

chart within the microscope. Each islet visualised was recorded on an islet count scoring sheet. At the end of the counting procedure, each count was normalised using a standardised calculation to determine the IEQ. An example of the scoring sheet used with values for the IEQ calculation is reflected below (Figure 7). Once each count was completed and IEQ determined, the fragmentation index was calculated by dividing the number of islets counted in each sample by the IEQ calculated for that sample (IPN/IEQ). To determine the significance of the findings, a Tukey's multiple comparisons test was performed using GraphPad Prism 10 (San Diego, USA).

OXCIT

OXFORD CENTRE for ISLET TRANSPLANTATION

OXSOP 5.6.5i
ISLET COUNTING RECORD

HP			Date:			Time:			
	<50	50-100 Size 5	100-150 Size 6	150-200 Size 7	200-250 Size 8	250-300	300-350 Size 9	350-400 Size 10	>400
CMRL T									
CMRL B									
UW T									
UW B									
		x0.167	x0.65	x1.7	x3.5	x6.3	x10.4	x15.8	x22.8
Total IEQ				x Multiplication Factor			=		

Figure 7: Islet Counting Record Sheet
Example of islet counting record sheet used during islet counting at each sample collection. Specific values for the calculation of IEQ are displayed in the appropriate column for calculation reference.

2.3.3.2 Islet Viability

At all collection time points, the islet viability was scored as per OXCIT SOP 5.6.3i. At each collection time point, 100 μl of the sample was collected in a biopsy dish (Thermofisher) from the top and bottom wells of each respective medium. Islets were centred in the dish by swirling the dish, then excess media was removed and replaced with 1 ml of HBSS. A selective staining solution was made up using 500 μl ethidium bromide (EtBr) (Bio-Rad), and 10 μl fluorescein diacetate (FDA) (Sigma) in a 1.5 ml Eppendorf tube (Eppendorf). 200 μl EtBr/FDA stain was added to the biopsy dish with HBSS and islets and left for 5 minutes, protected from light, to allow islets to stain. Islets were then recentred in the dish, and 70-80% of the media was removed and replaced with 1 ml HBSS. Using an Olympus microscope (Zeiss) and ZENworks software (Zeiss), islets were assessed for viability and imaged. Findings were recorded on an OXSOP 5.6.2 Islet Viability Staining sheet (Figure 8). As with the counting protocol, statistical analysis was completed using GraphPad Prism 10 by way of the Kruskal-Wallis test, using Dunn's multiple comparisons test.

OXCIT

OXFORD CENTRE for ISLET TRANSPLANTATION

OXSOP 5.6.2
ISLET VIABILITY STAINING

	HP:			Date:		Time:	
	ISLET VIABILITY: FLUORESCEIN DIACETATE/ETHIDIUM BROMIDE						
	90-100%	80-90%	80-70%	70-60%	60-50%	40-50%	<40%
CMRL							
UW							
Mean Viability %							

Figure 8: Islet Viability Staining record sheet
The record used when determining the viability of islets at each sample collection point (15-20 islets tallied in each sample for calculation of overall viability of sample).

2.3.3.3 Sample Purity

At all collection time points the purity of the sample was assessed via differential dithizone staining. The outcome of this assessment reflects the ratio of exocrine tissue to islets within the sample at the time of collection.

2.3.4 Statistical Analysis

Data is presented as mean ± standard deviation (SD). The normality of the data was determined by the Shapiro-Wilk test. Statistical significance was determined by way of ANOVA using Tukey's multiple comparisons test. Sample purity significance was determined using Kruskal-Wallis – Dunn's multiple comparison test. Statistical analysis was completed using GraphPad Prism 10 (San Diego, USA). Significance was recorded for results with *p*-values of less than 0.05.

2.4 Results

Donors (n=5) were selected using the established clinical isolation acceptance criteria for OXCIT. Using histological assessments, IEQ, islet number (or count), islet viability, and sample purity was evaluated for each sample collection for both mediums. Statistical analysis was completed using GraphPad Prism 10, and Tukey's multiple comparison test.

2.4.1 Specific Donor Demographics

N=5 donors were procured from 4 clinical islet preparations and 1 research islet preparation (Table 4). For additional, and subsequently omitted donors (n=2), data reflected that a poor islet isolation product (poor tissue quality and/or low viability) does not recover in either CMRL or UW during the culture period following isolation (discussed further in Chapter 5, see Table 10 and appendix Figures E1-E3).

	HP2302	HP2304	HP2307	HP2311	HP2340	Mean ± SD
Age, y (Mean ± SD)	52	53	48	35	36	44.8±8.7
Gender (M: F)	F	F	F	M	F	1:4
BMI (Mean ± SD)	23	32	34	33	32	30.8±4.4
CIT (hours)	12	5	7	6	5	7.0±2.9
Donor Type (DBD: DCD)	DBD	DBD	DBD	DBD	DBD	5:0

Table 4: Sample set (n=5) donor demographics for Chapter 2
*CIT, Cold Ischemia Time, *DBD, Donor by Brain Death, *SD, Standard Deviation. Mean and SD were determined with the Wilcoxon test.

2.4.2 IEQ, Count, and Fragmentation Index

Isolated islets were cultured in either CMRL or UW solution. Islets were collected every 24 hours, up to 72 hours, and assessed for count, fragmentation, viability, and purity. There was a significant loss in IEQ seen in the collection of islets in CMRL at 48 hours, when compared to the fresh sample (*p<0.05; Figure 9). There was also a significant difference in the IEQ of islets suspended in CMRL at 72 hours and UW at 72 hours (*p<0.01; Figure 9). Overall, there were more islet particles counted in UW at 24, 48, and 72 hours, when compared to CMRL samples collected a 24, 48 and 72 hours (Figure 10). There was a significant difference (*p<0.05; Figure 10) in the

number of islets suspended in CMRL at 48 and 72 hours in comparison to those islets suspended in UW at 48 and 72 hours, respectively (Figure 10). There was no significance detected in the fragmentation index of islets in either condition (Figure 11).

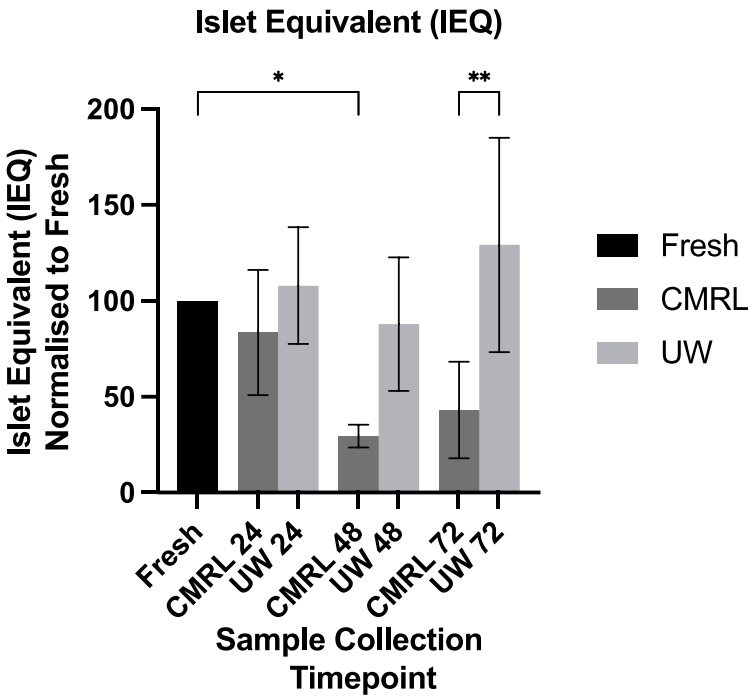


Figure 9: Islet Equivalent (IEQ)
Islet Equivalents were determined for each sample collection manually using the technique described in the methodology of this chapter. There was a significant loss in IEQ after 48 hours in CMRL, compared to the fresh islet sample (*$p<0.05$). There was a significant difference in the IEQ among CMRL and UW samples at 72 hours (*$p<0.01$). Statistical Significance was determined by way of ANOVA using Tukey's multiple comparisons test.

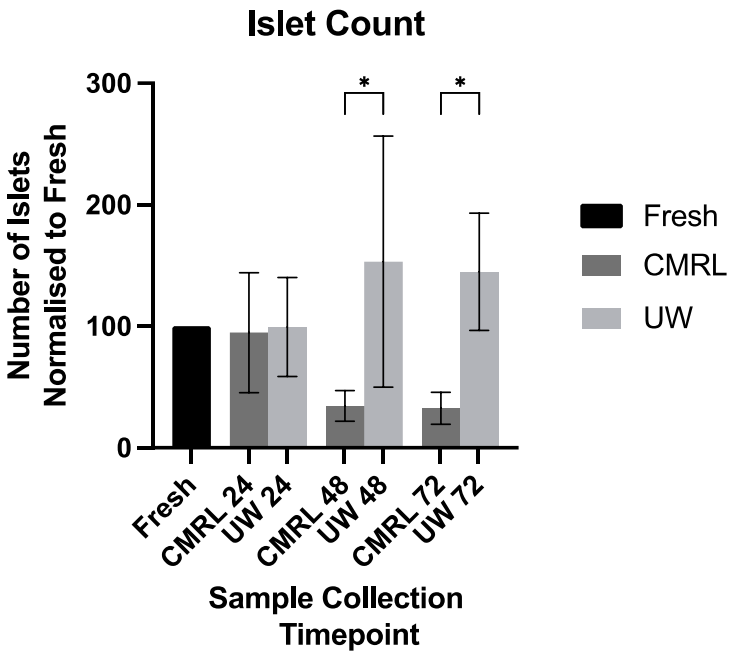


Figure 10: Islet Count

This graph displays the number of islets in each media (CMRL and UW) after isolation and 24, 48, and 72 hours in culture. There is a significant difference in the number of islets seen in CMRL at 48 hours and UW at 48 ($p<0.05$*) and 72 hours ($p<0.05$*). Statistical significance was determined by way of ANOVA using Tukey's multiple comparisons test.

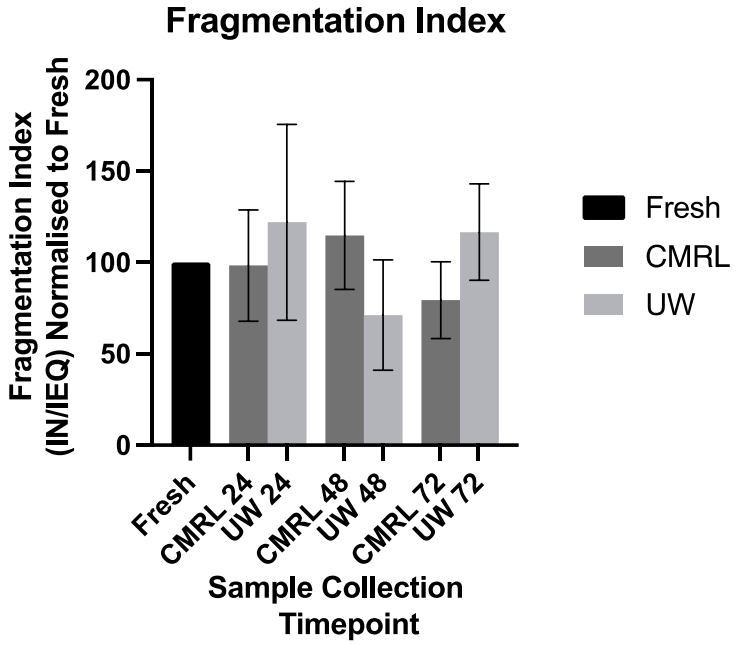


Figure 11: Fragmentation Index
There was no significance in Fragmentation Index detected among the two groups as determined by way of ANOVA using Tukey's multiple comparisons test.

2.4.3 Islet Viability

Islet viability significantly declined at 48 hours in CMRL, compared to the fresh sample (*p<0.0001; Figure 12). A significant decline in the viability of islets in CMRL at 72 hours (*p<0.0001; Figure 12) compared to the fresh sample was also detected. There was no significant decline in the viability of fresh samples of islets stored in UW for up to 72 hours. There was a significant difference between islets cultured in CMRL at 48 hours and islets stored in UW at 48 hours (*p<0.01; Figure 12). Lastly, there was a significant difference in viability detected at 72 hours between islets suspended in CMRL compared to UW (*p<0.01; Figure 12). Viability imaging showed a decline in islet viability of islets cultured in CMRL up to 72 hours, with visible central necrosis that was seen through the microscope shown in some imaging (Figure 13).

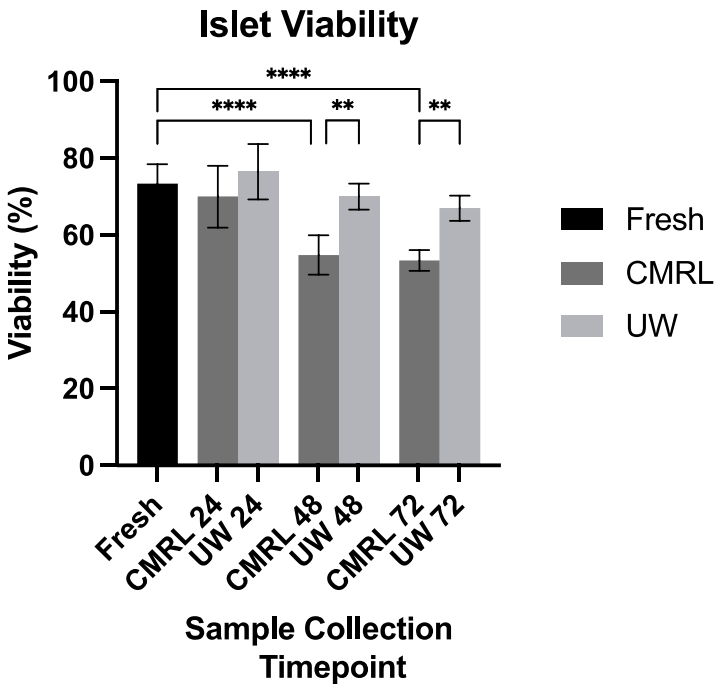


Figure 12: Islet Viability

There was a significant loss of viability seen in islets suspended in CMRL at 48 hours compared to fresh islets (*p<0.0001), and at 72 hours in CMRL compared to fresh islets (*p<0.0001). A significant difference in viability was seen in islets suspended in CMRL at 48 hours and islets suspended in UW at 48 hours (*p<0.001), and a significant difference in viability between the islets suspended in CMRL versus UW was detected at 72 hours as well (*p<0.001). Statistical significance was determined by way of ANOVA using Tukey's multiple comparisons test.

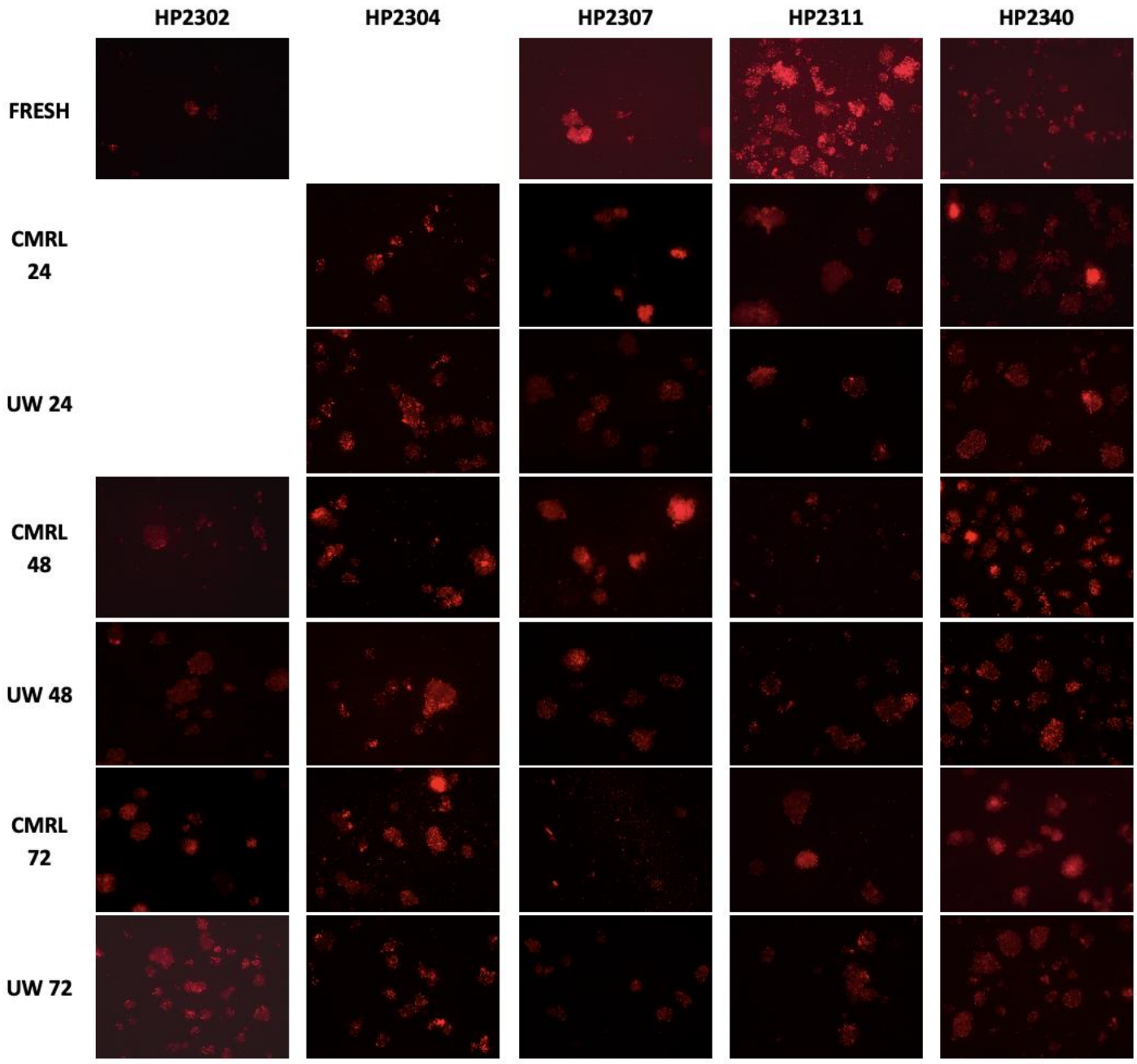


Figure 13: Islet viability imaging at each collection time point
Images reflect the sample assessed for viability at each collection time point, in either media, for the cohort of donors (n=5) included in this thesis. Viability staining was achieved using a selective (EtBr/FDA) stain. Imaging for the 'HP2302 24-hour' sample collection in both media and the 'HP2304 Fresh' sample was not completed due to microscope imaging software malfunction.

