Standardization of Islet Isolation and Transplantation Variables

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Abstract

Currently, the transplantation of islets of Langerhans is a viable means to maintain control of blood sugar levels and reduce the risk of hypoglycemia in defined populations with brittle type 1 diabetes mellitus or those requiring pancreatectomy. However, the process of islet isolation is highly variable and not all isolations result in islet numbers or quality suitable for transplantation.

This thesis aimed to improve transplantation success through optimization and standardization of the isolation process and to identify pretransplant variables associated with early islet engraftment.

A previously disregarded enzyme activity, tryptic-like activity (TLA), has been identified to influence pancreas digestion efficiency and islet isolation success in both the preclinical and clinical situations. For human pancreases, islet isolation success rates improved from 0% in the lowest TLA group to over 50% in the highest TLA groups without affecting islet quality. These findings should help standardize evaluation of enzymes for clinical islet isolation.

A closed, automated, pump-made gradient system was compared to the open, manual method for islet separation. No differences were observed in expected gradient volumes, islet yields or total purities between the two methods. The pump-made gradient system successfully removed manual influences on density gradient production while fulfilling regulatory requirements for closed system processing.

Islet quantification was evaluated with computer-assisted digital imaging analysis (DIA) and a semi-closed assessment system. By using the DIA system method, which measures islet purity and pellet volume instead of manual counting methods, variation in islet counts and purity reduced by almost half.

By using a transplant outcome measurement of C-peptide adjusted by blood glucose and creatinine, we identified four pretransplant factors that affect early transplant outcome. Of the four factors, one was related to the organ transport time, one to function of the islets, and two to the transplanted tissue volume. When these four factors were put into a predictive model, it accounted for about 40% of the transplant outcome.

The work contained in this thesis identifies and optimizes a number of critical elements related to islet isolation and transplantation protocols.

Keywords: Islet isolation, standardization, enzyme, gradient separation, digital imaging analysis, DIA, transplantation outcome, islet transplantation, prediction

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“What we think, or what we know, or what we believe is, in the end, of little consequence. The only consequence is what we do.”

John Ruskin (1819 - 1900)

For my family
Till min familj
Für meine Familie
A családomnak
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIR&lt;sub&gt;arg&lt;/sub&gt;</td>
<td>Acute insulin response to argentine</td>
</tr>
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<td>APS</td>
<td>Automated purification system</td>
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<tr>
<td>BAEE</td>
<td>N-benzoyl-L-arginine-ethyl ester</td>
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<tr>
<td>BD</td>
<td>Brain death</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<td>C-peptide</td>
<td>Connecting-peptide</td>
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<tr>
<td>CI</td>
<td>Collagenase class I</td>
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<td>CII</td>
<td>Collagenase class II</td>
</tr>
<tr>
<td>CIT</td>
<td>Cold ischemia time</td>
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<tr>
<td>CV%</td>
<td>Coefficient of variation × 100%</td>
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<tr>
<td>ΔCP/GCr</td>
<td>Change in C-peptide × glucose&lt;sup&gt;-1&lt;/sup&gt; × creatinine&lt;sup&gt;-1&lt;/sup&gt; ratio</td>
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<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
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<td>DMC</td>
<td>Dimethylcasein</td>
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<tr>
<td>DIA</td>
<td>Digital imaging analysis</td>
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<tr>
<td>DTZ</td>
<td>Dithiocarbazone, dithizone</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EP</td>
<td>European parliament</td>
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<tr>
<td>GMP</td>
<td>Good manufacturing practices</td>
</tr>
<tr>
<td>HbA&lt;sub&gt;1c&lt;/sub&gt;</td>
<td>Hemoglobin A&lt;sub&gt;1c&lt;/sub&gt;</td>
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<tr>
<td>HTK</td>
<td>Histidine-tryptophan-ketogluterate</td>
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<td>IAK</td>
<td>Islet after kidney</td>
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<td>IE</td>
<td>Islet equivalent</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IVGTT</td>
<td>Intravenous glucose tolerance test</td>
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<td>MCP-1</td>
<td>Macrophage chemotactant protein-1</td>
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<td>MMTT</td>
<td>Mixed meal tolerance test</td>
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<tr>
<td>NP</td>
<td>Neutral protease</td>
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<tr>
<td>PP-cells</td>
<td>Pancreatic polypeptide cells</td>
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<tr>
<td>PZ</td>
<td>4-phenylazobenzoyloxy carbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SGM</td>
<td>Standard gradient maker</td>
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<td>SUIT</td>
<td>Secretory units of islet in transplantation</td>
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<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
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<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
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<tr>
<td>TF</td>
<td>Tissue factor</td>
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<td>TLM</td>
<td>Two-layer method</td>
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<td>UW</td>
<td>University of Wisconsin solution</td>
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Introduction

Diabetes is a widespread and increasingly prevalent disease. Currently, β-cell replacement therapy via transplantation of islets of Langerhans is a viable means to maintain control of blood sugar levels and reduce the risk of hypoglycemia in defined populations of patients with brittle type I diabetes mellitus or those requiring partial or whole pancreatectomy. The process of islet isolation across centers suffers from variability and, despite important advances, remains to be standardized. Standardization of the isolation process, quality control parameters, quantification before transplant, and even variables associated with transplant outcome are needed for meaningful comparisons between labs.

In this thesis some variables affecting islet isolation success, evaluation of end product, and short-term islet engraftment are uncovered, addressed and evaluated. A previously disregarded enzyme activity, tryptic-like activity, has been identified to influence pancreas digestion efficiency and islet isolation success in both the preclinical and clinical situations. An effectively closed, automated pump system for the consistent and flexible creation of density gradients for separation of islet from non-islet tissue was developed. Islet quantification as evaluated with computer-assisted digital imaging analysis and introduction of a closed system that allows for the evaluation of the entire preparation at once is presented. Establishment of a hereto relatively unused transplant evaluation parameter, ΔCP/GCr, was used to identify donor, islet isolation, and quality control variables associated with early graft function in an islet after kidney patient population.

Through standardization, isletologists will be better equipped to advance the field more cheaply and efficiently with greater security and certainty. This thesis addresses aspects critical to the standardization of human pancreas processing for islet transplantation as well as some of those essential for evaluation of transplant function in the clinic.
Aims

General aims
The work contained in this thesis was carried out to improve reproducibility, safety, and reliability of islet isolation parameters and identify variables affecting transplantation outcome through standardization and adherence to good manufacturing practices where relevant.

Specific aims

Paper I
- To characterize efficient enzyme blends by evaluating the effect of a tryptic-like activity on rat and human islet isolation outcome
- To evaluate the effect of tryptic-like activity on islet viability, morphology and function in vitro and in vivo

Paper II
- To standardize the generation of continuous density gradients for use in human islet purification using an automated, closed system gradient making procedure

Paper III
- To identify sources of evaluation error in the quantification of islet isolation products
- To improve upon the widely used standard manual counting procedure with a GMP-friendly islet evaluation method

Paper IV
- To identify factors predictive of early islet engraftment in islet after kidney transplant patients as measured by the change in pre- to post-transplant C-peptide/(glucose × creatinine) ratios
- To create a best-fit model for prediction of early islet engraftment
Background

History of diabetes mellitus

From antiquity to insulin

“Diabetes is a dreadful disease, not very frequent among men, being a melting down of the flesh and limbs into urine. The patients never stop making water and the flow is incessant, like the opening of aquaducts.”

Areteaus of Cappadocia, ca. 100 AD

Descriptions of diabetes can be traced back to antiquity. In Thebes, Egypt, a document that came to be known as the Ebers Papyrus from circa 1535 BC, found between legs of a mummy, contains the first known description of a condition to what today could be called diabetes. It describes a patient where the body has “shrunken with disease” and suggests remedies “for the suppression of his thirst (and) for curing his mortal illness” (1). Sushruta an Indian surgeon from around 600 BC described a condition that produced “madhumeha” or honey-like urine. He also advised for the sedentary to become more active in the pursuit of curing the disease (2). In China about 400 BC in the Yellow Emperor’s Canon on the Traditional Chinese Medicine, the oldest Chinese medical book, a condition called “XiaoKe symptom”, meaning weight loss due to thirst is described. The first authoritative description of diabetes is from the 2nd century AD when the physician Areteaus gave the condition the name “diabetes” meaning “siphon”, used to describe the excessive flow of urine associated with the disease (see quote above).

The Latin term *mellitus*, which means “sweet honey”, was added to the name diabetes as introduced by Thomas Willis in 1674 (3). In 1776, Matthew Dobson noted that serum was sweet in taste as was the urine and concluded that the sweetness was due to sugar after boiling down 2 quarts of urine (4). The organ responsible for diabetes mellitus (DM) was long thought to be the kidney due to the excessive urination. Thomas Cawley, upon autopsy of a patient with diabetes, noted a shrunken pancreas with stones, however, no particular connection to diabetes was raised at the time of the report in 1788 (5). In 1797, John Rollo described different sugar levels in the urine of a patient with diabetes based on the type of foods ingested. An “animal” diet (i.e. meat) was recommended to reduce sugar excretion (6).

Paul Langerhans, in his 1869 doctoral thesis, described for the first time round groups of cells spread out at regular intervals throughout the pancrea-
tic parenchyma. He himself had no knowledge of the function of the cell clusters he described (7). The name “islets of Langerhans” was given by Frenchman Edouard Laguesse in 1893 in recognition of Langerhans’ thesis work (8).