2.4.4 Sample Purity

Sample Purity was maintained in both groups for up to 72 hours. There was no significant difference in the purity of CMRL or UW samples (Figure 13).

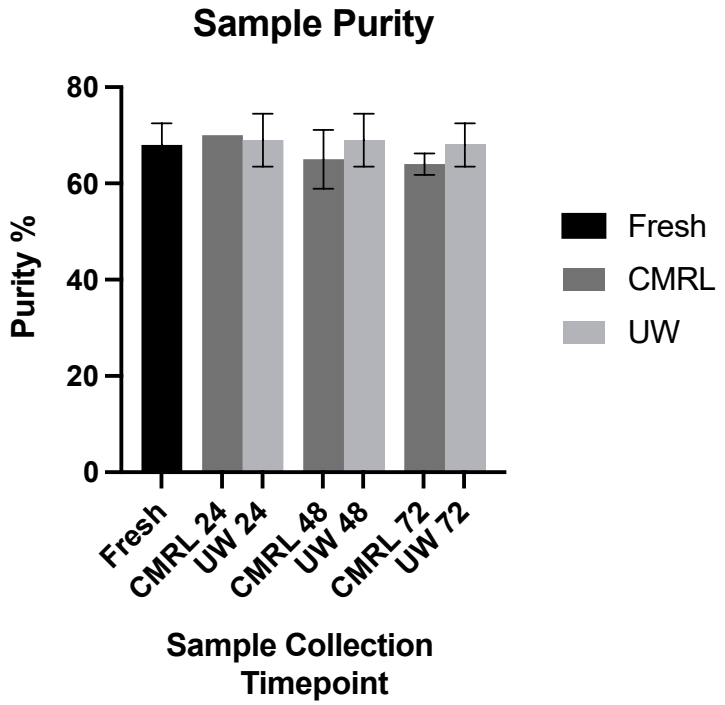


Figure 14: Sample Purity
There was no significance detected for Sample Purity among the two groups as determined by way of ANOVA using Tukey's multiple comparisons test.

2.5 Discussion

To achieve successful islet transplantation following the islet isolation procedure islet preparations must be assessed and meet the requirements for islet counting, viability, and purity. Each of these assessments may help to indicate the quality of the transplant product, and thus the success of islet function and survival in vivo. Previous research has demonstrated that UW is superior to CMRL for islet storage at 4°C [97]. Whilst it is well-known that UW cold storage is

better for islet storage, the underlying mechanisms surrounding this are lacking. The decline in the number of islets seen in CMRL is indicative of the progressive cell death that was visualised in each sample at each subsequent collection time point. It is well-studied that islet culture in this medium is associated with a loss of islets, however, the cause of this loss has not been concluded. The high significance detected in UW and CMRL groups for islet viability also indicates the increased cell death in those islets suspended in CMRL for up to 72 hours. It is important to better understand what the cell BM components look like at each collection point to conclude factors that may contribute to superior islet survival in UW. As elaborated on above in the section 'ECM of The Human Pancreas', each key matrix protein plays an imperative role in islet function and structure. The various components of the islet BM are involved in important functions of the islet including the secretion of insulin via β-cells. To ensure the islets transplanted are functioning as needed the human islet BM must remain intact during the islet isolation, and the following culture period. It is also possible that better preservation on the islet BM will allow for better reconstitution of the BM in vivo, potentially leading to better engraftment survival.

2.6 Conclusions

UW is superior to CMRL for the storage of islets following isolation. This is demonstrated in the islet assessment data shared above and has been determined by groups previously. The route cause for this is not understood, and further investigation into the human islet BM at each of these conditions may shed light on the key to better islet survival during the post-isolation recovery period.

Chapter 3

The Native Pancreas

3.1 Introduction

The ECM within the native pancreas is comprised of multiple proteins that aid in the regulation of β-cell proliferation, differentiation, and insulin secretion [100]. This intricate network of proteins and proteoglycans contains collagens, elastin, fibronectins, and laminins. During islet isolation, collagenase isoforms and NPs are used in conjunction to digest the pancreatic ECM. It is well known that this process causes a considerable amount of stress to the desired pancreatic islets. To ensure damage to islets is minimalised, there is close monitoring of enzymatic and mechanical digestion progress during the isolation process. It is imperative that there is minimal disruption to the islet BM structure during this phase of the isolation process and during the succeeding post-isolation culture period. A comprehensive understanding of the native pancreas environment is crucial to optimising the survival of these cells in vitro and once transplanted, in vivo.

3.2 Chapter Aims

The aim of investigating ECM proteins within the native pancreas is to understand the existence of each protein prior to pancreatic islet isolation. Protein presence before isolation compared to post-isolation may aid in the understanding of islet survival and function during this phase. The localisation and volume of each protein to individual islets may indicate better islet survival during pancreas digestion, as well as better recovery during the post-isolation culture period.

3.3 Methods

3.3.1 Whole Tissue Lysate

Approximately 20mg of frozen pancreatic tissue were homogenised in 250 µl of RIPA buffer (Sigma Aldrich), see appendix, Table C1, for composition, containing protease inhibitor cocktail (PIC) (Complete™ Mini Protease Inhibitor Cocktail, Roche). The Eppendorf tubes (Thermofisher Scientific) used for the homogenisation were low protein binding. One steel homogenisation ball was added to each tube (Retsch UK limited, Hope Valley, UK). Homogenisation was completed using a Retsch mixer mill (Retsch UK limited). Four x 60-second homogeniser cycles were completed, at a frequency of 25. Between each cycle, the tissue was cooled on dry ice for 30 seconds. Samples were centrifuged at 14,000 rpm for 5 minutes, and frozen at -20°C until use.

3.3.2 Immunolabelling of Pancreatic Tissue Sections for ECM Proteins and Insulin

Using a cryostat (Leica) frozen pancreatic tissue sections were prepared and collected on Superfrost glass slides (ThermoFisher), then frozen at -20°C until use. Slides were defrosted and washed with Tris-Buffered Saline (TBS) (Thermo Scientific) for 3 minutes to remove any excess optimal cutting temperature compound (OCT) (Sigma). 2.5% paraformaldehyde (PFA) (Sigma) was added to each section (150 µl/section), and the slides were then incubated in a wet chamber for 5 minutes. The slides were then washed in tap water with continuous flow for 5 minutes, and subsequently, TBS for 3 minutes. 150 µl blocking buffer; 1/10 goat serum (Vector Laboratories) supplemented with TBS (Sigma-Aldrich) for proteins laminin, laminin-α5, and perlecan, was added to each section to eliminate unspecific binding of ECM proteins. For collagen IV

immunolabelling, 150 μl blocking buffer; 5% swine serum (Vector Laboratories), supplemented with TBS, were added to each tissue section. Slides were incubated within a wet chamber for 30 minutes at room temperature. For all proteins, after 30 minutes, the blocking buffer was tipped off the slides and the primary antibody was applied to each section. 150 μl primary antibody (Guinea pig anti-human insulin; OCDEM, University of Oxford, Donkey anti-collagen IV; Bio-Rad, Rabbit anti-laminin; Abcam, Mouse anti-laminin-α5; Abcam, and Mouse anti-perlecan; Abcam) (appendix, Table C2, for host, dilution, and catalogue number) diluted in TBS was added to each tissue section prior to incubation in wet chamber at room temperature for 1.5 hours. Following incubation, slides were washed in fresh TBS for 4 minutes, 3 times. Secondary antibodies insulin (Donkey anti-guinea pig Alexa Fluor 594; Jackson Immunotech), collagen IV (Donkey anti-goat Alexa Fluor 488; Life Technologies), laminin (Goat anti-rabbit Alexa Fluor 488; Life Technologies), perlecan and laminin-α5 (Goat anti-mouse CFL; Santa-Cruz)) were diluted in TBS, in light protected environment (see Appendix, Table C1, for host, dilution, and catalogue number). 150 μl secondary antibody was added to each tissue section. Slides were incubated within a wet chamber at room temperature, protected from light, for 30 minutes. After incubation, slides were washed in TBS tanks as stated after primary antibody incubation. Using Vectorshield Hard-set Anti-fade (ThermoFisher) mounting media, slides were mounted with 22mm by 50mm coverslips. Slides were left for 30 minutes at 4°C to allow setting. In a dark room, slides were imaged using an Olympus microscope (Zeiss) with ZENworks software (Zeiss).

3.3.3 Enzyme-linked Immunosorbent Assay (ELISA)

The quantification of specific ECM proteins was completed using ELISAs. Proteins investigated from native pancreas samples include collagen IV, pan-laminin, laminin-α5, and perlecan (see appendix, Table C3, for manufacturer information and catalogue number). For each ELISA, 10 μg of homogenous pancreatic tissue lysate was loaded per well, as determined by bicinchoninic acid (BCA) assay (Peirce BCA protein quantification kit, Thermofisher Scientific). Each ELISA was run following the protocol provided by the manufacturer. The numerical value of specific ECM protein detection was analysed using GraphPad Prism 10 (San Diego, USA).

3.4 Results

Donor (n=6) samples were collected from clinical isolation preparations. The donor demographics from the cohort used for this analysis are reflected in Table 5 below. Histological and quantitative conclusions reflect the presence of ECM proteins: collagen IV, laminin, laminin-α5, and perlecan detected in the native pancreas. To quantify the amount of each protein, ELISAs were performed for 4 donors (Figure 15). Findings suggest the protein, collagen IV, is the most abundant of the four proteins examined (45.23 $\pm$ 9.51 ng/ml). The quantities of laminin (4.07 $\pm$ 0.72 ng/ml), laminin-α5 (1.54 $\pm$ 0.75 ng/ml), and perlecan (0.04 $\pm$ 0.004 ng/ml) were much lower than detected for collagen IV (Table 6). Immunolabelling of pancreatic tissue sections complimented the ELISA data and demonstrated the presence of each ECM protein within the ECM scaffolding throughout the native pancreas and both layers of the human islet BM (Figure 16).

Native Pancreas Donor (n=6) Demographics	
Donor Demographic	**Donors (n=6)**
Age, y (Mean ± SD*)	44 ± 12.26
Age, y (Median (Range))	49 (25-55)
Gender (M: F*)	4:2
BMI (Mean ± SEM)	27.17 ± 3.79
CIT, h (Mean ± SEM)	6.54 ± 1.08
Donor Type (DBD: DCD)	6:0

Table 5: Donor Demographics for Chapter 3
Donor demographics include HP identification number, age, body mass index (BMI), cold ischemia time (CIT), donor type, cause of death, and gender.
*SD, Standard deviation
*M: F, Male: Female

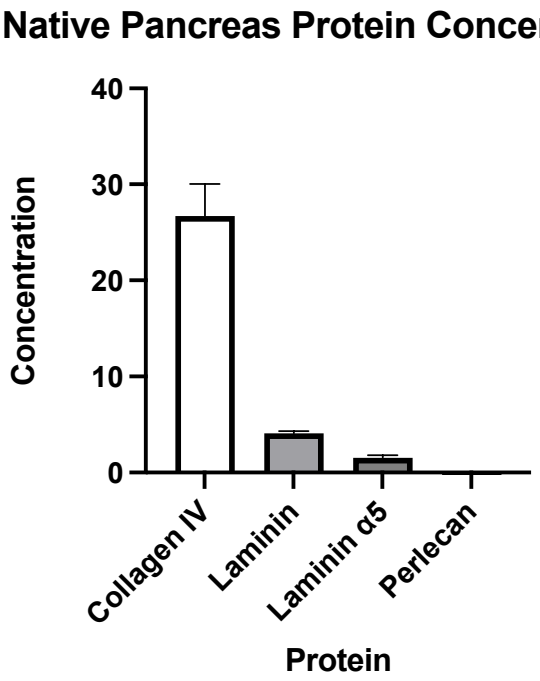


Figure 15: Native pancreas protein concentrations
Concentration of collagen IV, laminin, laminin-α5, and perlecan within the native pancreas from n=4 donors. Corresponding values are displayed in Table 6.

Concentration of ECM Proteins in The Native Pancreas		
ECM Protein	**Concentration, ng/ml (Mean ± SD) (n=4)**	**Concentration, ng/ml (Median (Range)) (n=4)**
Collagen IV	26.7 ± 10.27	23.33 (18.28- 41.26)
Laminin	4.07 ± 0.76	3.96 (3.21-5.20)
Laminin α5	1.54 ± 0.81	1.54 (0.75-2.37)
Perlecan	0.04 ± 0.004	0.04 (0.03-0.04)

Table 6: Concentration of specific ECM proteins for Chapter 2

Native pancreas tissue was assessed for the presence of ECM proteins including collagen IV, laminin, laminin-α5, and perlecan.

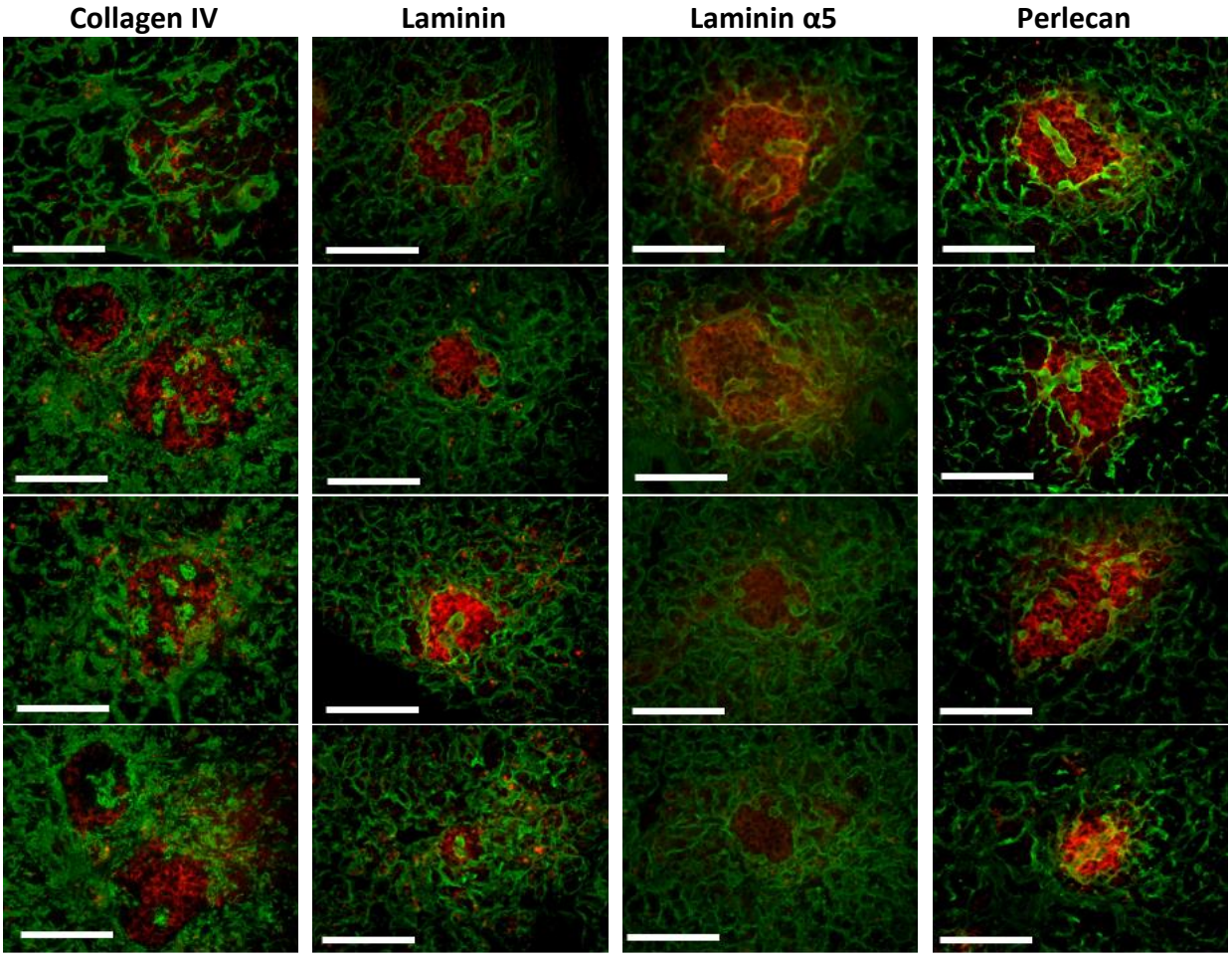


Figure 16: Immunolabelling for Specific ECM Proteins

Immunofluorescent labelling for collagen IV, laminin, laminin α5, and perlecan using green immunolabelling of respective protein and red immunolabelling of insulin. Green staining indicates ECM protein and red staining indicates insulin. Scale bars at 100 μm.

3.5 Discussion

Before considering how islet isolation impacts the structure of the islet BM, it is important to define the structure of the BM in the 'native' pancreas, prior to islet isolation. The studies in this Chapter demonstrate that collagen IV, pan-laminin, laminin-α5, and perlecan are all present in the ECM of the native pancreas, albeit at differing concentrations. These proteins serve as integral components of the BM structure, influencing cell function and survival [78, 101]. Collagen IV is recognised as a major component of the human islet BM, in both the peri-islet and vascular BM, that is largely involved with β-cell adhesion, motility, and insulin secretion [88, 102, 103]. Laminin's existence in the islet BM is understood to impact β-cell proliferation and insulin transcription [84]. Perlecan interacts with both collagen IV and laminins. This protein, along with collagen IV is detected within the vascular islet BM [88]. Histological and quantitative data from immunolabelling of specific BM proteins and respective ELISAs correspond to indicate key matrix protein presence. Although these proteins are detected in the duplex-layer structure of the islet BM in the native pancreas, enzymatic digestion may disrupt ECM protein composition. The use of enzymatic digestion to free islets from surrounding pancreatic ECM degrades the protein structure, thus impacting the superstructure of the overall membrane. Enzymatic dismembering of these proteins impacts BM construction, leaving behind a disorganized BM, and leaves islets vulnerable to further destruction and cell death in suboptimal conditions [74]. The presence of these proteins in differing quantities as demonstrated via ELISAs may imply the survival of certain matrices during islet isolation. Given the presence of collagen IV is in greater abundance than that of laminin, laminin-α5, and perlecan, this protein may demonstrate enhanced post-isolation survival. An evaluation of each ECM protein via histological and quantitative assessment should

be completed for islets after isolation, and throughout the subsequent culture or preservation periods, to better understand the existence of each protein considered above. Further deliberation surrounding the role of the pancreatic islet BM integrity in the success of islet transplantation considering graft success, insulin secretion, and cell survival should be evaluated in further studies.

3.6 Conclusions

This Chapter has demonstrated the existence of collagen IV, laminin, laminin-α5, and perlecan within the pancreatic islet BM of the native pancreas. The importance of each individual protein contributes to the overall structure and function of the islet BM. During islet isolation, the islet BM components are substantially disrupted. The loss of key matrix proteins in islet isolation and subsequent culture periods may impact islet function and survival both in vitro and in vivo. Further disruption of the BM has been shown when islets are culture in CMRL [74], which may result in cell apoptosis. Each protein demonstrated above may be referenced as a relative baseline to understand the effects of islet isolation, and subsequent culture and/or preservation periods on islet BM protein survival.

Chapter 4

Comparing the Islet Basement Membrane Integrity Following Culture /Preservation in CMRL and UW

4.1 Introduction

The human pancreatic islet BM is integral for islet survival and function both in vivo and in vitro [104, 105]. The human islet BM is comprised of a duplex structure including the endocrine BM, also known as the 'peri-islet BM', and the vascular BM. The organization of this duplex membrane structure suggests there is a separation between endocrine cells and the endothelial BM via an additional endocrine BM. Each membrane is made up of various ECM proteins that impact the pancreatic islet function and viability. For this thesis, the islet BM proteins investigated include pan-laminin, laminin-α5, collagen IV, and a specific heparan sulfate proteoglycan (HSPG) called perlecan. Each of these BM components is integral to various interactive ligands and the formation of the BM superstructure [75, 106]. As shown in Chapter 3, collagen IV is the most prevalent protein detected in all human BMs [107, 108]. Laminin chains are important to maintaining the integrity and shape of the BM, via cytoskeletal remodelling, contractility, and cell adhesion. In the human islet BM, laminin-α5 is detected in both the endocrine and vascular BM [109]. This distinguishing factor makes laminin-α5 an attractive protein to investigate to understand the extent of disruption to the islet BM during islet isolation and subsequent culture periods [109]. In humans, perlecan is suspected to impact β-cell function, thus loss of this protein may be associated with a decline in insulin secretion [101]. During the pancreas digestion phase of the islet isolation procedure an enzyme blend of collagenase and neutral protease work to free islets from the surrounding ECM. This results in significant islet BM disruption.

There is a mandatory period (24-48 hours) following islet isolation during which islets are cultured to allow for islet recovery and facilitate the mobilisation of the intended transplant recipient. The

current gold standard media, in which islets are suspended for culture, is CMRL media with supplementation (see Table A4 in the appendix for complete culture media composition). It is well studied, however, that this post-isolation islet culture in CMRL-based media is associated with a substantial islet loss [69, 72, 97, 110, 111]. Research has investigated the use of several media for islet storage following isolation including CMRL, University of Wisconsin (UW) solution, and PRODO standard culture media at various temperatures (4°C, 27°C, and 37°C) [66]. However, the impact of culture/preservation under these conditions on the islet BM has not been explored. As determined by count, viability, and purity assessments, UW is a more superior media for maintaining islet number and viability for up to 72 hours. Whilst the reason for this is unknown, this hypothesis suggests that preserving human islets in UW cold storage solution following islet isolation may better maintain the islet BM, compared to islet culture in CMRL, where the islet BM is progressively lost during the culture period [74]. The preservation of the human islet BM during the post-isolation recovery period may increase islet survival and function, allowing for better quality, and quantity of islets for the recipient.

4.2 Chapter Aims

Chapter 2 demonstrated that UW is significantly superior for maintaining islets during the post-isolation culture period, for up to 72 hours of culture. The underlying reason for this remains unclear, however, it is possible that UW better supports the critical islet BM post-isolation. The islet BM does not recover when islets are placed in CMRL post isolation, and indeed further decline has been demonstrated during the 72-hour culture period [74]. This chapter aims to

investigate the impact of human islet preservation in UW cold storage in comparison to the gold-standard culture in CMRL during the post-isolation recovery period on the human islet BM. This chapter promotes the need for better post-isolation islet storage by investigating the maintenance and integrity of the islet BM under the proposed conditions.

4.3 Methods

4.3.1 Islet Suspension and Preparation for Immunolabelling of BM Proteins

As displayed in Chapter 2 (Figure 6) islets were suspended in either UW or CMRL, plated in 6-well plates, and then stored at 4° C and 37° C, respectively. At each collection time point, islets were suspended in PFA, embedded in OCT, and cryosectioned onto glass slides for immunolabelling of specific BM proteins.

4.3.1.1 Islet Suspension in PFA

Upon sample collection after the islet isolation procedure, then 24-, 48-, & 72-hour time points, approximately 1,000 islets were collected and placed in a 1.5 ml Eppendorf tube (Eppendorf). Following centrifugation, 1 ml of supernatant was removed and frozen at -20°C for future use. Remnant supernatant was removed, discarded, and then replaced with 1 ml PFA. Samples were labelled with the relevant HP number, collection time point, and date, then stored at 4°C until use.

4.3.1.2 Embedding Islets in OCT

PFA-embedded islets from each collection time point were centrifuged for 1 minute at 1500 rpm. The PFA was removed from each sample and replaced with 1 ml PBS using gentle agitation to wash the islets. In total, 3 complete PBS washes were performed. For visualisation purposes, 20 μl eosin (1%, VWR) was added to each sample and left for 2-3 minutes to stain the islets pink, followed by 2 complete PBS washes. Remnant PBS was discarded and replaced with 1 ml ice-cold 20% sucrose (Sigma), and samples were left at 4° for 1 hour. After 1 hour, samples were spun down at 1500 rpm for 30 seconds, and the supernatant was removed, leaving 20 μl of volume in the Eppendorf tube with the islets. Using a 5 cm x 5 cm piece of foil, a small mould was made. A small volume of OCT (Sigma) was added to the bottom of the foil mould, which was then placed on dry ice to set. Islets were placed on top of the mould, containing the set OCT. OCT was added to the top of the islets, in a circular motion to ensure concentration of the islets toward the centre of the foil. Once distributed, the embedded islets were placed on dry ice to freeze. Samples were stored at -20°C until cryosectioning.

4.3.1.3 Cryosectioning of OCT Samples

OCT-embedded islets from each collection time point were cryosectioned (Leica) to 15 μm and collected on Superfrost glass slides (Eprendia). Once dried, slides were frozen at -20°C until use for immunolabelling of islet BM proteins.

4.3.2 Immunolabelling of Human Islets for BM Proteins

One slide per each collection time point was thawed in TBS (Thermo Scientific) before a hydrophobic barrier pen (Vector Laboratories) was used to encase the samples. Once the pen had dried, 150 μl of pepsin antigen retrieval (Abcam) was added to each tissue section and slides were incubated at 37°C for 45 minutes in the human tissue incubator. After 45 minutes, slides were washed under running tap water for 5 minutes before completing 3 TBS washes in TBS tanks. A permeabilisation and blocking buffer made up of 500 μl swine serum (5%, Vector Laboratories) and 20 μl triton (Sigma Aldrich) supplemented with 9.5 ml TBS, was added to each slide (150 μl/ tissue section) and slides were incubated at room temperature in the incubation chamber for 30 minutes. A 5-minute TBS wash was completed at this point to remove excess triton from the slides. Primary antibodies were made up as per Table D1 (see appendix) using insulin (Guinea pig anti-human; kindly provided by Dr Anne Clark, from OCDEM labs, University of Oxford) and respective ECM proteins, collagen IV (Goat anti-collagen IV; BioRad; 134001), laminin (Rabbit anti-laminin; Abcam; ab11575), laminin-α5 (Mouse anti-laminin alpha 5; Abcam; ab77175), and perlecan (Mouse anti-perlecan; Santa Cruz; sc-377219). 150 μl of primary antibody was added to each tissue section, and slides were placed in the incubation chamber for 1.5 hours at room temperature. Slides were washed 3 times in TBS tanks. Secondary antibodies were made up as per Table D1 (see appendix) using insulin (Donkey anti-guinea pig Alexa Fluor 594; Jackson Immunotech) and relative binding immunofluorescent secondary antibody for each protein; collagen IV (Donkey anti-goat Alexa Fluor 488; Life Technologies; A11055), laminin (Goat anti-rabbit Alexa Fluor 488; Life Technologies; 110088), perlecan (Goat anti-mouse CFL; Santa-Cruz; sc-362257), and laminin-α5 (Goat anti-mouse CFL; sc-362257)). Slides were incubated for 30

minutes in the incubation chamber, protected from light, and then washed 3 times in TBS tanks. Excess TBS was carefully removed from slides. Slides were mounted using Vectorshield and refrigerated at 4°C for 1 hour before imaging.

4.3.2.1 Imaging of Immunolabelling of Proteins

Prepared slides were imaged using ZENworks software (Zeiss). Slides were imaged using the respective channels, revealing green and red staining of the cells. For each collection time point, 3-10 islets were imaged (dependent on islet availability). These images were then scored for staining intensity on a scale of 0-3 (0= no positive staining, 1= minor staining, 2= moderate staining, 3= high/abundance of staining) for each protein. Scoring was completed by two operators (in a blinded fashion) and compared for accuracy.

4.3.3 Preparation of Islets for Protein Concentration Quantification

4.3.3.1 Islet Suspension in Hypotonic Buffer with Protease Inhibitor Cocktail (PIC)

At each sample collection time point slets were collected and pelleted as per the '4.3.1.1 islet suspension in PFA' protocol above. The supernatant was removed and stored at -20°C until use. Islets were washed three times in 1 ml HBSS. Following the final wash, HBSS was removed and replaced with 200 μl complete hypotonic buffer (180 μl hypotonic buffer (ThermoFisher Scientific) supplemented with 30 μl PIC (ThermoFisher Scientific), see appendix Table(s) D2 and D3). Samples were stored at -20°C until use.

4.3.3.2 Protein Extraction with RIPA Buffer

Islet samples collected at each collection time point and suspended in a complete hypotonic buffer with PIC were thawed gently on wet ice. Islets were then spun down via centrifuge at 2,000 rpm for 1 minute. The supernatant was removed and discarded from each sample, then islets were re-suspended in 200 µl of RIPA buffer. Samples were placed back on the ice and shaken on a plate shaker (Stuart) for 30 minutes. To ensure islets did not settle in RIPA buffer, samples were manually agitated vigorously throughout the 30 minutes. Samples were sonicated (Fisher Scientific) for 5 seconds at 90 watts. Following sonification samples were centrifuged at 13,000 rpm for 2 minutes. The supernatant (cell lysate) from each sample was removed and placed in a pre-cooled Eppendorf tube. Any remnant debris was frozen along with the cell lysate samples at -20°C until use.

4.3.3.3 Bicinchoninic Acid (BCA) Protein Assay

The protein concentrations of sonicated islet samples were determined using Bicinchoninic Acid assay (BCA). BCA Standards and working reagents were prepared using the manufacturer protocol (Pierce BCA Protein Assay Kit). 10 µl of each standard and 5 µl sample (diluted 1:2 with RIPA buffer) was pipetted into a 96-well plate (Thermo Scientific). 200 µl of working reagent was added to each well, and the plate was placed in an incubated plate shaker at 37°C for 30 minutes. The plate was then cooled to room temperature and absorbance was measured at 562 nm using a VERSAmax microplate reader (Molecular Devices) and SoftMax Pro software (Molecular Devices).

4.3.4 Enzyme-Linked Immunosorbent Assay (ELISA) for Laminin

All reagents, working standards, and samples were prepared as per the Laminin ELISA Kit protocol provided (Abcam). 100 μl of prepared standards and samples were distributed to appropriate wells in the provided 96-well plate. The plate was sealed and incubated for 90 minutes at 37°C. Contents of each well were removed, and any excess sample was agitated out of the plate by snapping the plate onto blue roll tissue. 100 μl of biotinylated anti-human laminin antibody was added to each well, the plate was sealed, and incubated for 60 minutes at 37°C. Contents were discarded, and the plate was washed with 300 μl TBS for about 1 minute, a total of 3 times. 100 μl of Avidin-Biotin-Peroxidase Complex was added to each well and the plate was sealed and incubated for 30 minutes at 37°C. After this incubation, the plate was washed as in previous steps 5 times. 90 μl of TMB colour-developing agent was added to each well and the plate was incubated at 37°C, protected from light, for 20 minutes. 100 μl TMB Stop Solution was added to each well. Using a VERSAmax microplate reader absorbance was read at 450 nm immediately. Results were recorded using SoftMax Pro software.

4.3.5 Enzyme-Linked Immunosorbent Assay (ELISA) for Laminin Alpha-5, Collagen IV, Perlecan

The protocol for ELISA of BM proteins laminin-α5, collagen IV, and perlecan (CloudCorpe) are as follows. Standards and reagents for each kit were prepared as per the instruction manual provided with the individual kit. Samples were prepared using volumes determined by BCA assay. 100 μl of each prepared standard and prepared sample were added to a 96-well plate from each respective kit. The plate was covered with a plate sealer and incubated for 60 minutes at 37°C.

Then, after removing liquid from each well by blotting the plate onto absorbent blue roll tissue, 100 μl of Detection Reagent A was added to each well. The plate was sealed with a fresh plate sealer and incubated for 60 minutes at 37°C. Using the provided wash solution, 350 μl was added to each well using a multichannel pipette (Discovery Comfort). After 1-2 minutes the remaining liquid was removed by blotting the plate onto absorbent blue roll tissue once more. This wash was repeated a total of 3 times. 100 μl of Detection Reagent B was added to each well, the plate was sealed, and placed in the incubator at 37°C for 30 minutes. The wash described above was repeated here 5 times. 90 μl of Substrate Solution was added to each well, and the plate was sealed and incubated at 37°C, protected from light, for 10-20 minutes. 50 μl of Stop Solution was added to each well. Using a VERSAmax microplate reader and SoftMax Pro software, each plate was read at 450 nm immediately following the addition of stop solution to wells.

4.3.6 Statistical Analysis

All statistical analysis was completed using GraphPad Prism 10 (San Diego, USA). The normality of the data was assessed using a Shapiro-Wilk test. Normally distributed data was analysed by way of ANOVA, using Tukey's multiple comparisons test. Data that was not found to be normally distributed by the Shapiro-Wilk test was analysed using a Kruskal-Wallis test, and Dunn's multiple comparisons test. The operator scoring significance was determined using a Wilcoxon matched-pairs signed-rank test for each sample and all proteins.

4.4 Results

4.4.1 Immunolabelling of Islets for Specific BM Proteins

Studies were performed on islets that had been isolated from 5 donors. BM integrity was assessed via the immunofluorescent labelling of laminin, collagen IV, laminin-α5 and perlecan at each collection time point (fresh, 24, 48, and 72 hours) following culture in either UW or CMRL. Staining revealed the presence of collagen IV in all samples, however there were variations seen in the other proteins investigated among the two media. Immunolabel scoring completed by 2 operators for each protein varied for some images by factor of 1. Scoring was completed for islets visualised, depending on the volume of tissue available on each slide, from each collection time point for n=4 donors.

Laminin scoring reflected a higher average score among islets preserved in UW cold storage (Figure 17). There was a significant loss of laminin seen in the immunolabelling of CMRL samples at 72 hours compared to CMRL samples at 24 hours (*p<0.05; Figure 17). A significant difference was seen in the presence of laminin immunolabelling at 48 hours in CMRL compared to 48 hours (*p<0.05; Figure 17). The collagen IV image scoring reflected that there was minimal change to the average collagen IV presence seen in islet immunolabelling (Figure 18). The average score of images showing immunolabelling of laminin-α5 was below 1 for all time points (Figure 19). There was a significantly greater amount of perlecan staining seen on islets suspended in UW at 24 hours, compared to islets cultured in CMRL at 24 hours (*p<0.05; Figure 20). The operator scoring for perlecan was, on average, lower for islets imaged within the CMRL group, compared to the UW group (Figure 20). Operator 1 scoring was validated by a second operator using a blinded

approach. No significant difference was detected between operator scoring and variations in scoring were only seen as a score difference of 1 at any given point. A comparison between Operator 1 and Operator 2 scoring is provided below for each BM protein assessed (refer to Figures 21, 22, 23, and 24).