Unfortunately, Langerhans passed away in 1888, the year before the coupling of the pancreas to DM was realized. Joseph von Mering and Oskar Minkowski removed the pancreases of two dogs and, with the organs removed, the animals displayed the characteristics of human DM almost immediately. When placing a small portion of pancreas in a newly pancreatectomised dog, hyperglycemia (elevated blood sugar levels, a defining characteristic of DM) was avoided until removal or degeneration of the organ (9). In 1894, P. Watson Williams transplanted 3 small sheep’s pancreas segments under the skin of a young boy who died 3 days later. The boy’s pancreas noted as “small, shriveled in appearance” (10).

After analyzing the cadaver pancreases of those who had diabetes or pancreatic disease the pathologist Eugene Opie in 1901 made the connection of the damaged islets of Langerhans to the metabolic disorder (11). The “internal secretion” of the pancreas leading to diabetes was linked to the islets of Langerhans and the race to isolate the secretion was on. The name for the substance providing the internal secretion was given by Meyer in 1909 as “insuline” in reference to the Greek word insula meaning “island” (12).

The big discovery!

There were many failed attempts to isolate the internal secretion of the pancreas, some more successful than others, but it wasn’t until 1921 that the work leading to widespread therapy was initiated (13). In the hot summer of 1921, Fredrick G. Banting convinced James R. Macleod to give him some lab space and help from his technicians. In the end it was Charles H. Best who assisted Banting, both initially unpaid, who isolated the internal secretion of the pancreas. From May to the end of July they produced the initial results of injecting “internal secretions” of the pancreas into dogs (14). Eventually, they convinced Macleod to involve Joseph B. Collip, a biochemist, to assist in the purification of what was initially named “isletin”, later named “insulin” upon the recommendation of Macleod. On January 11, 1922, a 14 year old patient was the first patient to receive insulin injections, one in each buttocks, and went on to live to 27 years of age (15). In 1923, Banting and Macleod shared the Nobel Prize for their efforts. Banting immediately shared half of his prize with Best and Macleod half of his with Collip. Eli Lilly and Company was approached to scale up, refine production and make insulin available to the general public (15). Banting and Best sold the rights to insulin for $1 to the University of Toronto (which Best does not recall ever receiving) (16).
Forms of diabetes and epidemiology

Standardization of treating diabetes came already in 600 BC when Sushruta delineated between two major forms of the disease. In the 1880s Etienne Lancereaux dubbed them diabetes maigre and diabetes gras which mean diabetes of the “thin” and “fat”, respectively (17). It was not until 1979 that the classifications of type 1 (T1DM), previously called insulin-dependent DM or juvenile diabetes, and type 2 (T2DM), previously called non-insulin-dependent DM or adult-onset diabetes, came into being (18).

Of all patients with DM globally, roughly 10% have T1DM and the other 90% are considered to have T2DM. For the purposes of this thesis the focus of T1DM and its complications are considered, however effects of hyperglycemia as discussed in the next sections affect those with T2DM as well.

Type 1 diabetes mellitus

T1DM is typically attributed to destruction of the β-cells (19) the only cells in the body that produce insulin. The absence of β-cells and/or the inability to produce enough insulin requires the exogenous use of the hormone for alleviation of hyperglycemia and to confer long-term survival. T1DM can occur at any age but typically develops in children or young adults. The causes of T1DM remain largely unknown, and despite a number of theories (20), it remains unclear as to what exactly may trigger its development and, correspondingly, there is no known cure.

The incidence of T1DM is also increasing worldwide (21-25). Geographic and seasonal changes in incidence of T1DM have been observed (26). Ethnicity also associates with differences in rates of diagnoses within the same country (27). Depending on the population considered, T1DM incidence is anywhere from 0.1 (China and Venezuela) to 37 cases per 100,000 (Finland and Sardinia) (28). In another study considering the Finnish population, in 1980 there were 31.4 cases per 100,000 per year (age-adjusted incidence) whereas in 2005 this rose to 64.2 per 100,000 per year (29) and the rate of the increase of incidence is about 4% annually (29, 30). Contrary to the Finnish reports is a recent Swedish report wherein the population demographics point to a tapering of T1DM incidence in the last decade (31).

Type 2 diabetes mellitus

T2DM is characterized by both insulin resistance and dysregulation of glucose metabolism. It is associated with age, obesity and physical inactivity among others (27). The latest data from the United States Center for Disease Control concerning prevalence of diabetes estimates 25.8 million or 8.3% of the US population has diabetes, both diagnosed and undiagnosed (27). T2DM incidence is also increasing at an alarming rate not only among adults but also in cases involving children (30). Increased physical activity and diet restrictions are typically prescribed, as is insulin in some cases.
Complications of diabetes
Living with chronic hyperglycemia can lead to many serious and life threatening complications and morbidity (32). Some complications include destruction of microvasculature leading to retinopathy, nephropathy, slow wound healing, and in some severe cases blindness, dialysis and/or amputation. Since mortality has been related to the progression of renal dysfunction (33) treating diabetes may prolong lifespan. Diabetes is a major risk factor for cardiovascular disease as well (34). Neuropathy and loss of feeling over time, reduced life-expectancy (32) and quality of life (35) are unfortunate consequences of diabetes for many. Intensive insulin treatment with the goal of normoglycemia can reduce the risk for some of these complications but can lead to increased episodes of hypoglycemia in some cases (36). Hypoglycemic episodes, seen most often in long-standing T1DM patients with glycemic lability, which if serious enough and left acutely untreated, can be life threatening. In cases where frequently low blood sugars are encountered a condition known as hypoglycemia unawareness can develop. In hypoglycemia unawareness the patient does not recognize dangerously low blood sugars and the body’s own counterregulatory mechanisms are insufficient to mount a sufficient response (37) which can lead to diabetic coma and/or death (32).

Costs of diabetes
As diabetes incidence increases so will the economic costs, which are already staggering. In 2007 in the US alone, diabetes accounted for $174 billion in healthcare costs (27). That equates to $580/person/year in the US, less than the cost of a full gym membership. Compared to non-diabetic individuals a person with T2DM has three-fold higher healthcare costs, a person with T1DM six- to seven-fold higher (38). There is also a personal cost associated with diabetes, possibly life-threatening hypoglycemia, and is the source of worry in this patient group (39).

Transplantation as therapy for diabetes
Pancreas versus islet transplantation
Since the first pancreas transplantations in humans were performed 1966 (40), over 30,000 vascularized pancreas transplantations have been performed (41). Pancreas transplantation is usually offered to patients who have total loss of β-cell function and even in some cases of T2DM (42). Pancreas transplantation is associated with a high level of β-cell functional capacity and typically the patients have immediate benefits of normalized glycemia (43). Risks of pancreas transplantation mainly include morbidity and mortality associated with the surgical complications (44). These patients require lifelong immunosuppression.
A less invasive alternative to pancreas transplantation is islet transplantation. Recipients of islet allotransplants can enjoy reduction of glycemic lability, including reduction of insulin requirements and reduced episodes of hypoglycemia even five years after transplant (45, 46). Measurements of hemoglobin (Hb)A1c, which is an indication of long-term (~3 month average) glycemia, typically increase with diabetes onset (47). Indeed, reduced HbA1c levels that are observed in the majority of islet recipients (45, 46, 48). Subjects with diabetes undergoing dialysis may have beneficial effects in terms of reduced macro- and microangiopathy as well as increased long-term survival if a kidney transplant is combined with a simultaneous islet transplant (48). Islet graft recipients currently require immunosuppression.

The negative side effects of immunosuppression must be weighed against what is gained by the transplant. Current immunosuppressive regimens can cause side effects such as mouth ulcers, anemia, diarrhea, hypertension, deterioration of renal function, and more (45, 49, 50). Despite these side effects, overall patients’ quality of life measurements indicate a positive outlook towards islet transplantation as diabetes therapy (51).

A necessity of islet transplantation is the proper use of scarce organs for transplantation purposes (52). In whole pancreas transplantation, organs from larger donors are associated with increased risk of graft failure (53) whereas they are preferred for islet cell processing (54). Allocation of organs deemed non-ideal for whole organ transplantation yet acceptable for islet processing can yield optimal distribution of available tissues (52).

Cost analysis of islet versus pancreas transplantation
Cost analysis of islet and whole pancreas transplantation has been performed (55, 56). In a study of a Swiss-French islet transplant consortium, cost for islet transplantation was slightly higher than pancreas transplantation (56). A major conclusion of that analysis was that “a better reproducibility of islet processing will be essential for the diffusion of this technology” (56). Less than 20% of the pancreases they accepted resulted in transplantation (56), a number lower than current transplantation rates at other centers (57-59).

Indications for islet transplant
Patients qualifying for islet transplants are usually over 18 years of age and present with labile diabetes despite intensive insulin management, they have had diabetes a minimum of 5 years, have documented hypoglycemia unawareness or related autonomic non-responsiveness, absence of stimulated C-peptide (<0.2 ng/ml) to arginine stimulation test, and progressive secondary complications). Patients receiving islet transplants should also have stable mental health, no history of non-adherence to prescribed medications, have a body mass index (kg/m²) <30 and be free of a host of other confounding conditions (e.g. pregnancy, hepatitis B or C positive, other infections or malignancies) (60).
Islet autotransplantation

Islet autotransplantation for intractable pancreatitis or other cases requiring pancreatectomy can prevent surgically induced severe diabetes without the need of immunosuppressive drugs (61-67). Auto- and alloimmunity or immunosuppression may play critical roles in the observed potency of transplanted islets (68, 69). The gradual loss of islet allotransplant survival over time could be due to the immune insults experienced by the islets (70-73) and cases of islet autotransplantation allow a special insight into this phenomenon (68). Cellular immunity is not the only explanation of differences between auto- and allotransplantation outcomes. There could be islet viability differences from living donors compared to brain dead donors (74) not to mention logistical differences between the two methods.