4.4.1.1 Immunolabelling of Islets: Operator 1 Image Scoring

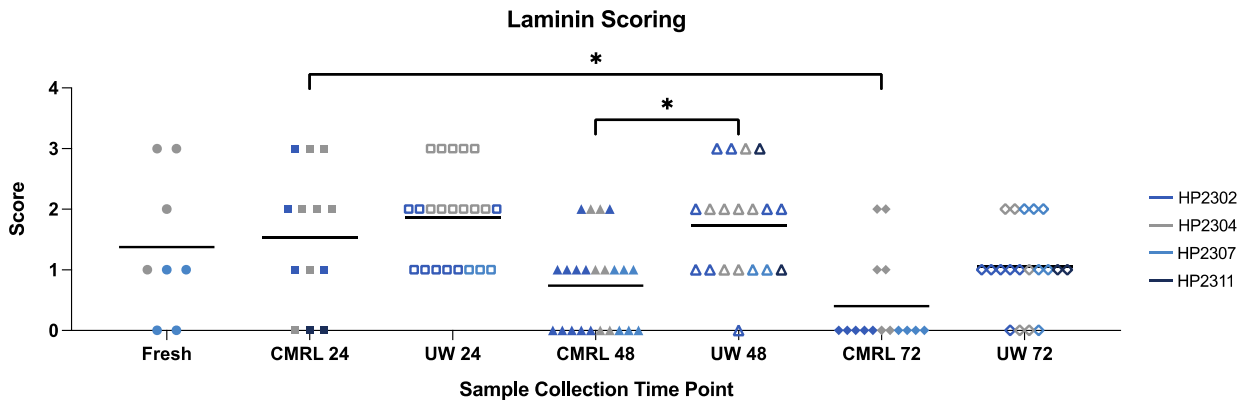


Figure 17: Islet scoring for immunolabelling of laminin
Laminin immunolabelling scoring for Operator 1. Immunolabelling of islets that were procured after isolation and subsequently at 24, 48, and 72 hours after culture/preservation in CMRL or UW, respectively. Statistical significance was determined using Kruskal-Wallis test and Dunn's multiple comparisons test. The number of islets scored for each collection point by n=4 donors varied due to tissue availability visualised on each slide.
(*p<0.05)

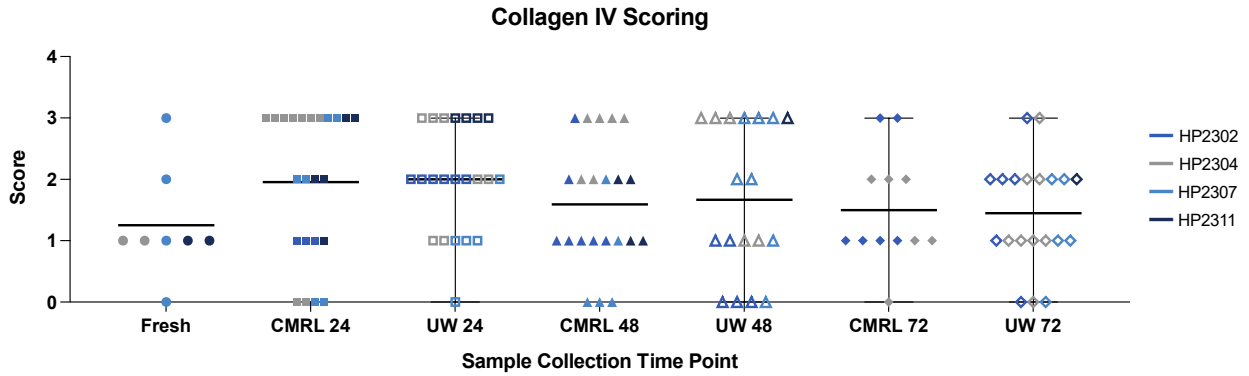


Figure 18: Islet scoring for immunolabelling of collagen IV

Collagen IV immunolabelling scoring for Operator 1. Immunolabelling of islets that were collected after isolation and subsequently at 24, 48, and 72 hours after culture/preservation in CMRL or UW, respectively. Statistical significance was determined using Kruskal-Wallis test and Dunn's multiple comparisons test. The number of islets scored for each collection point by n=4 donors varied due to tissue availability visualised on each slide.

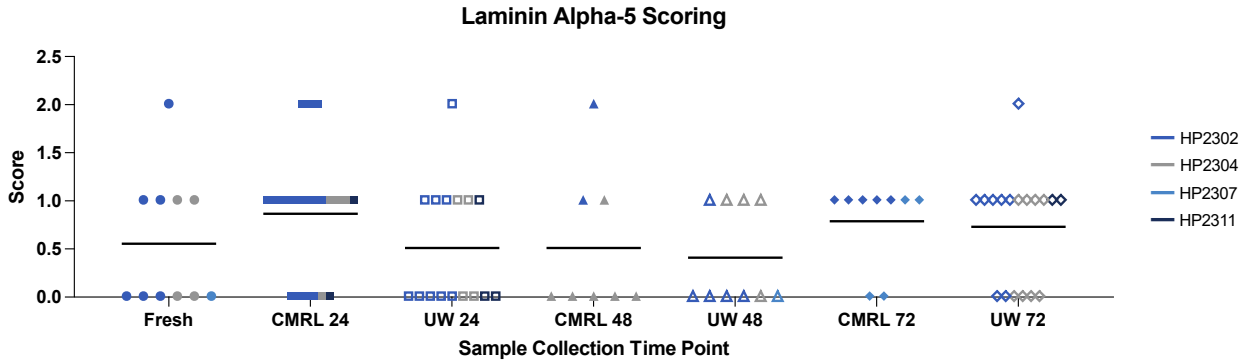


Figure 19: Islet scoring for immunolabelling of laminin-α5

Laminin-α5 immunolabelling scoring for Operator 1. Immunolabelling of islets that were collected after isolation and subsequently at 24, 48, and 72 hours after culture/preservation in CMRL or UW, respectively. Statistical significance was determined using Kruskal-Wallis test and Dunn's multiple comparisons test. The number of islets scored for each collection point by n=4 donors varied due to tissue availability visualised on each slide.

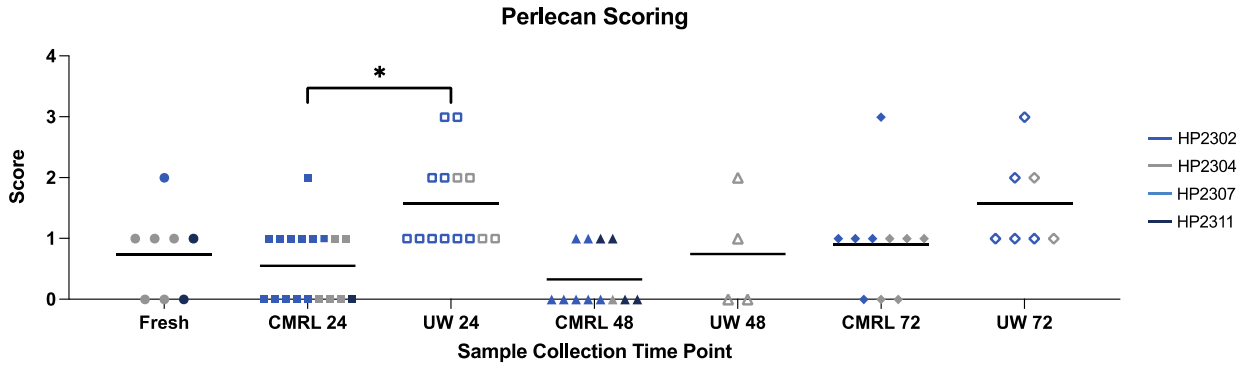


Figure 20: Islet scoring for immunolabelling of perlecan

Perlecan immunolabelling scoring for Operator 1. Immunolabelling of islets that were collected at the conclusion of isolation and subsequently at 24, 48, and 72 hours after culture/preservation in CMRL or UW, respectively. Statistical significance was determined using Kruskal-Wallis test and Dunn's multiple comparisons test. The number of islets scored for each collection point by n=4 donors varied due to tissue availability visualised on each slide.
(*p<0.05)

4.4.1.2 Operator 1 and Operator 2 Scoring Comparison

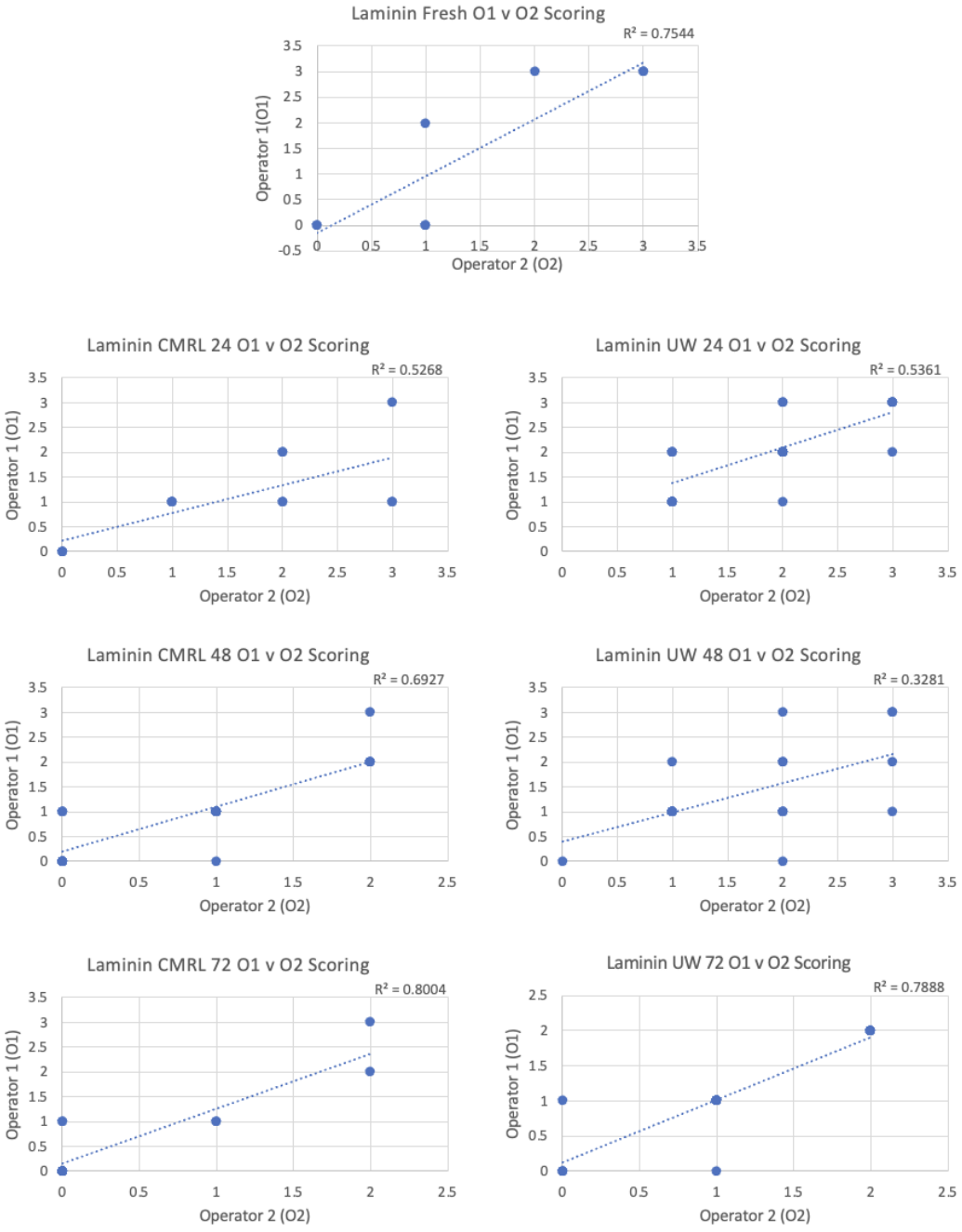


Figure 21: Laminin operator scoring comparison
For islet scoring, reproducibility was assessed using a blind approach with 2 operators. Each graph reflects the scoring of islets from each collection time point for laminin. R^2 value is included on each graph. No significant differences in operator scoring were recorded for any group (see Table D4 in the appendix for relevant p-value(s)). Statistical significance was confirmed using a Wilcoxon matched pairs signed rank test.

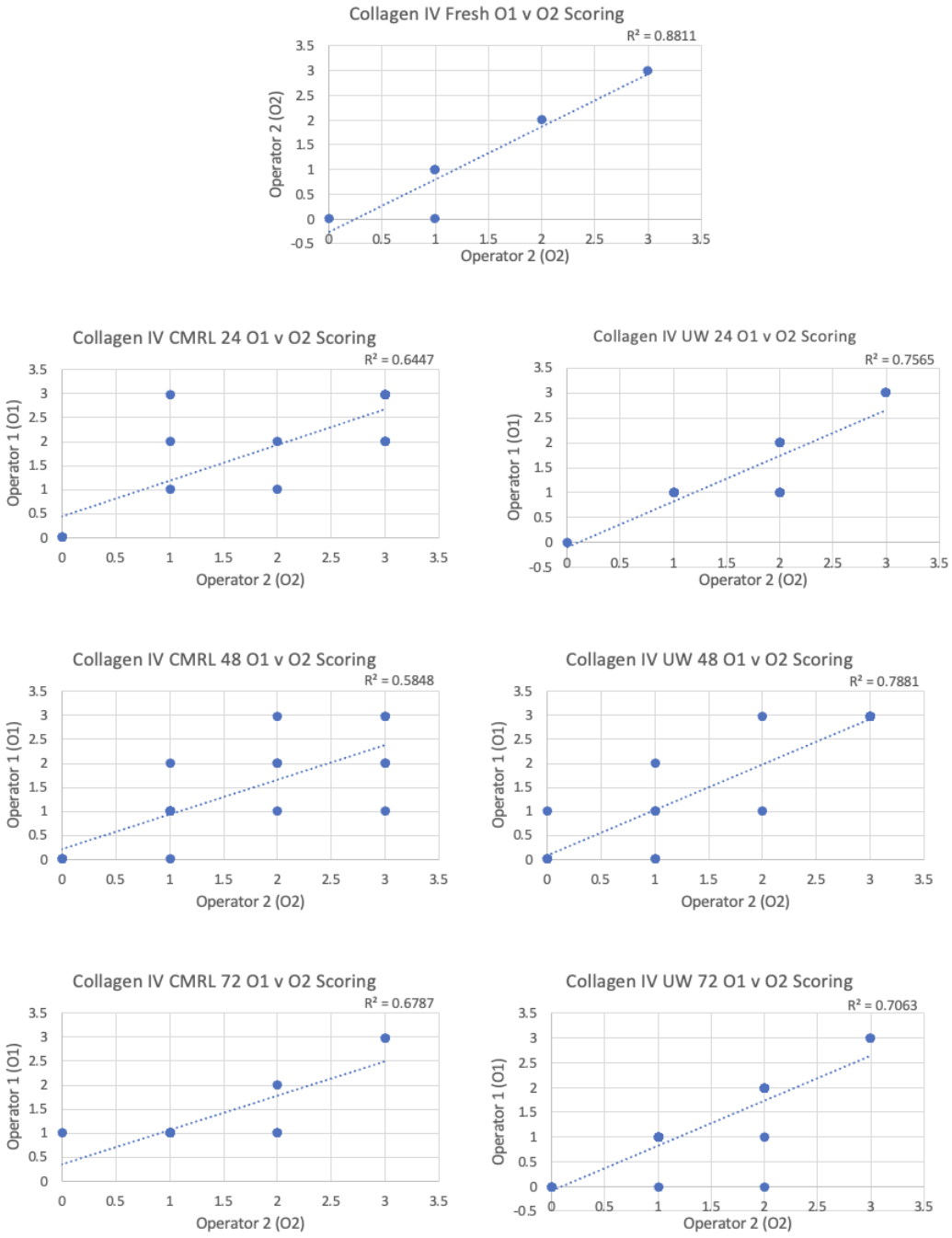


Figure 22: Collagen IV operator scoring comparison

For islet scoring, reproducibility was assessed using a blind approach with 2 operators. Each graph reflects the scoring of islets from each collection time point for Collagen IV. R^2 value is included on each graph. No significant differences in operator scoring were recorded for any group (see Table D4 in appendix for relevant p-value(s)). Statistical significance was confirmed using a Wilcoxon matched pairs signed rank test.

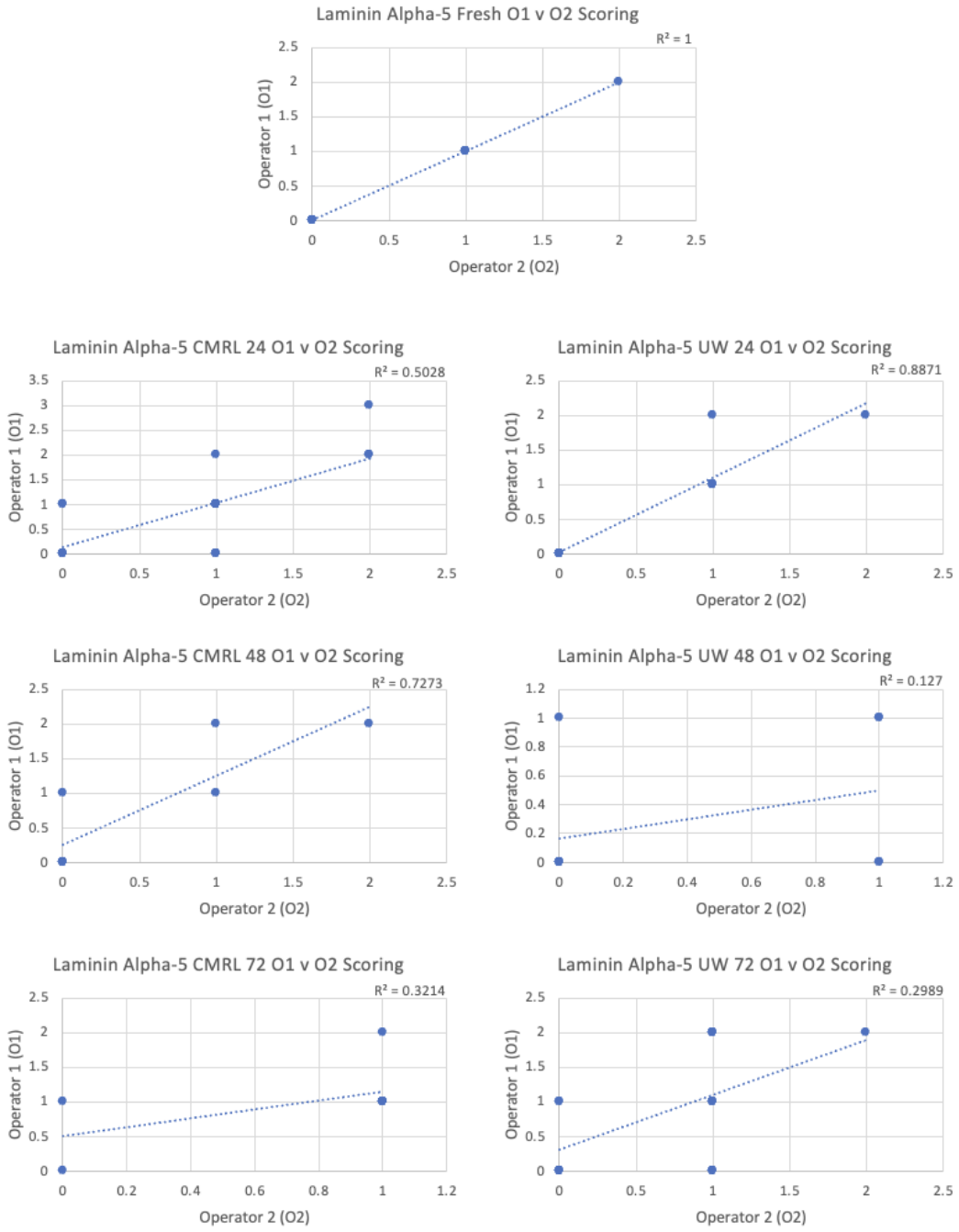


Figure 23: Laminin-α5 operator scoring comparison

For islet scoring, reproducibility was assessed using a blind approach with 2 operators. Each graph reflects the scoring of islets from each collection time point for laminin-α5. R^2 value is included on each graph. No significant differences in operator scoring were recorded for any group. (see Table D4 in appendix for relevant p-value(s)). Statistical significance was confirmed using a Wilcoxon matched pairs signed rank test.

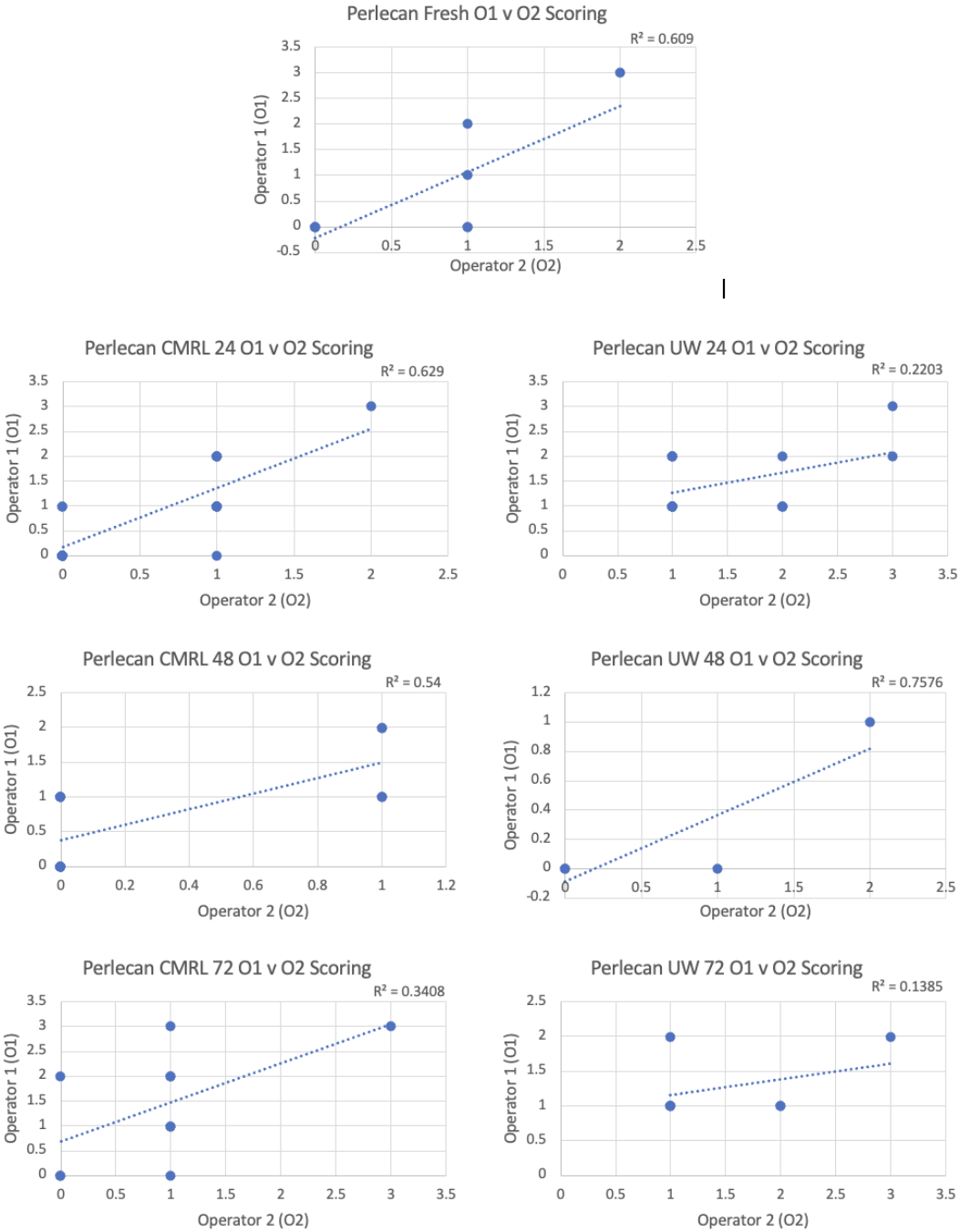


Figure 24: Perlecan operator scoring comparison

For islet scoring, reproducibility was assessed using a blind approach with 2 operators. Each graph reflects the scoring of islets from each collection time point for Perlecan. R^2 value is included on each graph. No significant differences in operator scoring were recorded for any group. (see Table D4 in appendix for relevant p-value(s)). Statistical significance was confirmed using a Wilcoxon matched pairs signed rank test.

4.4.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Protein-specific ELISAs were used to determine the concentration of each matrix protein. There was a significant loss of laminin in islets suspended in CMRL after 48 hours (*p<0.01; Figure 25) when compared to the fresh sample. The loss of laminin in islets suspended in CMRL at 48 hours was not reestablished by 72 hours, where another significant decrease was detected in comparison to the fresh sample (*p<0.05; Figure 25). Outliers in data can be attributed to a sampling error during the initial plating of islets in each condition. Collagen IV was maintained over the 72-hour culture/preservation period, with no significant changes detected in any group (Figure 27). Laminin-α5 was maintained in UW cold storage for up to 72 hours, however, there was a significant decline of the protein detected in the islets suspended in CMRL at 72 hours (*p<0.05; Figure 29) when compared to the fresh sample. There were no significant changes in the amount of perlecan recorded in any samples (Figure 31).

4.4.2.1 Enzyme-Linked Immunosorbent Assay (ELISA) for Laminin

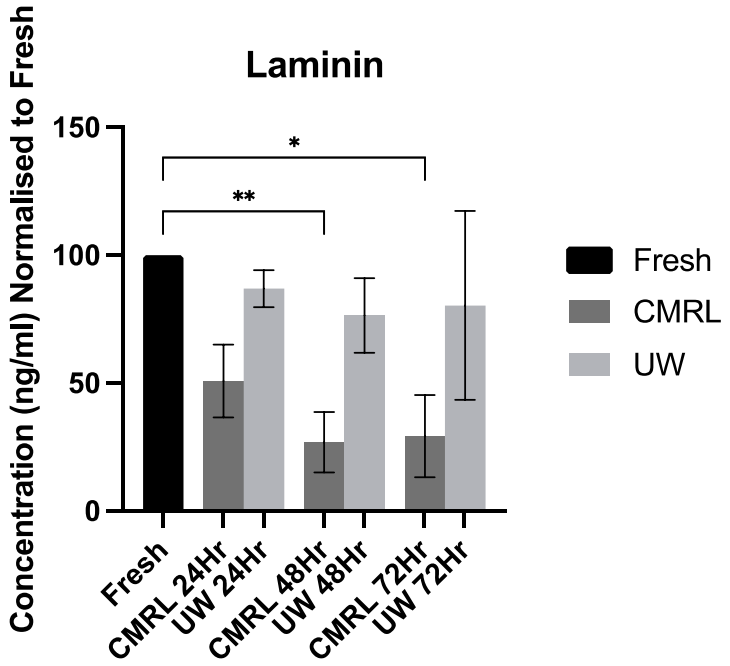


Figure 25: Laminin concentration determined by ELISA
The concentration of laminin detected for each sample collection point (n=4) was determined via enzyme-linked immunosorbent assay (ELISA). There was a significant decline in the concentration of laminin in islets cultured in CMRL at 48 hours, compared to fresh samples (*p<0.01). The significant loss of laminin in islets cultured in CMRL was not reestablished at 72 hours, as the significant decline from fresh was maintained (*p<0.05). The statistical significance was completed by way of ANOVA using Tukey's multiple comparisons test.

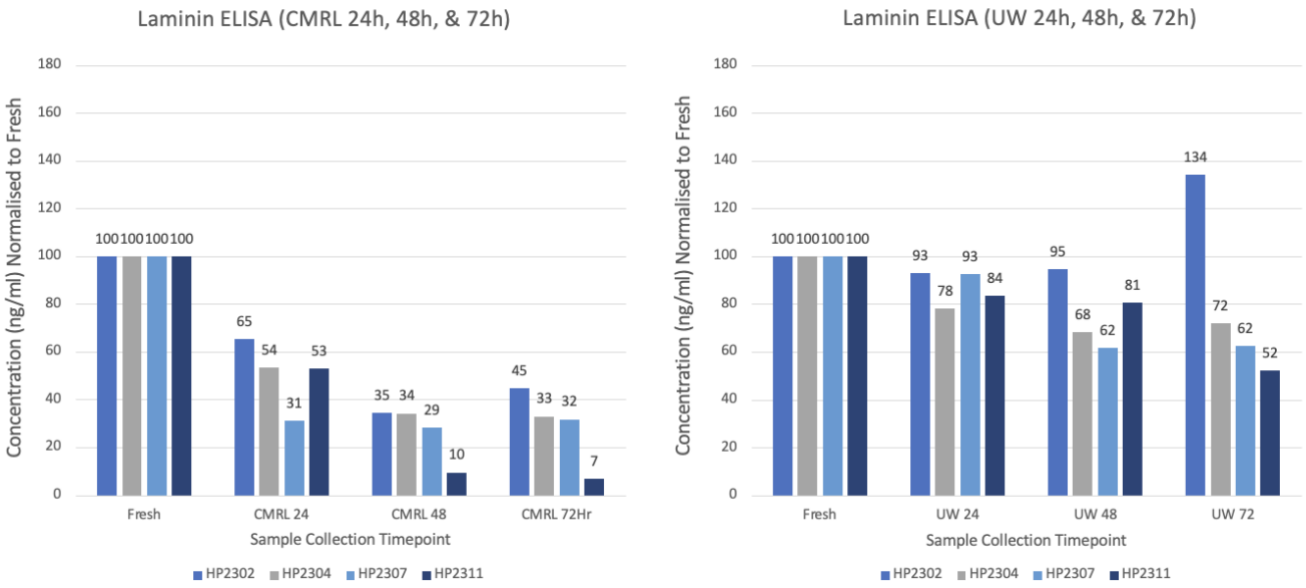


Figure 26: Laminin concentration for donors (n=4) for each collection point
These graphs represent the concentrations of laminin found in each donor from the ELISA assay, at each time point, in either medium. There was more laminin in islets from each donor in samples stored in UW, compared to the same donor sample cultured in CMRL.

4.4.2.2 Enzyme-Linked Immunosorbent Assay (ELISA) for Collagen IV

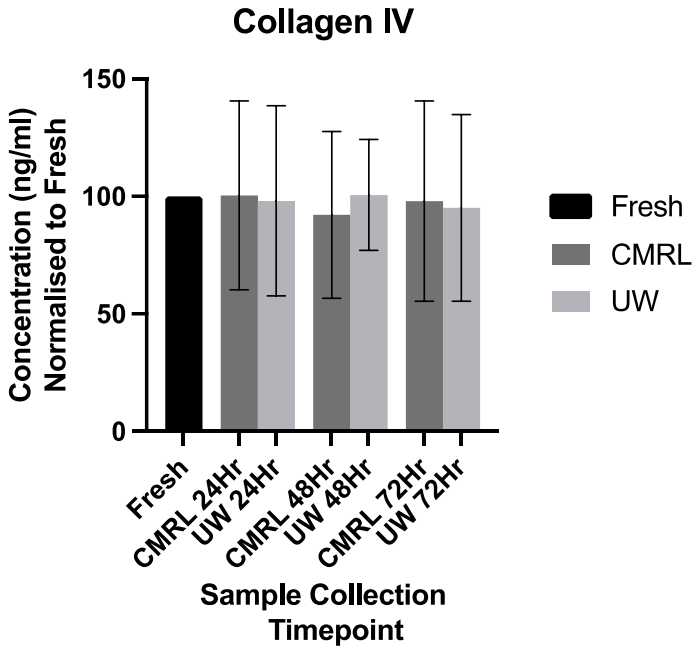


Figure 27: Collagen IV concentration determined by ELISA.
The concentration of Collagen IV was determined by enzyme-linked immunosorbent assay (ELISA). The matrix protein, collagen IV, was maintained in both media for up to 72 hours. Statistical significance was confirmed by way of ANOVA using Tukey's multiple comparisons test.

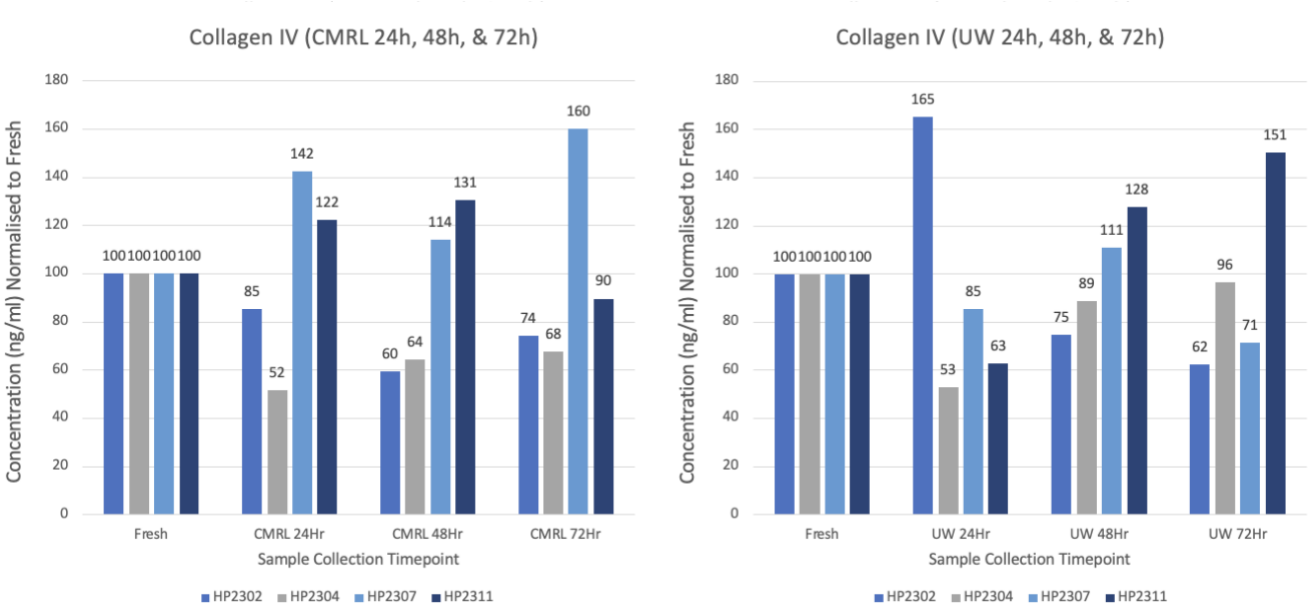


Figure 28: Collagen IV concentration for donors (n=4) for each collection point
Here, collagen IV concentration for each donor is recorded for each collection point, in both mediums. There was variation in collagen IV concentration detected for each donor, however overall, collagen IV was maintained up to 72 hours in both CMRL and UW.