Goals of islet transplantation

Initial results of islet transplantation focused on insulin independence as the primary endpoint. Freedom from exogenous insulin is the ultimate goal for transplant recipients. However, for the subset of patients qualifying for islet transplantation, labile, autonomic unresponsive T1DM is life-threatening (75). Glycemic control yields increased confidence and freedom from psychological stresses associated with progressive diabetes (76). Indeed, despite continuing with exogenous insulin after receiving an islet transplant, HbA1c levels can be maintained at acceptable levels without episodes of hypoglycemia (46, 77). Suggested goals of achieving metabolic control with islet transplantation (78), not just insulin independence, are gaining momentum in the quest to provide for as many patients as possible (79).

Islet transplantation

Short history of clinical islet transplantation

The introduction of insulin therapy revolutionized the treatment for diabetes patients and prolonged the lives of millions of people. However, in certain cases of uncontrollable, so called brittle diabetes, intensive insulin therapy is recommended to be used with caution (48). For these patients, uncontrollable blood sugars and hypoglycemia unawareness remain a persistent, potentially lethal threat. These patients are ideal candidates for islet transplantation as a therapy for their disease (33).

The first human islet transplant took place at the University of Minnesota in 1974 (80). Initial success was low, and between 1990-1998 only 12% reached insulin independence for more than one week (81). Despite protocol improvements no real breakthroughs came until 2000 when the group in Edmonton reported insulin independence in 7 consecutive patients with an average follow-up of almost one year (82). The so-called Edmonton protocol
involved transplanting a marginal mass of purified islets from two to four
pancreases to patients on an immunosuppression regime free of glucocorti-
coids and reduced dosage of calcineuren inhibitor (82).

After the release of the Edmonton protocol an explosion of research fa-
cilities and transplantation activity ensued (83). Funding increased and an
international trial of the Edmonton protocol ensued involving 10 centers
from the US, Canada and Europe (45). Developments in islet isolation (dis-
cussed in detail later), transplantation and immunosuppression protocols
continue to improve the clinical procedure.

Islet isolation procedure

The complex islet isolation process is notoriously inconsistent, partly due to
the empirical nature of progress in the field and, partly due to lack of stan-
dardization of the process itself. Identifying variables critical to islet iso-
lation success and improving process standardization is made even more chal-
lenging considering the regulations associated with production of biologicals
for clinical use.

Regulatory considerations

Regulatory bodies such as the United States’ Food and Drug Administration
(FDA) and the European Parliament (EP) were established with the goal,
among others, to prevent the spread of diseases to the general population by
controlling the production, traceability, quality and safety of food, cosmetics
and therapeutics as well as, where appropriate, potency testing of prospec-
tive drugs, organs, tissues or cells used for therapeutic applications (84). For
instance, in the US the regulations governing islet cell processing for trans-
plantation are extensive as islets fall under different regulatory categories; a)
biologic b) drug c) somatic cell therapy (85). EP rules also require the main-
tenance of good manufacturing practices (GMP) conditions for the manufac-
ture of islets as outlined in directive 2006/17/EC Annex IV and detailed in
the COM-directive from 2006.

Both the FDA and EP state that the exposure of product at any time to the
atmosphere, a so-called “open system”, must be in a controlled environment
of the highest grade possible. The exposed product should preferably be in a
Class 100 (Class A for EP) environment. This corresponds to a maximum
load of 100 particles larger than 0.5 μm in a cubic foot (ft³) of air or for EP
regulations at maximum 3,500 particles no larger than 0.5 μm in a cubic
meter (equivalent to 99 particles/ft³). To reach such high levels of safety and
cleanliness GMP facilities are designed according to strict standards (60).
**Implications of GMP regulations for islet production**

Costs associated with building a GMP cell production facility can prevent the establishment of new islet transplant centers. Even low cost approaches such as the modification of existing lab space to achieve GMP level processing can be prohibitive for centers looking to offer islet transplant therapy (86).

To avoid the costs and maintenance of clean rooms necessary for open system processing, some clinically available therapies use closed system technologies. In contrast to open systems, which include any open bottle, spike ports, luer ports or any other exposure to the environment, closed system technology avoids any opening to the surrounding environment and meets GMP regulations for exposure of product. Such technology is commonly used in blood banks with tubing welders to make validated connections between bags. Other branches of cell therapy incorporate closed system technology with the intention of facilitating the transition from research to the clinic (87-89).

Despite stringent precautions of GMP facilities to maintain sterility, contamination of human islet preparations is occasionally observed (90). A majority of contaminations are limited generally to organ transport, prior to GMP facility processing, or the early steps of the isolation process (91-93). Even when contamination is present, some patients expressed no signs of infection, perhaps due to prophylactic antibiotic administration (93, 94). In contrast, some preparations with negative cultures have led to infection as well as those with positive cultures (94). Such *de novo* contamination during islet processing is rare (90, 92) but can even occur under GMP conditions (93). It should be kept in mind that major surgeries and transplantation procedures are performed on a regular basis in operating theaters with exposure to a much less regulated environment.

**Closed system islet isolation**

In the pursuit of islet isolation standardization and development one should consider closed system technology to improve safety and compliance with GMP. As mentioned above, the enormous costs that are associated with realizing a GMP lab from scratch favor the introduction of closed systems when possible. Due to the complex nature of large-scale islet processing, technologies that fulfill functional requirements for the use in blood banks, such as apheresis, elutriation or customized systems, have not been implemented in clinical islet transplantation so far (95, 96). Despite some movement of the islet field toward closed system processing (59, 95-97), a fully closed system has yet to be presented for all steps at one center. Major efforts were made in the presented studies to create standardized systems compliant with closed system processing that were flexible enough to allow for the most demanding requirements in the field (98, 99).
Islet donors and organ transport

Islet donor criteria
Presently, the main source of islets for transplantation to patients with T1DM comes from brain-dead, cadaveric donors (83). Living donor distal pancreatectomy and islet allotransplantation has been performed once in the post-Edmonton protocol era with initial insulin independence in both the patient and the recipient (100).

Significant variability of organ donors (101, 102) and islet isolation cost makes selection of optimal pancreases for islet isolation a critical challenge (58). O’Gorman et al. (58) sought to meet that challenge and have reported on the standardization of cadaveric, brain-dead pancreas donors for islet isolation. The literature is full of support (103) for their summary of donor criteria which includes age (104-112), cause of death (110, 112, 113), cold ischemia time (CIT, time of organ harvest to organ processing) (59, 108, 110, 112), body mass index (BMI, kg/m²) (54, 59, 107, 112), procurement team (59, 107, 111, 113, 114), length of hospital stay (111) (reference 115 cites reduced islet function with longer hospitalization) (115), serum amylase (59, 112, 116), use of vasopressors (59), donor blood glucose (110, 111), and donor medical and social history (111). More recently, Hubert et al. reported preprocurement acute insulin response to argentine (AIRarg), an in vivo functional test of β-cell mass, to be predictive of isolation success in humans (117).

Organ transport
University of Wisconsin solution (UW) or the two-layer method (TLM, a layer of UW on top of high-oxygen concentration perflurocarbon) have been the dominant methods of pancreas preservation in recent years. The debate of which organ preservation solution is better with regard to islet isolation success remains inconclusive (112). Smaller studies of UW versus TLM preservation methods identified the TLM as optimal (113, 118-120) whereas no beneficial effect of TLM storage was observed in two larger studies (121, 122). The largest analysis of pancreas preservation methods (n=802) found similar islet isolation success rates of UW or TLM transported pancreases but identified a third transport solution, histidine-tryptophan-ketoglutarate (HTK), as negatively associated with islet isolation success (112). A large scale analysis with Custradiol pancreas preservation has yet to be performed.

Enzymes for islet isolation
Successful preparation of isolated islets of Langerhans was first achieved by Claes Hellerström using microdissection on rodent pancreases (123). Islets isolated in this fashion typically express high viability, however, the tedious technique is impractical for large-scale islet isolation.
The initial use of collagenolytic enzymes for islet isolation was performed by Moskalewski using a crude collagenase product from *Clostridium histolyticum* for the dissociation of guinea pig pancreata (124). In the years that followed, Moskalewski’s procedure was continuously refined for the isolation of islets from the human pancreas (125-128) but a significant advance in terms of isolated islet yield was the production and use of highly purified and defined enzyme blends (128-130).

Experiments in rats indicated that only collagenase class I (CI) and class II (CII), representing seven different isoforms, and an unspecific protease, such as neutral protease (NP), must be present to obtain a complete liberation of islets from within the pancreatic acinar tissue of the pancreas (131-133). For that reason, commercial enzyme production has aimed in the recent past on purifying crude enzyme products, consisting of twelve or more different enzymes and other bacterial products (134), to the highest possible extent. Impurities that have to be removed include bacterial endotoxin, which is related to increased cytokine production in human peripheral blood mononuclear cells and correlates negatively with engraftment in a rat islet transplantation model (135, 136). Another component that has to be carefully titrated is NP, which can reduce islet morphological and functional integrity, in contrast to purified collagenase that does not seem to have a detrimental effect on islet viability if overdosed (137, 138).

In spite of the efforts to provide highly purified enzyme blends, lot-to-lot variability in terms of isolation efficiency remains a problem that has persisted from the very beginning of enzymatic islet isolation until now (124, 139, 140). This variability was partially explained by a lot-dependent degradation of CI observed in retrospective high-pressure liquid chromatography analysis resulting in different ratios between CI and CII (139, 140). Prospective studies in rats confirmed that the ratio between CI and CII not only influences the efficiency of islet release from the pancreas but determines also the amount of NP that is required for efficient pancreas dissociation (141, 142). In humans the CI/CII ratio also was observed as an important parameter in effective enzyme batches (143).