4.4.2.3 Enzyme-Linked Immunosorbent Assay (ELISA) for Laminin-α5

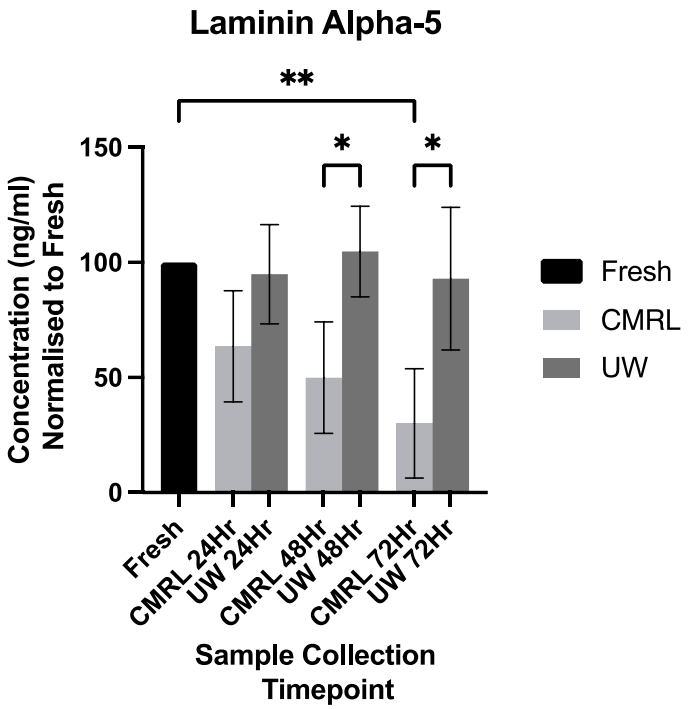


Figure 29: Laminin-α5 concentration determined by ELISA.
The concentration of Laminin-α5 in all samples was determined through enzyme-linked immunosorbent assay (ELISA). A significant loss of laminin-α5 was detected in islets cultured in CMRL at 72 hours following islet isolation (*p<0.01) compared to the fresh sample. Significantly less laminin-α5 was detected in islets cultured in CMRL at 48 and 72 hours, compared to islets suspended in UW at 48 and 72 hours, respectively (*p<0.05). Statistical significance was confirmed via ANOVA using Tukey's multiple comparisons test.

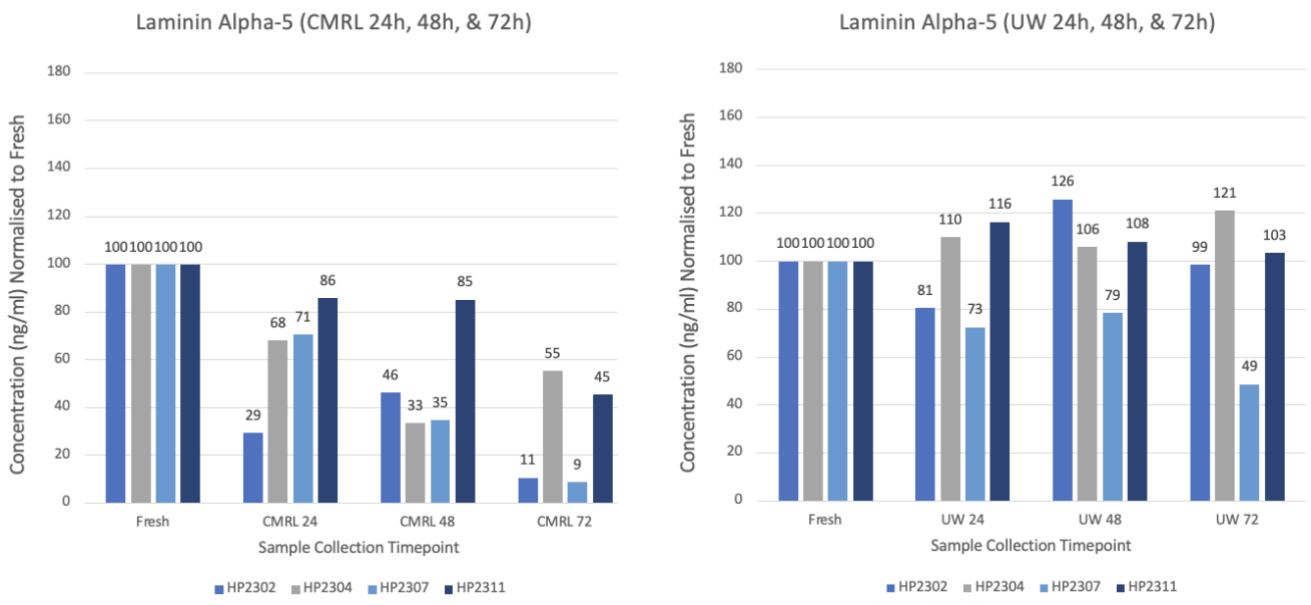


Figure 30: Laminin-α5 concentration for donors (n=4) for each collection point
These graphs display the concentrations of laminin-α5 detected in each donor, in CMRL and UW storage, for up to 72 hours. There is a steady decline in the concentration of laminin-α5 detected in islets stored in CMRL (with respect to one outlier), which was not seen in islets stored in UW.

4.4.2.4 Enzyme-Linked Immunosorbent Assay (ELISA) for Perlecan

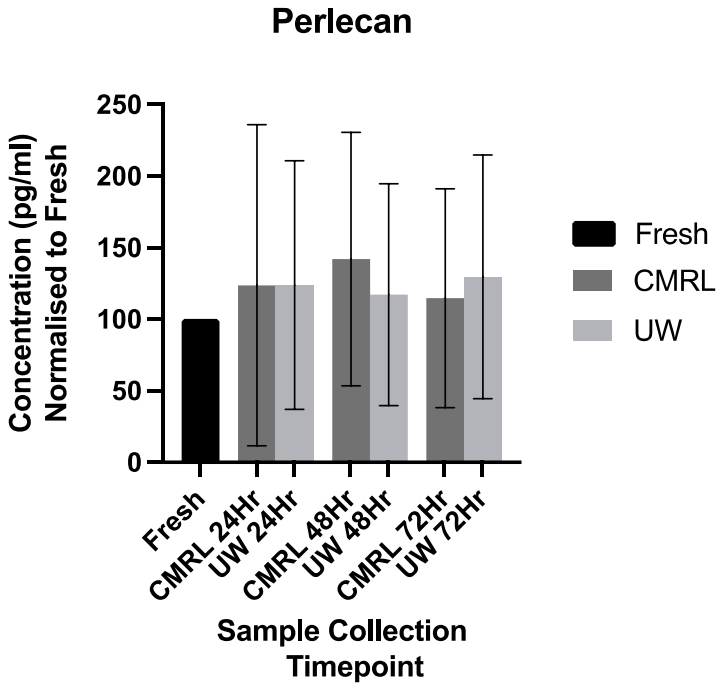


Figure 31: Perlecan concentration determined by ELISA
The concentration of perlecan was determined by way of enzyme-linked immunosorbent assay (ELISA). Findings concluded that the protein, perlecan, was maintained in both groups for up to 72 hours. Statistical significance was confirmed by way of ANOVA via Tukey's multiple comparisons test.

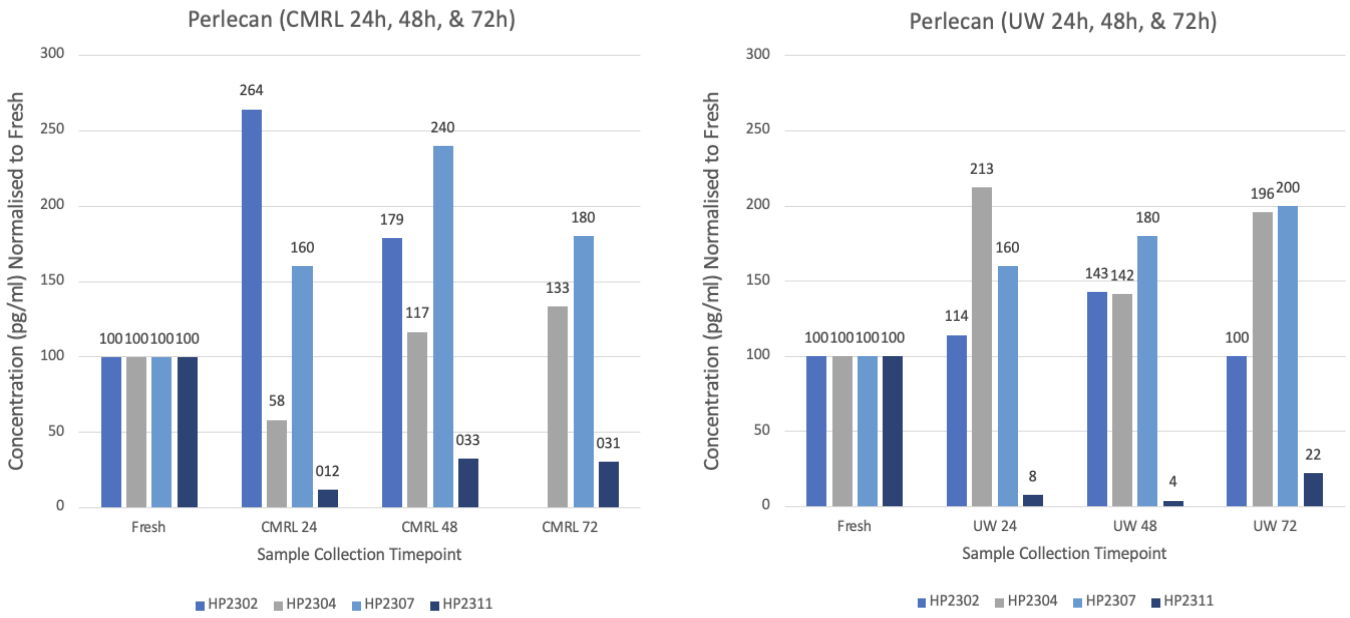


Figure 32: Perlecan concentration for donors (n=4) for each collection point
These graphs represent the concentrations of perlecan detected for each donor, at each sample time point for islets stored in either CMRL or UW up to 72 hours. Variation is seen among donors (n=4).

4.5 Discussion

This chapter aimed to investigate the impact of islet culture/preservation on key matrix proteins within the human islet BM. Chapter 2 demonstrated that islets are significantly better preserved in UW than in CMRL during the 72-hour culture period following isolation. The islet BM is very important for islet survival and function. As discussed in Chapter 3, various BM proteins are detected surrounding the vascular and peri-islet BMs in human islets. Previous work has shown the human islet BM to be inadequately supported during culture in CMRL [74]. For this thesis, it

is the hypothesis that UW better preserves the islet BM, which may contribute towards the overall survival of islets, at a molecular level, during this recovery period.

The investigation of human islet culture/preservation serves to improve the understanding of the maintenance of the islet BM during this period. This will help us to challenge the long-standing global assumption that CMRL is the most appropriate culture media for the culture of human islets prior to transplantation. Collection of samples for each donor after the pancreatic isolation procedure, and at 24-, 48-, and 72-hours following islet isolation provides insight on the impact of extended periods of recovery time following islet isolation. Recognising the significant loss of islets during culture in CMRL [74, 111], the conditions of post-isolation islet storage must be optimised to maintain a transplantable yield and viable islets.

As reflected in other studies, preservation of islets at 4°C in UW during the mandatory recovery period has been shown to prevent islet loss compared to culture in CMRL [69, 91, 97, 112]. The findings above, combined with understanding the impact of the human islet BM on islet survival and function suggest that the integrity of the basement membrane is better preserved in UW, leading to a greater yield of viable islets up to 72 hours.

At each sample collection point, the islets collected were analysed to assess the presence of BM proteins. Proteins assessed include those investigated within the native pancreas in Chapter 3, laminin, laminin-α5, collagen IV, and perlecan. The concentrations of each protein varied for each collection time point and respective media suspension at each point, however, overall UW proved

to be a superior media for the preservation of laminin and laminin-α5 up to 72 hours. As laminins are critical to the survival and function of cells in vivo, it is important to recognise the significant loss of pan-laminin and laminin-α5 detected in the CMRL islets at 72 hours. There was not a significant difference in the presence of collagen IV in islets in CMRL versus UW up to 72 hours, which may be due to the abundance of protein within the islet BM. Minimal perlecan detection in islets stored in both CMRL and UW may be indicative of unrecoverable damage due to enzymatic digestion via the collagenase/neutral protease blend.

The islet scoring data, done by two operators, shows that the presence of BM protein, laminin, is better maintained up to 72 hours in UW cold storage solution when compared to CMRL. This data supports the findings of the quantitative BM protein ELISA, which showed significant declines in the amount of laminin detected in islets cultured in CMRL for up to 72 hours. There was also a significant loss of laminin-α5 in islets cultured in CMRL, which was not seen in UW. As a BM component that is said to be essential to β-cell adhesion, proliferation, and insulin secretion [113], it is pertinent that this protein is maintained. Other matrix proteins collagen IV and perlecan remained stable or were minimally detected, respectively, in all collection time point samples. The implications of this protein loss can include loss of islet structural support as collagen IV and laminin act as the primary scaffold for other BM proteins including perlecan.

Previous studies have investigated cold storage for the preservation of islets following islet isolation. Findings of these studies showed that although islets suspended in CMRL at 4°C at 24 hours showed better survival than those in CMRL at 37°C at 24 hours, there was an even more significant decline in the morphology and function of islets stored in CMRL at 4°C at 48 hours

compared to CMRL at 37°C at 48 hours [97]. This same decline was not detected in islets stored in UW at 4°C at 48 hours. Based on this conclusion, it is likely that the components of the UW cold storage solution may be responsible for the enhanced islet preservation, rather than the temperature at which islets are stored during this period. This data suggests that improved integrity of the human islet BM could be one reason for the enhanced survival of islets in UW, as demonstrated in Chapter 2.

Whilst the findings portray the outcomes of islet storage in UW up to 72 hours, there is limitations to the study including the outcome of resuspension of islets in CMRL following islet culture. Due to the existing Na/K+ ratio of UW cold storage solution, this media cannot be transplanted directly into recipients. Resuspension of islets in CMRL may cause disruption to the islet BM due to change in storage media components and temperature. Further research may investigate the impact of resuspension in CMRL on islet survival, and even more, islet BM integrity, by resuspending islets that have been stored in UW cold storage in CMRL and incubating these islets for an appropriate time to regain physiological activity.

4.6 Conclusions

UW better preserves pan-laminin and laminin-α5 BM proteins up to 72 hours following islet isolation. Although CMRL is the gold standard media, more optimal mediums and culture conditions may exist to increase cell survival. Successful islet preservation in UW for up to 72

hours lends a larger, more viable product yield than under current culture conditions. By enhancing the outcomes of islet culture/recovery more islets may be available to transplant, increasing the likelihood of successful engraftment during transplantation.

Chapter 5

Conclusions and Recommendations for Further Work

5.1 Introduction

Current islet culture systems remain suboptimal for supporting islets during the post-isolation mandatory culture period. It is estimated that between 10% and 50% of extracted islets are lost during culture when utilising the current gold standard CMRL-based media [66]. Islets undergo considerable stress during the islet isolation procedure, which includes the partial enzymatic removal of the BM that surrounds and supports human islets [78, 80, 99]. The islet BM provides support that influences survival and function both in vivo, and in vitro [74, 77, 80]. Previous research has demonstrated that the islet BM is not re-established during islet culture and that certain BM proteins continue to further deteriorate in standard CMRL culture systems, over a 72-hour period [74]. Due to the importance of the BM in islet function and survival, it is reasonable to assume that enhancing the preservation of the islet BM would facilitate better function and survival of islets both in vitro and in vivo. UW has been demonstrated to better support islets during the post-isolation period [66, 69, 92, 93]. However, no previous literature has investigated the impact of UW storage on BM survival. This thesis therefore aimed to determine if a UW system was superior to the current CMRL system for preserving the islet BM post-isolation.

5.2 Aims of Chapter

- Chapter 2 aimed to provide a comparison between the efficiency of CMRL medium and UW cold storage in maintaining islets during the post-isolation culture period. Assessments to determine this include islet count, IEQ, islet viability, and sample purity

recorded at the conclusion of islet isolation, and 24, 48, and 72 hours following fresh collection and sample plating.

- Chapter 3 aimed to show the key matrix proteins within the native pancreas to understand the abundance and structure of each protein prior to islet isolation.
- Chapter 4 aimed to understand the impact of human islet preservation/culture in UW, and CMRL, respectively, on the human islet BM through the assessment of key matrix proteins collagen IV, laminin, laminin-α5, and perlecan via protein quantification and immunolabelling of proteins.

5.3 Thesis Conclusions

5.3.1 Overall Thesis Conclusions

The following table (Table 7) includes a summary of key findings from Chapter's 2, 3, and 4 of this thesis.

Chapter	Key findings
2. Pancreatic Islet Isolation, Collection, and Post-Isolation Assessment	• There is a significant loss of IEQ in islets cultured in CMRL up to 72 hours. • Loss of IEQ is not seen in islets suspended in UW for up to 72 hours. • The number of islets remaining after storage in CMRL is significantly lower than islets stored in UW at 48 hours. • Islet viability significantly declined in islets suspended in CMRL for up to 72 hours. • Islet viability was relatively maintained in islets suspended in UW for up to 72 hours.

	• UW storage is superior to CMRL storage for survival of islets for up to 72 hours following islet isolation.
3. The Native Pancreas	• Key ECM proteins detected within the native pancreas include laminin, laminin-α5, collagen IV, and perlecan. • Collagen IV exists within the native pancreas in the greatest abundance, followed by laminin, laminin-α5, and perlecan, respectively.
4. Comparing the Islet Basement Membrane Integrity Following Preservation/Culture in UW and CMRL	• Immunofluorescent staining revealed a significant loss of laminin in islets suspended in CMRL after 72 hours, compared to those suspended in CMRL after 24 hours. • There was significantly greater perlecan staining visualised on islets suspended in UW at 24 hours, compared to islets in CMRL at 24 hours. • Blinded operator scoring comparison was not significant, representing reproducibility. • The concentration of laminin detected in islets declined significantly after 48 hours in CMRL and was not re-established after 72 hours. • Collagen IV was maintained in both media for up to 72 hours. • A significant decline in the concentration of laminin-α5 was detected in islets suspended in CMRL at 72 hours, compared to fresh islets. • There was a significant difference in the concentration of laminin-α5 in islets in CMRL at 48 and 72 hours compared to islets in UW at 48 and 72 hours, respectively.

Table 7: Key findings of individual thesis chapters

5.3.2 Implications for Culture in CMRL or UW of Poor Islet Isolation Product

Isolated islets from 2 additional donors (Table 8) were collected for inclusion in this study. The islets were processed as per the sample plating, collection, and post-isolation assessments

described above. The islet counts and viability data reflected a poor isolation product will not recover in CMRL nor UW culture systems following islet isolation. These results reflected that there was a large decline in islet number and viability after only 24 hours in CMRL. While the islet preparations were better maintained in UW, there was still a decline in islet count, IEQ, and viability seen in both samples at 48 hours, with a further decline by 72 hours (see appendix E, Figures E1-E3).

	HP2338	**HP2339**
AGE	51	39
GENDER	F	M
BMI	30	30
CIT (HOURS)	7	9
DONOR TYPE	DBD	DBD

Table 8: Donor Demographics for Chapter 5
Donor demographics of excluded donors.

5.4 Discussion

Islet cell transplantation is a minimally invasive intervention that can help to reduce hypoglycaemic unawareness in individuals with T1DM. The process of human islet isolation, in which the cells are procured for transplantation, is still suboptimal. The mandatory islet culture period following islet isolation has been shown to decrease islet function and viability under current conditions [8, 72, 112, 114, 115]. Previous studies have shown islet cell culture in UW cold storage solution to be superior to islet culture in CMRL [66, 69, 97], which is the current gold standard media for islet culture. The existing literature surrounding human islet cell survival in UW cold storage compared to CMRL following islet isolation proves that islet particle number,

viability, and IEQs are better preserved in UW compared to islets cultured in CMRL [66, 69]. Previous literature has explored whether the better survival of islets may be due to storage temperature, however findings showed the storage of islets in CMRL at 4° C was more detrimental to islet survival, as compared to currently used islet culture in CMRL at 37° C. The findings from this study build off these concepts and results to suggest an alternative explanation for better islet survival in UW cold storage. This research offers a novel understanding of the maintenance of the human islet BM during the mandatory islet culture period under 2 islet culture/preservation conditions. Findings from this research prove UW cold storage to be a superior storage system to CMRL culture for the maintenance of the human islet BM during the mandatory islet culture period. This will help to challenges current paradigms that CMRL is the best culture system as these findings provide a biological reasoning for the better survival of islet cells.

5.5 Limitations of study

One of the greatest limiting factors in islet isolation and transplantation is the lack of available donor organs for processing. For this study a sample size of 5 donors was achieved. Although 7 donors were procured for the use of this study, two of these donors were omitted due to poor material quality, as explained above. I have included a record of all 13 islet isolations attended throughout the course of this master's research for the intended use of this project, which can be found in the appendix of this thesis (Table E1).

5.6 Recommendations for further work

5.6.1 Explore Reconstitution of The Human Islet BM in *Vivo*

Understanding the impact on the islet BM of islets suspended in CMRL culture systems following islet isolations can indicate the reconstitution of the islet BM in vivo. One group has shown, using a rodent model that following the culture of islets, the basement membrane of pancreatic islets does reconstitute after re-transplantation. These findings, however, reflected a highly disorganised reconstitution of the islet BM in mice [115]. The disorganisation of the BM may reflect poor BM existence at the time of engraftment. Due to the importance of the islet BM for β-cell function, the disorganised reconstitution of the BM may hinder islet function. Considering this model, more optimal storage of islets prior to transplantation may help preserve the islet BM and increase BM reconstitution success as islets will be transplanted with an existing islet BM scaffolding.

5.6.2 Consider Extension of Storage Period (beyond 72 hours)

Within the United Kingdom, islet isolation and transplantation are governed by the Human Tissue Authority (HTA) under the Human Application Sector. Within this regulatory framework, it is not possible to culture islets for more than 48 hours without the establishment holding a HTA 'storage licence'. To be granted a storage licence, scientific evidence must be presented to the HTA, which clearly demonstrates that prolonging islet storage beyond 48 hours has no detrimental impact on islet survival and quality. Indeed, obtainment of a storage licence has certain logistical benefits, such as allowing the islets to be "reoffered" to other islet recipients, if

the intended recipient was to become unwell immediately prior to the transplant. The Oxford Islet Facility have encountered this scenario several times in the past, but due to the time restraints, it is not possible to reoffer the islets for clinical use, as it would not be possible to mobilise a second recipient before the 48-hour window elapsed.

The data presented within this thesis suggests that islets deteriorate after 48 hours when they are placed in standard CMRL culture media. This would therefore not support a successful application to the HTA for a storage licence. Given the improved islet survival under UW storage conditions, islets may be able to be preserved for longer than 48 hours. This data would support the application for a storage licence and would help to ensure that transplantable islets are not being lost to the time restraints that are currently placed on establishments that do not hold this licence type.

5.6.3 Resuspension in CMRL Before Transplantation

Although UW cold storage proves to be superior for maintaining the human islet BM in vitro, this medium would not offer suitability as an excipient, due to the ratio of sodium (Na) to potassium (K+) present in the solution. Additionally, as a cold storage system, the cell's metabolic activity is minimalised significantly. Considering the work presented in this thesis, future work within the group will focus on islet survival following the resuspension of islets in CMRL media prior to transplantation. Given islet survival declines after 24 hours in culture, resuspension of islets in CMRL for less than 24 hours may allow for islets to regain physiological activity without causing significant disruption to the islet BM, impacting cell viability and function. Resuspension in a

transplantable medium is important for the implementation of this storage method in clinical practice.

5.6.4 Explore Resolution of Na/K+ Ratio in UW Cold Storage Solution

The current Na/K+ ratio present in the commercially used Belzer's UW cold storage solution may not be used in vivo, as the high concentration of K+ induces cardiac arrhythmias. However, studies have explored altering the existing Na/K+ ratio to mimic more physiological conditions [116]. Further exploration of islet survival in an extracellular-like UW solution is necessary to determine the logistical outcome of using such media.

5.7 Conclusions

This thesis represents the first study to investigate the integrity of the pancreatic islet BM following islet isolation in UW cold storage solution for up to 72 hours. UW is a superior media for islet storage up to 72 hours following pancreatic islet isolation, compared to CMRL. The concentration of key BM proteins of islets stored in UW is greater at 72 hours, compared to the concentration of key BM proteins of islets stored in CMRL at 72 hours. The better preservation of the islet BM throughout the post-isolation recovery period may foster better engraftment, survival, and function of islets in vivo. The findings presented in this thesis, as well as recommendations for further work, will help increase the success of pancreatic islet transplantation for a subset of patients with Type 1 Diabetes.

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Appendices

Appendix A

(Chapter 1)

Donor Pancreas Acceptance Criteria		
Parameter	**Donor after Brainstem Death (DBD)**	**Donor after Circulatory Death (DCD)**
Cold ischemia time (CIT)	≤ 10 hours	≤ 6 hours
Age	22 - 60 years; for donors aged 22 -25 years, BMI must be > 27	22 - 50 years; for donors aged 22 - 25 years, BMI must be > 27
Body Mass Index (BMI)	23 – 40; for donors with BMI 23 – 25, age must be > 40 years	23 – 40: for donors with BMI 23 – 25, age must be > 40 years
History of pancreatitis	No	No
History of diabetes	No; HbA1c < 6.5% (< 48mmol/mol)	No; HbA1c < 6.5% (< 48mmol/mol)
Negative virology (except EBV*, CMV*, and Toxicology)	No viral meningitis; No positive test result for COVID-19	No viral meningitis; No positive test result for COVID-19
History of drug abuse	No evidence of IV drug abuse	No evidence of IV drug abuse
History of excess alcohol intake	No	No
Cardiac arrest downtime	≤ 30 minutes	≤ 30 minutes
Exercise caution if donor has: • **History of drug abuse** • **History of excess alcohol intake** • **High pancreatic amylase (>110; may indicate pancreatic damage** • **eGFR <60 (indication of poor kidney function)** **Decline organ if donor has:** • **History of intravenous drug use** • **History of malignancy (if patient has been clear of malignancy for >5 years then discuss potential acceptance)** • **Past social history that could increase likelihood of transmissible disease**		

Table A1: UK Islet Transplantation Donor Organ Acceptance Criteria

Summarized Islet Transplant Recipient Criteria	
Inclusion Criteria	**Exclusion Criteria**
Adults with well-established Type 1 Diabetes (insulin dependent ≥ 5 years, negative C-peptide)	Medical counter-indications, such as CVD
Intensive diabetes management impacting patient QOL	
Absence of insulin resistance	
≥ 2 episodes of severe hypoglycaemia requiring third party intervention within the last 2 years	

Table A2: UK Islet Transplantation Recipient Criteria

Bridge to Life, Belzer UW® Cold Storage Solution Components	
Component	**Volume**
Pentafraction	50 g/L
Lactobionic Acid	35.83 g/L
Potassium Phosphate monobasic	3.4 g/L
Magnesium Sulfate Heptahydrate	1.23 g/L
Raffinose pentahydrate	17.83 g/L
Adenosine	1.34 g/L
Allopurinol	0.136 g/L
Total Glutathione	0.922 g/L
Potassium Hydroxide	5.61 g/L
Sodium Hydroxide, 5N	5.0 mL/L
Water for Injection	q.s.

Table A3: Bridge to Life, Belzer UW® Cold Storage Solution Composition

Pan-Biotech, CMRL-1066 w/o: L-Glutamine, w/o: Phenol red, w: 2.2 g/L $NaHCO_3$ Composition	
Component	**Volume (mg/L)**
Calcium chloride x $2H_2O$	264.92
Potassium chloride	400.00
Magnesium sulfate x $7H_2O$	200.00
Sodium acetate x $3H_2O$	82.90
Sodium chloride	6800.00
Sodium dihydrogen phosphate x H_2O	140.00
Cholesterol	0.20
D (+)-Glucose anhydrous	1000.00

Glutathione (red.)	10.00
Sodium glucuronate x H_2O	3.88
Tween 80	5.00
Carboxylase x HCl	1.00
Coenzyme A	2.50
NAD	7.00
NADP sodium salt	1.00
UTP	1.00
L-Alanine	25.00
L-Arginine x HCl	70.00
L-Aspartic acid	30.00
L-Cysteine x HCl x H_2O	260.00
L-Cystine	20.00
L-Glutamine	0.00
L-Glutamic acid	75.00
Glycine	50.00
L-Histidine x HCl x H_2O	20.00
L-Hydroxyproline	10.00
L-Isoleucine	20.00
L-Leucine	60.00
L-Lysine x HCl	70.00
L-Methionine	15.00
L-Phenylalanine	25.00
L-Proline	40.00
L-Serine	25.00
L-Threonine	30.00
L-Tryptophan	10.00
L-Tyrosine	40.00
L-Ascorbic acid	50.00
P-Aminobenzoic Acid	0.05
D (+)-Biotin	0.01
D-Calcium pantothenate	0.01
Choline chloride	0.50
Folic acid	0.01
Myo-Inositol	0.05
Nicotinic Acid	0.025
Nicotinamide	0.025
Pyridoxal x HCl	0.025
Pyridoxine x HCl	0.025
2'-Deoxyadenosine x H_2O	10.00
2'-Deoxycytindine x HCl	11.60
2'-Deoxyguanosine	10.00

2'-Deoxythymidin	10.00
5-Methyl-2'-deoxycytidine	0.10
Flavin adenine dinucleotide Na_2-Salt	0.106
$NaHCO_3$	2,200.00

Table A4: PAN-Biotech, CMRL-1066 w/o: L-Glutamine, w/o: Phenol red, w: 2.2 g/L $NaHCO_3$ Composition

Appendix B

(Chapter 2)

2.2.3 GSIS Methodology

Following OXCIT SOP 3.2c, a GSIS assessment was completed on fresh islets collected at the conclusion of the isolation procedure, and on islets after 48 hours in the respective media. To evaluate islets suspended in UW at 48 hours, a sample was taken and resuspended in CMRL at 37°C for 6-8 hours. Due to the components of cold storage, this period was necessary to allow islets to become physiologically active prior to assessment. Using the pre-made Krebs Ringer Buffer (KRB) working solution, one 50 ml aliquot was thawed for use. In a 15 ml falcon tube (Appleton) 9.0 mL of KRB was aliquoted to create the KRB glucose-free (0 mM) solution. 30.6 mL of the thawed KRB working solution was transferred into another 50 ml falcon tube and supplemented with 110 μl of 100 g/L glucose solution (Sigma-Aldrich) for a final KRB basal glucose solution concentration of 2 mmol/L. The KRB stimulating glucose solution was created by transferring 10 ml of thawed KRB working solution into a 15 ml falcon tube and adding 400 μl of glucose solution for an overall solution concentration of 22.0 mmol/L. The cell lysis reagent was made using 47.5 ml of sterile water and 2.5 ml of Tween 20 (Sigma-Aldrich), then labelled, and stored at 4°C until use.

For the islet collection aspect of the protocol, islets were collected at the conclusion of isolation (fresh) and at 48 hours in each respective media (CMRL or UW). All media was equilibrated to 37°C using the microtitre plate shaker incubator (Stuart). Islet samples collected were sedimented via centrifugation for 3 minutes at 1000 rpm. Remnant supernatant was carefully

removed and replaced with 2000 μl of 2 mM-KRB. Islets were then transferred to 35 mm petri dishes (Fisherbrand). Using 2 pre-labelled 2 ml microtubes (Eppendorf) 10 islets and 15 μl of 2 mM-KRB were collected in each. The subsequent steps of the protocol elaborate the stimulation process using the different concentrated solutions described as prepared above.

The Islet equilibration (Ins-pre) phase is conducted by adding 985 μl of 2 mM-KRB to each tube. The tubes are then placed on the microtitre plate shaker incubator at 37°C for 30 minutes with continuous shaking at 250 rpm. After 30 minutes islets were settled for 3 minutes. 900 μl of supernatant was collected in a 1.5 ml microtube, labelled, and stored at -20°C until use. Next, the basal phase (Ins-2.1) was completed by refilling the 2 ml microtubes with 900 μl of pre-warmed 2 mM-KRB. Samples were incubated for 45 minutes at 37°C with continuous orbital shaking. Islets were left to settle for 3 minutes, then 900 μl of supernatant was collected, labelled, and stored at -20°C until use. The same steps were repeated for the stimulation phase (Ins-20), with respect to the addition of 22 mM-KRB, rather than 2 mM-KRB following the collection of sample supernatant and prior to 45-minute incubation. Similarly, these steps were repeated for the down-regulation phase (Ins-2.2), however replacing the 22 mM-KRB with 0 mM-KRB following collection of sample supernatant. After the last supernatant collection from the Ins-2.2 phase, cell disintegration was completed. 900 μl of ice-cold cell lysis reagent was added to each 2 ml microtube containing islets. Samples were sonicated for 20-30 seconds at 20 watts and stored at -20°C for later use.

2.2.3.1 Insulin ELISA

Using an insulin ELISA kit (Mercodia; 35454), samples prepared as per the GSIS protocol described above were assessed for insulin secretion. 25 µl of calibrators and samples were added to respective wells. 100 µl of enzyme conjugate was added to each well, and the plate was incubated for 1 hour at room temperature on a plate shaker (Stuart). The plate was washed 7 times using prepared wash buffer, and excess contents were removed by snapping the 96-well plate against blue roll until dry. 100 µl TMB substrate was added to each well and the plate was incubated at room temperature for 15 minutes. 50 µl of stop solution was added to each well and the plate was read using a VERSAmax plate reader (Molecular Devices) at an absorbance of 450 nm. GSIS ELISA results were analysed for significance using GraphPad Prism 10 (San Diego, USA) by way of analysis of variance (ANOVA). A Tukey's multiple comparisons test was run to detect differences in glucose stimulation index between the islets suspended in UW, compared to those suspended in CMRL.

GSIS Results

Due to the nature of the sample, the results of the GSIS assessment were inconclusive. Fellow researchers within the OCDEM, who also worked on islets from these preparations, reported that the islets were not adequate for GSIS assessment due to immediate disintegration upon warming (which is required for GSIS). The results of the GSIS assessment run for this thesis are reported below.

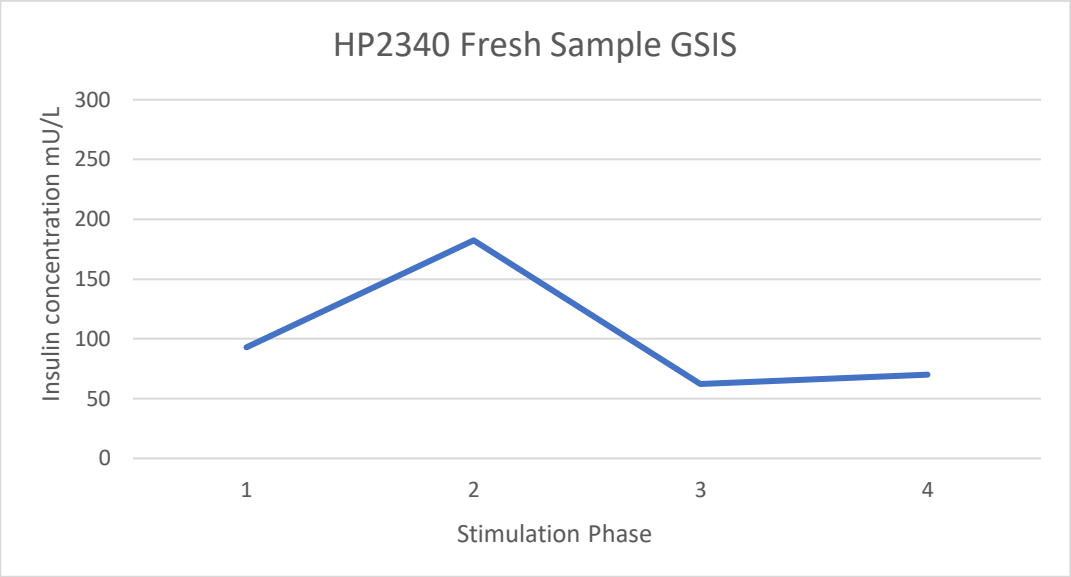


Figure B1: Glucose stimulated insulin secretion test results determined by ELISA for HP2340 fresh sample
Stimulation phase key: 1= Ins-pre, 2= Ins-2.1, 3= Ins-20, 4= Ins-2.2.