However, all these findings do not explain the drop in islet isolation outcome and transplant activities observed worldwide subsequent to the replacement of one product (Liberase HI) by another one (Serva NB 1) that fulfills the guidelines for quality assurance in clinical islet transplantation (57, 144). Since assays used by individual labs provide analytical data different from those of the manufacturers (137, 145) it was hypothesized that besides collagenase and neutral protease other additional enzymes that are not listed on the certificate of analysis may play a significant role in pancreas digestion. In fact, other enzymatic activities have been reported to act as a key component in the isolation of porcine islets (146).

Recently, there have been advances in enzyme products from a number of manufacturers (147-150) as well as novel assays proposed to measure more
accurately non-degraded, active forms of CI (151). The importance of intact or degraded/truncated CI (100 kDa) for efficient pancreas dissociation is debatable as conflicting reports have emerged (148, 152). The importance of NP and the role of endogenous pancreatic enzymes involved in dissociation of the pancreas remain somewhat ambiguous yet undoubtedly significant (133).

Islet purification

The native pancreas is mainly composed of acinar, ductal, and vascular tissue, representing the exocrine pancreas, whereas islets, as the endocrine component, represent on average 1.3% of the pancreas which equates to 0.5 to 1.3 ml of tissue (153). After enzymatic dissociation of the pancreas the vast majority of the resulting digest is still composed of exocrine tissue.

Studies in rodents and humans have shown that pure preparations are desirable when graft function and patient well-being are considered. In rodents crude digest or increased exocrine contamination leads to decreased graft function or engraftment compared to pure islets (154). In humans, large packed tissue volumes are thought to be at least partially responsible for a host of severe complications encountered during intraportal infusion early in the history of islet transplantation (99, 154, 155). Transplanting purified islet tissue is currently the standard procedure in the field of human islet transplantation (82, 105, 156).

Current methods of islet purification include the use of a COBE 2991 cell separator and that of continuous density gradients (99, 155, 157, 158). This method allows for large tissue volumes, such as those attained from pancreas digestion, to be loaded and separated in a single procedure (155, 157). The technology to produce a large-scale continuous density gradient for islet separation on a COBE 2991 was established about 20 years ago (99, 155) and despite some changes to islet separation equipment and materials, such as COBE refrigeration (159) and osmolarity modifications (160-162), it still represents the current standard for human islet purification (45). The equipment for generation of density gradients is composed of an open system standard gradient maker (SGM). The SGM consisting of two beakers in which media of different densities are poured (see Figure 4 in the Materials and methods section). The density solutions are then mixed using a manually controlled magnetic stir plate with the magnet in the heavy solution chamber (155). The importance of proper mixing was also stressed in the first paper using continuous density separation for islet purification (Figure 1) (155). Since the technique is now used worldwide, there are a variety gradient maker models, magnets and, of course, different personnel responsible for magnet stirring speeds to generate the gradients. The open chambers, often reused, are not conducive to closed system processing.
Figure 1. Experiment showing the importance of proper mixing of solutions from the original publication employing continuous density gradients for large-scale islet isolation [ref. (156)]. Open circles (○) shows proper mixing and closed circles (●) represent poorly mixed solutions. The figure is reproduced with permission.

Various density solutions are used for continuous density production including Ficoll (59, 158), a University of Wisconsin solution (UW)/Ficoll mix (162, 163) and iodixanol-based medias (156, 164). Optimal densities for isopycnic human islet separation vary. The Minneapolis group has shown that separation densities for islets prepared for autotransplantation were found to be higher than that of islets for allotransplantation by about 0.015 g/ml (98). Although there are exceptions (57), functional separation densities used to isolate clinical islets have a range of about 0.025 g/ml and can be anywhere from 1.060 to 1.116 g/ml (98, 162, 165). A major limitation of the standard SGM is that it must use the density range corresponding to that of the heavy and light solutions mixed.

Once the islets have separated on the density gradient the preparation needs to be collected. To dilute the density solutions used for the separation collection is done using a wash solution. The preparation is typically collected in 12 to 20 fractions of about 20-30 ml each (59, 162). With progressing steps in the collection process, density solution gets heavier, and since the inherent density of exocrine is heavier than endocrine tissue, it becomes progressively impure. Similar purities are combined, washed, and either transplanted immediately or cultured. The islet purification procedure poses a major challenge to achieving a closed system for islet isolation.

Islet culture
Isolated islets were for a long time transplanted immediately post-isolation (45, 80). However, increasing evidence that islet culture is beneficial for islet
function, despite the possible loss in number, has changed standard practice (166-168). The introduction of a period of culture is advantageous in many respects as it allows time for transport of patients to transplantation centers, allows time for administration of prophylactic immunosuppression in islet recipients, provides a substantial opportunity for islet preparation evaluation, and may reduce immunogenicity of the preparation (169).

**Flasks vs. bags**

The cultured tissue concentration is strictly controlled to avoid overseeding which may lead to hypoxic damage due to competition for oxygen. Even large (>100 μm diameter) islets by themselves have been reported to have necrotic cores (170, 171).

Culturing islets in bags may be beneficial for islets in many respects. Since islets do not remain suspended in culture media they sink to the bottom of the culture vessel. Compared to petri dishes or culture flasks, gas-permeable bags can improve oxygen transfer directly through the islet/vessel interface while receiving nutrients from the media above (59), a theory also put in practice with silicon rubber membranes (172). Clinically established bags produced in high volume yield advantages of low cost, convenient transition of islet culture to bags used clinically and GMP-friendly, semi-closed culture conditions (59). Culture in bags is a natural precursor to final preparation of islets for transplantation which routinely occurs through gravity infusion with islets in bags (173).

**Islet preparation quantification**

The establishment of the islet equivalent (IE) as a unit of volume measurement initiated standardization of islet quantification (174). Since then, the large majority of centers pool pure and impure fractions into culture flasks, retrieve samples from each, and then trained counters manually evaluate the sizes of up to (and sometimes beyond) 200 irregularly-shaped individual islets microscopically. Evaluation of islet purity, number, size distribution, islet fragmentation, entrapment in exocrine tissue and day-to-day changes in these values is considered important for some clinical islet programs (105, 175, 176).

**Sampling validation**

A common problem in the evaluation of an islet preparation is the inconsistency of sampling. Intra- and inter-technician variability seen in sample evaluation is often high (177) even when a large sample number is taken. Unlike single cell suspensions, an even distribution of pancreatic tissue particles is difficult to obtain due to their relatively large size and size distribution. Large dilution factors compound error in the inherently variable assessment of islet number, size distribution and purity. Defining a method for sampling that produces consistent results between samples and technicians,
as well as between centers, would tremendously benefit islet quantification comparability.

Despite standardization efforts variability in counting is large (178). To minimize manual influences automated or computerized techniques have been developed to assess islet preparations (179-187), some technically advanced and costly (188, 189). A major obstacle to achieve accurate and completely automated islet evaluation is variability in the staining of islet tissue, which can make it difficult to identify tissues appropriately.

One major advantage of more readily available imaging equipment is the adherence to documentation requirements according to GMP regulations (178). A simple, intuitive, affordable program to aid in evaluation of islet sample purity, counting and size distribution has been developed at our lab in Uppsala (190) and elsewhere (189). These programs could prove a valuable tool to standardize islet evaluation and to aid intra and inter-lab comparisons of islet counts and purity.

Islet isolation quality control
Regulatory bodies require tissue identity, purity, potency and safety to be carried out on clinically bound tissues. Meeting these requirements for islets can be met with relatively simple assays.

*Islets of Langerhans – what do they do and what should we test?*

In healthy individuals, islets of Langerhans are popularly known as the organs primarily responsible for maintaining levels of physiologically optimal blood glucose levels. The islets themselves are composed of a number of cell types which respond to various environmental stimuli, primarily blood glucose levels. Islet cell types include β-cells, α-cells, δ-cells, ε-cells, PP-cells and more (endothelial cells, pericytes, grehlin). β-cells produce insulin (C-peptide as a byproduct) and compose between 28-75% of the cells in an islet (191). The glucagon producing α-cells make up about 10-65% of the islet, δ-cells which manufacture somatostatin constitute from 1.2-22% (191) and remaining cells <2%.

The kinetics of β-cell insulin secretion in response to glucose is characterized by a biphasic release (Figure 2). This biphasic pattern is seen in both native pancreas and in isolated islets, implying that mechanisms involved are inherent properties of islets themselves (192).
By measuring the biphasic pattern with the glucose-stimulated perifusion assay, it is possible to visualize insulin release over time. The perifusion assay has an advantage over static glucose stimulation methods since it provides information about intracellular events that would otherwise be missed. Important markers of efficient packaging of ready-release insulin at the cell periphery, mobilization of insulin granules as well as transport of these granules to the cell surface are among the events visualized. Appropriate insulin release means all physiological machinery is in place and functioning well. Dysfunction of insulin secretion can depend on a variety of mechanistic variables, everything from the first step of efficient glucose sensing (193) to pathways involved in insulin exocytosis (194).

Islet insulin content is another parameter thought to be associated with islet quality. Brandhorst et al. have shown that insulin content depends on the donor pancreas but does not change drastically despite the insults administered during islet isolation (195). By measuring the insulin content a functional reserve of insulin may be relevant considering the metabolic demands of the recipients.

Other assays measuring metabolic activity or viability including oxygen consumption rate (OCR) (196), glucose-stimulated increased increment of OCR (197-199), ATP/ADP (200, 201), variations of glucose-stimulated insulin secretion (45, 156, 201, 202), cytometric β-cell viability (203) and combinations (201) of these methods have yet to be validated as predictive of graft function in humans, however, some are promising.

Cytokines
Cytokine expression can alert islet scientists of the inflammatory status of the tissue to be transplanted. For instance, macrophage chemotactant protein-1 (MCP-1) and tissue factor (TF) expressed by islets are related to post-transplant cross-linked fibrin levels, a marker that coagulation has occurred (204, 205). Islet TF expression as part of the instant blood mediated inflammatory reaction is associated with the unfavorable outcome of short-term
post-transplant clinical C-peptide release (206). Donor islet interleukin (IL)-10 is associated with islet graft survival and function (73). The role of IL-6 is ambiguous as it has immunomodulatory effects protecting islets (207) and at the same time it may contribute to diabetes development (208). IL-8 is an inflammatory cytokine associated with neutrophil migration and can be induced by hypoxia, steroids and IL-1β, its expression an indication of islet damage (209). The above mentioned cytokines are those pertaining to this thesis but it should be known that there are a number of others (167).

Measuring clinical success

Measures of clinical success initially focused on insulin independence. However, the life-threatening aspects of brittle diabetes (those aspects qualifying patients for transplant) include diabetic coma and severe glycemic lability, conditions greatly solved to a large extent even with partially functioning islet grafts (46, 77). A shift in the field from obtaining insulin independence to a more therapeutic approach to obtaining normoglycemia has occurred recently (79). To best use available tissue, determination of islet engraftment and prediction of transplantation success (however measured) are hindered by a number of variables not yet standardized or validated.

Engraftment endpoints

Effective prediction of how an individual islet preparation will function after transplantation has remained an elusive goal (210), in part due to the wide array of available measures for graft function. Endpoints of graft success have been related to insulin independence rate (118, 156, 211), β-score (212), secretory units of islet in transplantation (SUIT) index (213), C-peptide × glucose⁻¹ and C-peptide × glucose⁻¹ × creatinine⁻¹ ratios (CP/GCr) (214), HbA₁c (77), fasting and stimulated C-peptide (215), oral glucose tolerance test, mixed meal tolerance test (MMTT), intravenous tolerance tests with acute insulin response to glucose or AIRₜₐₓ (166, 216, 217) and more (218, 219). Recently, the Pittsburgh group, in addition to a number of the above mentioned tests, measured IE per unit insulin reduction as reported by Deng et al. to be about 24,000 IE/U. The same study (166) and another (48) measured plasma C-peptide-to-creatinine ratio to account for possible renal dysfunction in their islet after kidney (IAK) populations.

C-peptide is perhaps the best candidate to measure functional islet capacity. C-peptide, which is released in conjunction with insulin in a 1:1 ratio (Figure 3), is recognized as the best measure for functional insulin secretory capacity according to the American Diabetes Association (220). C-peptide positivity in islet recipients has been associated with relief from hypoglycemia and reduction of glycemic lability despite the continuation of exogenous insulin therapy (77). Assays for both insulin and C-peptide are widely available but C-peptide benefits from a longer half-life in circulation making it a
more stable measurement parameter (221). Furthermore, fasting (>8 hr without food intake) C-peptide levels are highly correlated to the 90-minute MMTT peak C-peptide values (222). There could however be cross-reactivity with circulating split proinsulin leading to higher than actual C-peptide readings (66) but with modern assays this phenomenon has largely been dealt with (220).

![Figure 3](image.png)

**Figure 3.** The processing of proinsulin to insulin and C-peptide which are released in a one-to-one molar ratio. Image reproduced from ref. (192) with permission.

Since C-peptide is cleared via the kidney, renal function should also be accounted for, especially in patients suspected of possible kidney dysfunction at the time of transplant (166, 214) or even long afterwards. Measurements of creatinine or albumin clearance are two options for evaluating renal function (223). Plasma creatinine is easily attained from the same blood samples as those taken for C-peptide and glucose. By combining C-peptide levels in relation to both glucose and creatinine concentrations as proposed by Faradji et al. (214), the CP/GCr provides a relatively simple, standardized measure of islet graft function.

**Reported predictors of transplant success**

The ability to predict transplantation outcome evades islet scientists (210). There are however some hints as to the most important factors. Numerous reports have correlated the number of IE transplanted (83, 105, 214, 217, 224) or the ϒ-cell mass (225), donor age (109, 224), and CIT (226) to transplant function. Most of those studies however did not account for renal function. In light of the nephrotoxic effects some immunosuppressive agents
have on transplant recipients (227) it may be prudent to introduce such a measure to account for renal clearance dysfunction.
Materials and methods

Please refer to the appropriate section in the respective papers for descriptions of materials and methods. More detailed descriptions for some methods are described below.

**Pump-made gradients**

Instead of using the SGM with its two-chamber, magnetic stirring system (Figure 4) (155) a pair of computer-controlled pumps were used to create density gradients. Bags containing either light or heavy density solutions were steriley welded to a pumpable tube, which was again steriley welded to the COBE bag set. Each pumpable tube passed through a pump and by controlling the pump speed, the volume of each solution could be controlled to yield desired densities and volumes of each density. Mixing of high and low density solutions in this system occurs when the solutions reach the spinning COBE bag. Observations with dyed solutions led to this finding.

A schematic of the pump-made density gradient system and appropriate separation of islet and non-islet tissue is shown in Figure 5.

![Figure 4](image-url)  
*Figure 4. The open standard gradient maker (SGM) system with two chambers for holding heavy and light density solutions. The tube between the chambers allows light density solution to flow into the heavy density solution chamber while being mixed by a magnet.*
**Figure 5.** The effectively closed, pump-made gradient system. From L to R; computer program controlling the two pumps for heavy and light density solutions, pumps and bags for light and heavy solutions, COBE 2991 with spinning bag, visualization of densities in bag and islet/exocrine separation in the light and heavy portions of the density gradient.

**DIA evaluation**

The DIA program was used in papers I, II, and III and for some data in paper IV. The DIA macro used in conjunction with Leica Qwin software was developed according to standards in the field (174) and utilized capabilities for quantification that are otherwise impractical without computer-assisted evaluation.

The macro logs information relevant to operator and sample information, fulfilling some aspects of traceability as advised according to GMP. However, the entered data is not secured by password, electronic signature or contra-signing, a requirement of data used for clinical data document security.

Pancreatic tissue when stained with dissolved dithizone (DTZ), a zinc-chelator, preferentially dyes the endocrine portions red (228). The program works by first allowing the user to select red-stained tissue for islet quantification, then, other colors (white or lighter colors) for purity quantification, allows some editing to remove bubbles or other unwanted objects and automates analysis.

Values reported by the program include date analyzed, sample ID, operator ID, purity based on area of islet to total tissue, purity based on individual particle volume contributions to islet and total tissue, number of islet particles, number of non-islet particles, size index (IE/number islets), fragmentation index (accounting for perimeter/area measurements for each particle), separation into the classical size range categories established in 1990 (174) (plus a 20-50 \( \mu \)m range) and color selection data values for the islet and non-islet color selections.
The fact that the program is not completely automated depends on the fact that there is a differential staining of islets. This confounding factor limits the extent of automation of the software used to analyze images of islets stained with DTZ using light microscopy.
Results and discussion

The goal of the islet isolation process aims to yield the maximum number of islets with optimal viability for transplantation to patients for alleviation of the serious complications associated with loss of glycemic control. Variability in all steps of the isolation process, from donor care to islet isolation to recipient care, can affect this result (see Background). The work presented in each paper/manuscript aimed to improve aspects of the standardization of islet isolation and transplantation variables to better optimize relevant protocols and improve standardization.

Identification of a previously unrecognized enzyme activity (Paper I)

One of the most critical steps in obtaining enough islets for transplantation is the identification of efficient pancreatic digestive enzymes (105, 111, 229). Due to inconsistent results and lot-to-lot (and intra-lot) variations from commercially available enzyme products (230), further characterization of enzymatic factors influencing human islet isolation success are urgently required.

A number of enzymatic activities are measurable even in purified enzyme blends including digestion of the substrate BAEE which reflects tryptic or tryptic-like activity (TLA) [depending on protocol used (146)]. The TLA, a contemporarily ignored enzyme activity, was evaluated in a rat model and subsequently for human islet isolation.

The standardization of the evaluation of TLA required activities to be related to the context of collagenase parameters (BAEE-U/PZ-U activities ratio and CII/CI ratios). The BAEE-U/PZ-U ratio is defined as the TLA-ratio. Neutral protease (NP) levels were also adjusted according to guidance from previous experiments. For the rat experiments this was accomplished with a fixed ratio of CII/CI and constant and appropriate levels of NP.

Evaluation of enzymatic digestion of rat pancreas

A Lewis rat model was first used, employing sequentially increasing TLA-ratios from 1.3 to 10%. In the rat model, increasing TLA-ratios correlated
with significantly decreased digestion times with no difference in islet viability, morphology or yield. Purity for 1.3% TLA-ratio isolated islets was lower than that of 5.0 or 10% TLA-ratio islets. In vitro testing for insulin content was lower for the 10% TLA-ratio islets compared to 1.3% islets yet functionality testing as measured by stimulation indices were similar for all groups. No difference in in vivo functionality was seen in the different TLA-ratio isolated rat islets as all mice from each group that were transplanted under the kidney capsule were cured from streptozotocin-induced diabetes until nephrectomy. These experiments warranted testing human pancreas digestion since functional viability remained intact yet regarding islet yields there were TLA-ratio dependent differences.

Evaluation of enzymatic digestion of human pancreas

Controlling the ratios of collagenase classes and NP for human islet isolation was important to ensure that comparisons were valid when considering different TLA-ratios. As such, collagenase CII/CI ratios were restricted to a narrow range and NP levels were adjusted to appropriate levels. TLA-ratios were increased stepwise from 1.3% to a maximum of 12.6%.