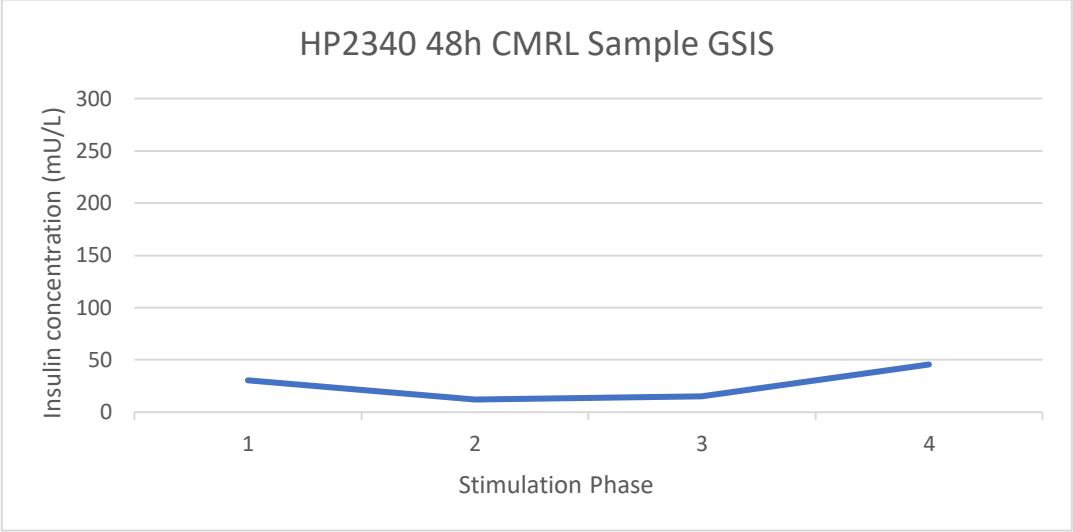


Figure B2: Glucose stimulated insulin secretion test results determined by ELISA for HP2340 48-hour CMRL sample
Stimulation phase key: 1= Ins-pre, 2= Ins-2.1, 3= Ins-20, 4= Ins-2.2.

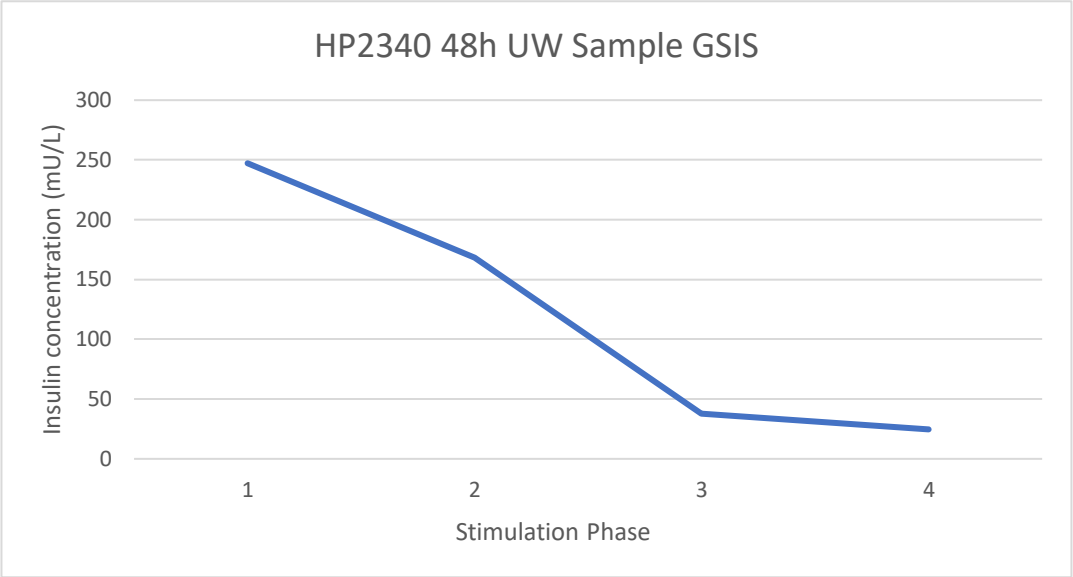


Figure B3: Glucose stimulated insulin secretion test results determined by ELISA for HP2340 48-hour UW sample

Stimulation phase key: 1= Ins-pre, 2= Ins-2.1, 3= Ins-20, 4= Ins-2.2.

Appendix C

(Chapter 3)

Sigma-Aldrich, RIPA Buffer
Component
150 mM NaCl
1.0% IGEPAL® CA-630
0.5% sodium deoxycholate
0.1% SDS
50 mM Tris

Table C1: Sigma-Aldrich RIPA Buffer composition

Protein	**Blocking buffer; dilution; catalogue; manufacturer**	**Primary antibody; dilution; catalogue; manufacturer**	**Secondary antibody; dilution; catalogue; manufacturer**	**Insulin primary antibody; dilution; catalogue; manufacturer**	**Insulin secondary antibody; dilution; catalogue; manufacturer**
Collagen IV	Swine serum; 5%; S-4000; Vector Laboratories	Goat anti-collagen IV; 1:100; 134001; Bio-Rad	Donkey anti-goat A488; 1:500; A-11055; Life Technologies	Guinea pig anti-human insulin; 1:100; In house produced antibody kindly provided by Dr. Anne Clark, OCDEM, University of Oxford	Donkey anti-guinea pig A594; 1:500; 134515; Jackson Immunotech
Laminin	Goat serum; 10%; S-1000; Vector Laboratories	Rabbit anti-laminin; 1:300; ab11575; Abcam	Goat anti-rabbit A488; 1:500; A-110088; Life Technologies	Guinea pig anti-human insulin; 1:100; In house produced antibody kindly provided by Dr. Anne Clark, OCDEM, University of Oxford	Donkey anti-Guinea Pig A594; 1:500; 134515; Jackson Immunotech
Laminin-a5	Goat serum; 10%; S-1000; Vector Laboratories	Mouse anti-laminin alpha 5; 1:300; ab77175; Abcam	Goat anti-mouse CFL; 1:100; sc-362257; Santa-Cruz	Guinea pig anti-human insulin; 1:100; In house produced antibody kindly provided by Dr. Anne Clark, OCDEM, University of Oxford	Donkey Anti-guinea pig A594; 1:500; 134515; Jackson Immunotech

Perlecan	Goat serum; 10%; S-1000; Vector Laboratories	Mouse anti-perlecan 1:300; sc-377219; Santa Cruz	Goat anti-mouse CFL; 1:100; sc-362257; Santa-Cruz	Guinea pig anti-human insulin; 1:100; In house produced antibody kindly provided by Dr. Anne Clark, OCDEM, University of Oxford	Donkey anti-guinea pig A594; 1:500; 134515; Jackson Immunotech

Table C2: Materials for immunofluorescent stanning of native pancreas tissue sections for ECM proteins with insulin

Protein	Supplier and (catalogue number)
Collagen IV	Cloud-Clone Corp. (SEA180Hu)
Total laminin	Abcam (ab119599)
Laminin-α5	Cloud-Clone Corp. (SEC078Hu)
Perlecan	Cloud-Clone Corp. (SEA565Hu)

Table C3: ELISA kit information for native pancreas protein quantification

Appendix D

(Chapter 4)

Protein	Permeabilization and blocking buffer; dilution; catalogue; manufacturer	Primary antibody; dilution; catalogue; manufacturer	Secondary antibody; dilution; catalogue; manufacturer	Insulin primary antibody; dilution; catalogue; manufacturer	Insulin secondary antibody; dilution; catalogue; manufacturer
Collagen IV	Swine serum; 5%; S-4000; Vector Laboratories *Supplemented with TBS and Triton	Goat anti-collagen IV; 1:100; 134001; Bio-Rad	Donkey anti-goat A488; 1:500; A-11055; Life Technologies	Guinea pig anti-human insulin; 1:100; In house produced antibody kindly provided by Dr. Anne Clark, OCDEM, University of Oxford	Donkey anti-guinea pig A594; 1:500; 134515; Jackson Immunotech
Laminin	Swine serum; 5%; S-4000; Vector Laboratories *Supplemented with TBS and Triton	Rabbit anti-laminin; 1:300; ab11575; Abcam	Goat anti-rabbit A488; 1:500; A-110088; Life Technologies	Guinea pig anti-human insulin; 1:100; In house produced antibody kindly provided by Dr. Anne Clark, OCDEM, University of Oxford	Donkey anti-guinea pig A594; 1:500; 134515; Jackson Immunotech
Laminin-a5	Swine serum; 5%; S-4000; Vector Laboratories *Supplemented with TBS and Triton	Mouse anti-laminin alpha 5; 1:300; ab77175; Abcam	Goat anti-mouse CFL; 1:100; sc-362257; Santa-Cruz	Guinea pig anti-human insulin; 1:100; In house produced antibody kindly provided by Dr. Anne Clark, OCDEM, University of Oxford	Donkey anti-guinea pig A594; 1:500; 134515; Jackson Immunotech
Perlecan	Swine serum; 5%; S-4000; Vector Laboratories *Supplemented with TBS and Triton	Mouse anti-perlecan 1:300; sc-377219; Santa Cruz	Goat anti-mouse CFL; 1:100; sc-362257; Santa-Cruz	Guinea pig anti-human insulin; 1:100; In house produced antibody kindly provided by Dr. Anne Clark, OCDEM, University of Oxford	Donkey anti-guinea pig A594; 1:500; 134515; Jackson Immunotech

Table D1: Materials for immunolabelling of human islets for BM proteins

ThermoFisher Scientific, Hypotonic Buffer
Component
20 mM Tris-HCl, pH 7.4
10 mM NaCl
3 mM $MgCl_2$

Table D2: Hypotonic Buffer composition

ThermoFisher Scientific, Protease Inhibitor Cocktail (PIC)
Component (concentration in 100x cocktail formulation)
100mM AEBSF•HCl
80μM Aprotinin
5mM Bestatin
1.5mM E-64
0.5M EDTA
2mM Leupeptin
1mM Pepstatin A

Table D3: Protease Inhibitor Cocktail (PIC) composition

Protein	Time Point	P-value
Laminin	**Fresh**	**>0.9999**
	CMRL 24	**0.1250**
	UW 24	**0.4531**
	CMRL 48	**0.3750**
	UW 48	**0.1719**
	CMRL 72	**0.2500**
	UW 72	**>0.9999**
Collagen IV	**Fresh**	**>0.9999**
	CMRL 24	**>0.9999**
	UW 24	**0.0625**
	CMRL 48	**0.3594**
	UW 48	**>0.9999**
	CMRL 72	**>0.9999**
	UW 72	**0.1250**
Laminin-α5	**Fresh**	**No differences**
	CMRL 24	**>0.9999**
	UW 24	**>0.9999**
	CMRL 48	**0.5000**
	UW 48	**>0.9999**
	CMRL 72	**0.5000**

	UW 72	**0.5078**
Perlecan	**Fresh**	**>0.9999**
	CMRL 24	**0.1250**
	UW 24	**>0.9999**
	CMRL 48	**0.0625**
	UW 48	**0.5000**
	CMRL 72	**0.2500**
	UW 72	**0.6250**

Table D4: Operator scoring p-values for each score comparison
Statistical significance confirmed via Wilcoxon matched pairs signed rank test.

Appendix E

(Chapter 5)

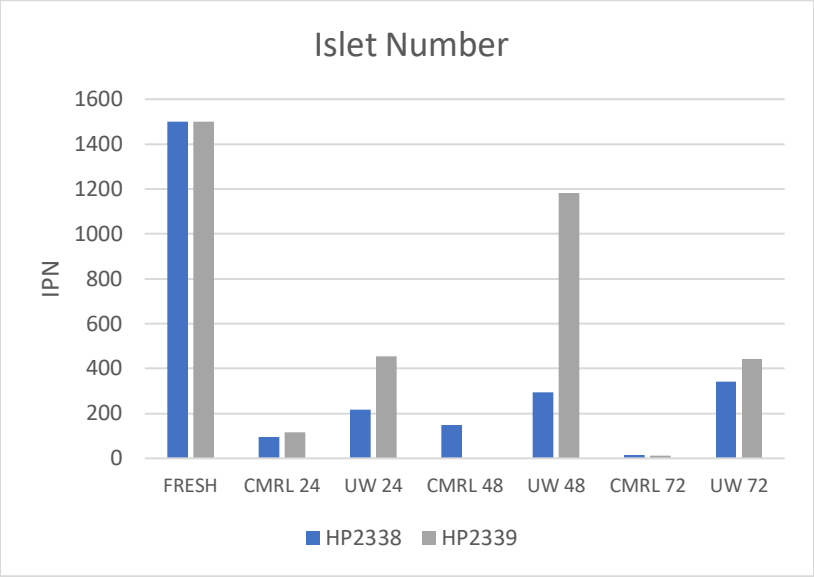


Figure E1: Islet count for omitted donors (HP2339 and HP2338) at each sample collection time point

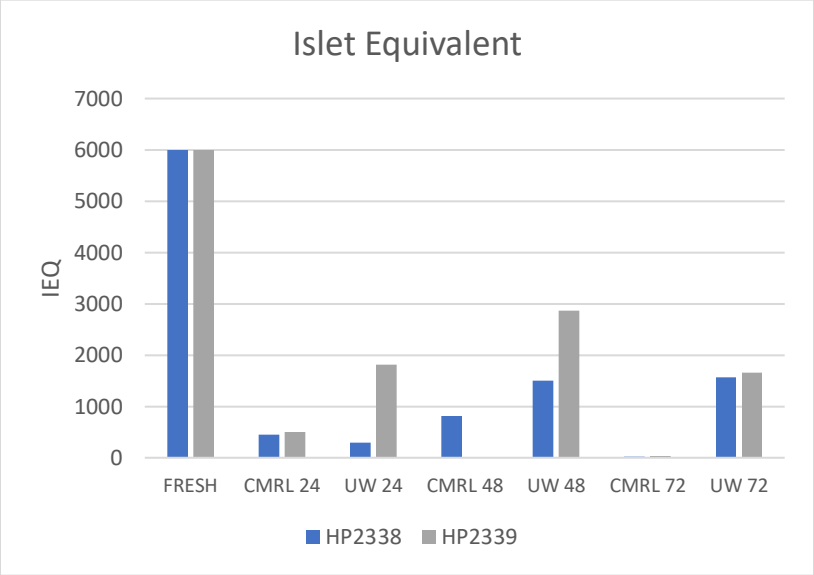


Figure E2: Calculated islet equivalent for omitted donors (HP2339 and HP2338) at each sample collection time point

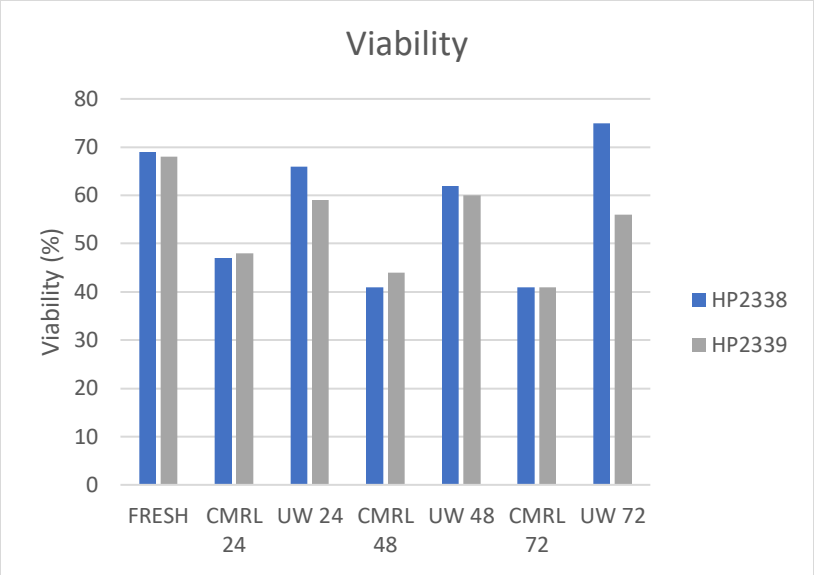


Figure E3: Islet viability for omitted donors (HP2339 and HP2338) at each sample collection time point

HP	Date	Outcome
2232	07/10/2022	Poor tissue quality, allocated to research after poor reassessment
2304	27/01/2023	Research: yield did not meet requirement for recipient.
2307	09/02/2023	Research: yield did not meet requirement for recipient.
2311	21/02/2023	Research: yield did not meet requirement for recipient.
2322	18/04/2023	Transplanted
2328	18/05/2023	No research consent, poor yield.
2330	03/06/2023	Stopped processing due to poor tissue digest.
2335	07/21/2023	Stopped processing due to decline by recipient transplant facility.
2337	04/08/2023	Transplanted
2338	17/08/2023	Research: yield did not meet requirement for recipient.
2339	12/09/2023	Research; insufficient islet viability and poor tissue quality.
2340	26/09/2023	Research; declined after islet isolation due to donor history of gestational diabetes
2343	06/11/2023	Transplanted

Table E1: Isolation attendance throughout the duration of my master's research October 2022-December 2023