Increasing TLA-ratios resulted in correlation to increased isolation success rates as measured by total islet yield and adherence to quality testing parameters. A low TLA-ratio of 1.3% was associated with long average digestion times and isolation failure. The highest islet equivalent (IE) count per gram pancreas weight was seen with the 9.1% TLA-ratio at almost 4000 IE/g (n=16). The dose response of the TLA-ratio did not cause differences in packed tissue volume nor percent digested pancreas. This indicates a more efficient release of islets from the surrounding exocrine tissue, not in the digestion of the macrostructure of the pancreas.

The quality parameters of post culture IE recoveries and purities were similar in all TLA-ratio categories. This indicated no effect of differences in this range of TLA-ratios on islet loss after culture. Islet size distribution was also similar in all TLA-ratio groups, a distribution matching closely that of initial studies evaluating the Liberase enzyme (129). Preliminary clinical data from pancreases isolated with TLA-ratio enzymes ranging from 5.7% to 12.6% in nine islet-after-kidney transplant patients showed no influence of different TLA-ratios on basal or stimulated C-peptide values, 0.57±0.10 and 1.87±0.54 ng/ml respectively.

The characterization of TLA in commercially available enzyme products for clinical islet isolation appears to play a critical role in terms of isolation efficiency in meeting criteria for transplantable islet preparations without sacrificing islet morphology, viability or function.
Progress in the field since Paper I was published

The effect of TLA on islet isolation is used by the lab in Uppsala as an important enzyme batch selection criterion. However, “test vials” of enzyme are still used in order to evaluate the pancreatic digestion quality before committing to large scale purchase. Excluding the pancreas acquisition fees, the enzyme is the single most expensive reagent in the islet isolation protocol and arguably the most critical component for success.

Our lab and other researchers have also focused on evaluation of other recently commercially available collagenase and neutral protease products (57, 147-150, 231). It is apparent that highly purified enzyme products meeting general safety, regulatory, purity and dosing guidelines can yield acceptable digestion kinetics and sufficient islet quality to warrant clinical use. The emergence of new enzyme products is good for islet isolation advancement.

As a side note, the introduction of TLA as presented awakens the need for evaluation of unrecognized enzyme activities. Assays measuring enzyme activities for use in human pancreas digestion are being developed (151) and will continue to help better characterize enzymes for controlled cell dispersion.

Regarding TLA, no formal articles on the effects or activities of TLA have been reported since the publication of Paper I (to date of submission of this thesis, April 2011). The specific mechanism of action of TLA remains elucidation.

Automated gradient making system (Paper II)

Density gradient creation is a technically complex procedure essential for large-scale islet purification with the intention of clinical transplant. The procedure has remained largely unchanged since its inception (99, 155), a testament to the durability of the methodology. However, the standard procedure of gradient making for islet purification is somewhat unsuitable for high-quality, high-reproducibility, good manufacturing practices (GMP)-level methodology due to manual variability and as a possible source of contamination.

As made evident in the first article describing the density gradient manufacturing procedure, which is still used today, mixing density solutions properly can dramatically affect the quality of the gradient (Figure 1) (155). This variability can come from a number of different parameters such as the chamber size, tube size connecting light and heavy density solution chambers, magnet model and spinning speed.

As an open procedure, this manually controlled method provides many opportunities for improvement when considering standardization and manufacture according to GMP procedures. We theorized that by controlling flow
rates of the individual density solutions that we could create reproducible and standardized gradients in a closed-system.

A closed, computer-controlled, automatic purification system (APS) was compared to standard gradient maker (SGM) methods for the generation of density gradients for human islet purification.

Functionality of pump-made gradients
Reliability of the technology needed to be established prior to use for human islet purification. To measure the gradient making efficacy of the APS, the expected volume of density gradients were compared to that of the SGM. For 400 ml gradients, the APS achieved a more complete recovery of expected gradient volume (98.2±2.0% vs. 90.0±1.1%, p<0.05) indicating that computerized dosing for density gradient manufacture is reliable.

To test the versatility of the APS we attempted to produce non-linear, continuous density gradients. The system readily constructed sigmoidal gradients, increasing volumes of either the heavy or light portions of the gradients. Unpublished observations include the creation of the UW/Ficoll gradient protocol from the University of Chicago (162) which creates linear densities between (1.063-1.074 g/ml) without prior mixing of the two densities of the UW (1.045 g/ml) and heavy Ficoll (1.100 g/ml).

Islet separation with pump-made gradients
To test the ability of pump-made gradients to separate islets of Langerhans from contaminating exocrine tissue, both the APS and SGM were used to create linear gradients. Both methods recovered similar overall islet equivalents (IE) and purities. There was no difference in the number of purified fractions used for tissue culture between SGM and APS.

Any change to the isolation procedure requires testing for functionality, viability and inflammatory status. Quality control parameters were similar in regard to perifusion glucose-stimulated insulin secretion stimulation indices and ADP/ATP ratio indicating viable, functional islets from both groups. Cytokine expression was not different for TF expression. Differences arose in the APS separated islets which expressed lower IL-6, IL-8 and MCP-1 versus SGM. Since the purest fractions were used for quality control purposes it cannot be ruled out that this affected in vitro test results due to higher islet purity in APS islets (72.2±4.5 vs. 80.9±3.9%, p<0.05). This observation is in-line with the SGM and APS insulin content per DNA measurements of 4.1±0.7 vs. 5.0±0.8 ng/ng (p<0.05), respectively. This small difference in purity could have implications for the cytokine expression levels (232, 233), however, the influence of minor changes in purity and the repercussions for islet quality control and clinical transplantation remain outside the scope of this investigation.
The closed, automated gradient making system proved a feasible and flexible system to solve problems associated with standardization of islet processing. The APS also resolves one of the major obstacles to realizing a closed system for the entire islet isolation process and in meeting GMP production requirements.

Progress in the field since Paper II was published (2008)
Adoption of this method has been initiated at two centers. Technicalities have slowed progress at one center and the other center in Leiden, Netherlands will likely implement the pump-made gradient system in their clinical islet production unit soon (Marten Engelse, personal communication, March 29, 2011). Plans to implement the procedure at the City of Hope in Duarte, CA, USA are underway.

Digital imaging analysis (DIA) and presentation of a GMP-friendly islet quantification technique (Paper III)
The standard used for quantification of islets after isolation and prior to transplantation involves manually sampled and counted aliquots, a procedure known for high variation, both subjective and technical in nature. Most centers have dedicated “counters” to ensure some level of reproducibility. Variation between counters at different centers remains high despite standardization efforts (178). Sampling variation can also contribute to uncertainty of counts (177). Methods of automated analysis of islet samples (179, 183, 188) have unfortunately not been widely applied. This could be due to cost of some apparatuses used (>100,000 USD in one case) or the complexity of software used to analyze images.

The challenge of adapting the islet quantification procedure to closed system technology is daunting. Consequently, in order to quantify islets in compliance with GMP practices, state-of-the-art clean rooms, expensive facilities and experienced personnel are required (234). Flask-based culture methods ubiquitously utilized by islet labs throughout the world pose a serious challenge to reaching a closed system islet isolation method.

Using customized, computer-assisted digital image analysis (DIA) macros, a purity and volume based (PV) system to evaluate islets in a GMP-friendly manner was evaluated with respect to counting variation and compared to that of standard manual methods.
DIA validation

The DIA system required calibration according to a standard suitable for islet evaluation. Our coauthors in Miami sent blinded pools of calibrated microspheres of known size distribution and quantity for assessment (177). The DIA program recorded aspects of microsphere size distribution, with values almost identical to expected, establishing the validity of the program to reliably record sizes accurately. The total islet equivalents (IE) of the prepared microsphere pools matched with expected values. Despite the accuracy of the system to evaluate microsphere sizes, the average percent coefficients of variation (CV%, CV% = standard deviation × average⁻¹ × 100%) of total IE values for the pools (n=15) was 21±10%, indicating large evaluation variation.

Computer-assisted DIA versus manual methods

DIA was compared to manual methods to determine the variation between evaluators. Variation in islet samples (n=14) as evaluated between 3 individuals reduced by almost half when using DIA for both IE (31% vs. 17%, p=0.002) and islet purity (20% vs. 13%, p=0.033). Although, the absolute values of IE and purity were not significantly different between the two methods. This data established that DIA can reduce variation between individuals compared to manual evaluation.

Accuracy in size distribution, performed better by computers than humans, allowed individual sizing of islets. The DIA used for this study accounted for individual islet particle area when calculating IE, avoiding grouping of particles into size categories as is customary with the standard, manual method. To assess the difference between the standard method to that of DIA, DIA analyzed IE (calculated based on individual sizes) were compared to IE calculated based on the size ranges (standard method). An observed 15% reduction of total IE using DIA corresponded well with a reduction of 16% seen in another similar DIA system (189). This means that current thresholds for transplantation of, for instance, 5,000 IE/kg should be revised to 4,250 IE/kg or 300,000 IE to 255,000 IE when using DIA from sampled pools for total IE estimation.

Evaluator versus sample variation

The identification of the source of the variation in islet evaluation can be important for methodologies aiming to increase quantification reliability. To examine contributions of counter versus sample errors, 14 pools of islets were sampled 5 times by one individual with extensive islet isolation and sampling experience. Each pool yielded a CV% value for IE and purity as analyzed by DIA. The average CV%s were, for IE counts 20.5%±7.5%, and
for purity 14.3%±5.7%. Then, 3 individuals analyzed 20 images (four pools worth of samples) to identify if evaluator differences were the cause of the high CV%s. However, with CV%s of 6.6±3.2% for IE and 5.8±3.2% for purity it was apparent that variation came from sampling.

Closed system islet evaluation

The measurement of an islet equivalent is a volume (174). Correspondingly, the total volume of the islet preparation should be able to be used for the quantification of transplantable endocrine mass. By knowing the purity and the pellet volume, a theoretical estimation of total IE should be possible. We set out to use the purity and volume (PV) of microsphere/tissue pellets in a GMP-friendly bag system to evaluate total IE. By performing analysis on the entire cell preparation we hoped to avoid large variations in outcome.

Standard curves of the microsphere pools were generated to establish volumes for known amounts of IE. The standard curves allowed translation of known, measured volumes to surface areas of pellets in bags.

A known amount of microspheres was mixed with exocrine tissue and evaluated with both the standard manual and PV methods. The values of expected IE did not differ with either method. However, manual IE resulted in a total CV% of 44.3% and a range spanning 258 k IE, whereas PV evaluation resulted in a CV% of 10.7% and range of 60 k IE, a remarkable difference. In this analysis, again, we saw major contributions of variation that appeared to come from sampling, not evaluators. Purity CV% values for each method were similar approximating 10.5% and differed from expected by +7% for the manual method and +3% for PV. Again, like the islet analysis, purity CV%s were nominally lower than those for IE.

A semi-closed islet evaluation system is presented which is able to reduce variation compared to standard manual methods. The major contribution of variation in islet evaluation appears to be sample related since evaluator errors were consistently lower in comparison. Variation in IE and purity values reduced by using DIA compared to standard manual methods. Islet quantification according to GMP by utilization of the PV method also reduced variation compared to manual methods and is the first such model allowing adherence to strict standards for clinical islet products.

Progress in the field since Paper III was published (2011)

This paper was recently published (less than two months ago). However, since publication an imaging modality for visualizing 3 dimensional islet volume and cellular content from histological whole pancreas sections has been reported (235). Although not used for islet isolation quantification it represents the fast-paced development of computer-assisted methodology for quantification and identification of tissues of interest.
Prediction of islet graft potency (Paper IV)

Predictive evaluation of islet transplant engraftment would benefit islet research in many respects. By knowing what variables affect individual islet transplantation function, the decision to transplant a particular batch can be made and thereby possibly avoid procedural complications, possible immunization and costs associated with unnecessary transplantation procedures. As mentioned earlier, a number of reports correlate to transplant success to various parameters, including the number of IE transplanted \((83, 105, 214, 217, 224)\) or the \(\beta\)-cell mass \((225)\), donor age \((109, 224)\), or CIT \((226)\).

Strict criteria are in place for clinical islet transplantation trials, such as that of used for the International Trial of the Edmonton Protocol, which required at least 5,000 IE/kg recipient weight \((45)\) for a transplant to take place. The Edmonton trial allowed numerous transplantations to the same patient in the hopes of reaching insulin independence \((45)\). Many other centers have adopted that same multi-transplant ideology. Upon subsequent transplantations this creates a problem in evaluation to determine which graft may function and which may not. Currently, such information can only reliably be taken from solitary islet transplants, a limitation in the quest to identify important predictive markers.

The islet isolation center in Uppsala is one of the most active in the world as part of the Nordic Network for Islet Transplantation \((236)\). As such, a considerable number of transplantations have been performed. To maximize the amount of information to be gained, the short term islet engraftment, as measured by the change in pre- and 28-day posttransplant islet graft function using the established C-peptide \((\times 100) \times \text{glucose}^{-1} \times \text{creatinine}^{-1}\) ratio \((\Delta\text{CP/GCr})\) was used. Usable data from 110 islet after kidney (IAK) transplantations were retrospectively analyzed using univariate regression of various donor, islet isolation, quality control and recipient variables. Similarly, using multiple, backwards-selection regression an optimized transplantable functional islet mass (TFIM) predictive model was generated to identify variables important for engraftment.
Variables analyzed include the following:

**Donor variables**
- Age
- Cold ischemia time (CIT)
- Days in intensive care
- Cause of death
- Sex
- Weight
- Body mass index (BMI)
- Number of other organs harvested for transplantation
- Local or distant donor hospital (all have individual organ harvest teams)

**Islet processing variables**
- Preservation solution
- Enzyme used
- Total islet equivalents (IE)
- Islet purity
- Total tissue volume
- Days cultured

**Quality control measures**
- Dynamic perifusion glucose-stimulated insulin secretion stimulation index
- Insulin content

**Recipient parameters**
- Age
- Sex
- Body weight
- IE/kg body weight
- Pretransplant insulin
- Pretransplant HbA\textsubscript{1c}

**Correlations to short term outcome**

No correlation of ΔCP/GCr for either the univariate or multiple regression analysis was identified for the donor or recipient age, sex and body weights, days in intensive care, cause of death, body mass index, number of other organs harvested for transplantation, local or distant harvest team, preservation solution, enzyme used, islet purity, days cultured, islet insulin content, and pretransplant insulin and HbA\textsubscript{1c}. Already in this “non-significant” list we can see a difference from the literature in that donor age did not associate to function as it has in smaller studies \[n=55\] for ref \((109)\) and \(n=8\) for reference \((224)\).

The variables associated with engraftment for both univariate regression (Spearman \(r^2\) and p-values shown) and the TFIM model were cold ischemia time (CIT) \((r^2=0.091, \ p=0.0015)\), islet equivalents (IE) \((r^2=0.130, \ p<0.001)\), total tissue volume \((r^2=0.050, \ p=0.025)\), and dynamic perifusion glucose-stimulated insulin secretion stimulation index (SI) \((r^2=0.090, \ p=0.0033)\). Each of the above mentioned parameters are easily attainable prior to transplantation. Individually, the amount of endocrine tissue transplanted had the highest correlation to short-term engraftment, a rather unsurprising \((83, \ 105, \ 214, \ 217, \ 224)\) but welcome observation. The IE, although significant, accounted for only 13\% of the transplant outcome variability.
Transplanted functional islet mass (TFIM) model

All variables were used to calculate and create the predictive TFIM model. The TFIM had an adjusted $r^2$ of 0.387 ($p<0.001$) meaning it explains 38.7% of the variation in $\Delta$CP/GCr. This is a substantial proportion considering the few parameters taken into account.

The TFIM model considers the 4 variables CIT, IE, total tissue volume, and SI and each alter the expected $\Delta$CP/GCr to varying degrees. Each can affect the others and, consequently, for the model to function properly all four variables are required.

The influence of each factor on $\Delta$CP/GCr gives us a better understanding of what they mean in reality, not just how significant something is. For instance, for every 100,000 IE transplanted or increase of 2 units SI, the TFIM increased by 0.13. It decreased by the same amount with 1250 µl tissue volume or about 20 minutes CIT between 4-8 hrs storage.

The confounding nature of the model is seen in the influences of IE and total tissue volume. IE is positively associated with outcome and total tissue volume (which inherently has an IE component) is negatively associated. Since such low volumes of tissue were transplanted the effect of the non-islet portion of the tissue volume likely plays a role in the negative contribution of total tissue. Indeed, the exocrine tissue can contain cytokine secreting cells and may harm the graft by enhancing proinflammatory pathways (232). The exact influence of the different exocrine components is not fully known but the TFIM model definitely suggests that whatever the influence, it most likely is not beneficial for early transplant function.

Cold ischemia time is a perpetual factor influencing islet isolation (59, 108, 110, 112) and transplantation success (226). Here, in regards to the TFIM model we can get an idea of how important it is. The $\Delta$CP/GCr reduces 0.13 for every 20 minutes in hours 4-8 after procurement, the same increment increase for 100,000 IE. The highest single transplant achieved a $\Delta$CP/GCr of 1.5, the equivalent of a little less than 4 hrs CIT. The TFIM is reduced even more with increasing cold storage time.

The SI, a measure of β-cell insulin secretory capacity, was higher in better functioning islet preparations. The capacity of isolated islets to secrete insulin efficiently and strongly in the presence of very high glucose levels reflects, essentially, expected islet functionality in the transplant situation. In this IAK population the $\Delta$CP/GCr as a measure of engraftment worked well. The main differences of this report to others measuring C-peptide/glucose (214) or C-peptide/creatinine (48, 166) is the larger number of transplants and the consideration of both glucose and creatinine to normalize serum C-peptide levels. CP/GC was reported initially by Faradji et al. (214) but not investigated further in their study due to its relative weakness to the simple C-peptide/glucose ratio. Their study of 22 patients included 4 with previous kidney transplants, all of which had “normal and stable renal
function” (214). Indeed, in our population with variable renal function no correlations were observed without adjusting for creatinine.

Despite the simplicity of the TFIM model a considerable portion of early islet graft outcome variability is explained by parameters easily attainable pretransplant. The TFIM provides a straightforward and potent tool to guide the decision to utilize a specific islet preparation for clinical transplantation.
Conclusions

Specific conclusions

Paper I
- Measurement of TLA ratio can supplement already reported enzyme activities for the identification of effective batches of enzyme for human pancreas digestion
- *In vitro* and *in vivo* function of islets were seemingly unaffected by different TLA-ratios

Paper II
- Removal of manual variation is achievable using a GMP-level, closed system, computer-controlled pump system for the generation of density gradients for the large-scale separation of islets from exocrine tissue

Paper III
- A GMP-friendly islet quantification system using DIA reduced variability compared to standard manual methods
- Sampling variation remains the main source of islet quantification error

Paper IV
- The simple ΔCP/GCr correlated early IAK transplant engraftment to measures easily attainable pretransplant
- An optimized TFIM model using 4 variables (1 donor-, 2 isolation-, and 1 quality control-related) explained almost 40% of the variation in engraftment

General conclusion
Reproducibility, safety, reliability and adherence to good manufacturing practices in islet isolation methods was achieved through work in Papers I-III and variables affecting engraftment were identified through standardization of outcome measures and a predictive model in Paper IV.
Critical considerations regarding research design and methods

Paper I
There are a number of confounding factors present in islet isolation not immediately translatable to other labs and, as such, standardization of all variables is not possible. For instance, the human pancreas is perhaps the best assay for enzyme evaluation yet at the same time maybe the worst. Variation comes from a number of sources, e.g. age, islet capsule variability (101), genetics, endogenous pancreatic enzyme secretions [possibly affected by CIT (133) and/or brain death (74)] islet processing variables, patient care prior to islet isolation, etc.

The fact that the ratio of females to males increased with increasing TLA-ratios cannot exclude the possibility that gender may have played a role in the results of the isolation success rates. Also, the highest TLA ratio group had the heaviest pancreases which may have influenced results.

Paper II
The volumes of APS made gradients for human islet separation were 400 ml and those of the SGM were 300 ml. As such it may be expected that a better separation may occur although no difference was observed. There was about a 15 minute delay in density gradient manufacture between the SGM and APS-made gradients, and which was produced first was not recorded although they did alternate. Comparisons of expected gradient used the same volume.

Paper III
The ability of each individual to measure the islet or exocrine portions of the images is subjective. However, when using the DIA software the differences in selection were minimized compared to manual methods as mentioned in Paper III.

The standard curve for the pellets in bags with a known amount of standardized microspheres is not ideal (Paper III, Fig. 3). There is a large spread of analyzed area in 150 k and 200 k IE populations. Also, the method of imaging the pellets in bags is not as standardized as it should be. Develop-
ment to further standardize this process and even automate digital pellet image evaluation is a top priority. As mentioned in the discussion of Paper III, confirmation of these results using even higher numbers of IE needs to be performed before acceptance of this method into the clinic.

Paper IV

Not all values of possible interest were taken into account in this analysis. Being a retrospective study this is one of the defining limitations of the presented work. Also, only pancreases taken for clinical transplant were considered. Negative corollaries of graft function, such as recipient autoantibody status (70, 71, 156) were not assessed.
Future perspectives

The ultimate goal for islet isolation technologies from cadaver pancreas donors is maximal yield and functional capacity of isolated and transplanted islets according to regulatory requirements. Restricting future perspectives to cadaver donor pancreases as a source for β-cell replacement therapy, some of the following developments may come to fruition.

Optimization of donor variables
As presented by Hubert et al. predictive markers for islet isolation success (117) are still being discovered. Other factors possibly influencing islet isolation or transplantation outcome might include brain death, as evidenced by islet potency reported in whole pancreatectomy, autotransplanted patients (68). Patient care immediately after brain death could be a major factor in improving organ quality and viability of tissue. Continuing development in transport solutions, improving oxygenation of hypoxic tissue will likely increase tissue recovery and potency (237).

Enzymatic islet isolation
Continued characterization of the actions of enzyme components on human pancreas dissociation will help us understand even better the critical processes involved in efficient islet release from exocrine tissue. Assays characterizing these activities and even quick histological analysis may be employed to determine the chances of isolation success.

Islet harvest
The work presented in this thesis will help realize a more standardized islet isolation process. Other procedural improvements to reduce variation between labs as well as further GMP processing include closing the system immediately post pancreas digestion. Lakey et al. have reported results with canine islet harvesting using an angled sluice device with seemingly good results. They effectively reduced media volume and concentrated digested tissue without centrifugation (95). However, this system was not adopted by the islet community and its production was subsequently dropped. Other methods to concentrate crude pancreas dilutions from greater than 5 L include the system presented by Klaffschenkel et al. that used the COBE 2991 cell processor (97). This method utilizes repeated centrifugations on packed
tissue, a stressful process for living cells. Manufacturing another device similar in principle to the sluice relying on the passive nature of large cell clusters to sediment rapidly out of solution would be optimal. Minimizing mechanical trauma most likely will improve cell viability and recovery as well as allow for online concentration of cell product.

_Loading pancreatic digest to the COBE 2991/tissue purification/islet collection_

The presented APS provides essential technology to completely close the entire islet isolation procedure. Unfortunately, the COBE bag set itself is not a validated closed system. Loading tissue into the COBE 2991 cell processor and subsequent collection of purified tissue is still performed with open systems. During this stage, unsterile equipment is in close proximity to surfaces in direct contact with product. A proposed bag system to load incubated tissue and subsequently collect the various separated fractions would allow for collection in a closed system, eliminating the need for open flasks or conical tubes.

In its current form the automation of density gradients could confer substantial benefits for centers that tailor density gradients to the specific density of individual pancreases (98, 163, 165, 238). Also, studies easily performed with the APS, such as preferentially heavy or light gradients have not been investigated.

_Islet quantification and culture_

The closed system quantification of islet tissue is presented in this thesis but there may be other methods that could do the same job. The use of optical coherence tomography (OCT), a technique that can create three dimensional reconstruction of objects up to 500 μm, could provide online yields from purification collection to during infusion on a per particle basis or, if OCT scanners become available, they could scan bags with the tissue sediment.

Frequent media change has been examined by the Lille group (239) with promising results. Online media change as employed in other cell expansion technologies (240) could also be employed with islets, reducing workload and, if the evidence from the islet center Lille is any indication, increase tissue viability and maybe even potency.

_Engraftment assessment_

The nature of multiple transplantations makes evaluation of engraftment and contributions of individual islet grafts to functional β-cell capacity difficult. Identifying the extent each islet preparation contributes to long-term function is still virtually impossible.
*Fully automated islet isolation*

The process of islet isolation and culture could one day be fully automated and optimized on a per pancreas basis. For this to occur, advances in all aspects of the presented isolation procedure will need to be made. It will require substantial human and material capital, dedicated doctors, technicians and engineers.
Diabetes mellitus is an increasingly prevalent and costly disease. Insulin injections are required for those with type 1 diabetes mellitus to survive long term. This is due to the destruction of the pancreatic β-cells, the only cells in the body that produce insulin. The β-cells are located in small cell clusters called islets of Langerhans found in the pancreas, and these islets can be transplanted from cadaver donors to type 1 diabetes patients. The use of donor pancreas organs for islet isolation and transplantation is one way to replace insulin producing islet cells. However, the process of islet isolation is highly variable and not all isolations result in transplantable islet numbers and quality. Additionally, the impact of individual islet preparations to their function in transplanted patients is not standardized to the extent possible. This thesis aimed to identify critical variables associated with islet isolation success and the evaluation of posttransplant function.

A critical step in the islet isolation process is the enzymatic breakdown of pancreatic tissue to release islets from surrounding tissues. Outcome variability is in large part due to enzyme variations. We introduce an as yet unidentified critical enzyme activity for the efficient digestion of donor pancreases for islet isolation. Changes in this enzyme activity led to marked differences in islet isolation success rates without sacrificing islet quality. These results should help standardize evaluation of enzymes for clinical islet isolation.

Another critical isolation step is the purification of the digested pancreas. The crude pancreatic digest is mainly composed of tissue that does not contain insulin-producing islets. Therefore, we use a separation technique based on density gradients to separate islets from the other tissue. To improve on the widely used manual method for gradient making we developed a computer-controlled pump system to create the desired density gradients using approved technologies. The pump-made gradients worked as well as the man-made gradients and did so with minimal effort.

Evaluating the number of islets after isolation and before transplantation is another source of variation. Using small samples, manual methods that are currently used to microscopically size each islet to obtain total islet yields are not reliable. We developed a digital imaging analysis system to measure islet yield and purity. The principle is that instead of counting each islet we can obtain similar numbers but with greater accuracy by measuring the total tissue pellet volume and islet purity. Indeed, by simply using digital counting
methods instead of manual methods, variation in counting reduced by almost half. By using the pellet volume/purity method, variation was also reduced compared to manual methods. Another benefit of the new method is that it is an easy and clean method.

Once we have an islet preparation we would like to know how it works in the transplanted patient. C-peptide is released with insulin so it works as a measure of insulin release. Both blood sugar levels and creatinine affect C-peptide levels and therefore we measure all three in order to obtain a proper measure of the transplant’s function. By using this measurement we identified four pretransplant factors that affect outcome. One factor was related to the organ transport time, one to function of the islets, and two to the transplanted tissue volume. When these four easily attainable factors were put into a predictive model, about 40% of the variation in the islet transplant outcome was explained.

The work contained in this thesis identifies and optimizes a number of elements critical to consistent islet isolation and transplantation success.

Ett av de viktigaste momenten i ö-isoleringsprocessen är den enzymatiska nedbrytningen av bukspottkörteln. Utfall i variationen är till stor del på grund av enzymvariationer. Vi har introducerat en ännu oidentifierad kritisk enzymaktivitet för effektiv nedbrytning av bukspottkörtlar för ö-isolering. Förändringar i den här nya enzymaktiviteten ledde till betydande skillnader i framgången av ö-isoleringen, utan att ö-kvaliteten försämrades. Mätningar av den här enzymaktiviteten kommer att ha betydelse för ö-isoleringsstabilisering.

Ett annat kritiskt isoleringssteg är reningen av den nedbrutna bukspottkörteln. Bukspottkörteln består till 98% av vävnad som inte innehåller öar med insulinproducerande celler. Därför används en separationsteknik baserad på densitetsgradienter för att separera öarna från övrig vävnad. För att förbättra den utbredda manuella metoden för att göra densitetsgradienter har vi utvecklat ett datorstyrt pumpsystem för att skapa önskade densitetsgradienter. De automatiserade datorstyrdra gradienterna fungerade lika bra som de tidi-}

let för manuella metoder, minskade variation nästan till hälften. Den största
nyttan med den nya metoden är att den också är mycket användarvänlig.

När vi har en ö-preparation, vill vi veta hur den kommer att fungerar i den
transplanterade patienten. Man kan göra det genom att mäta ett antal olika
parametrar. C-peptid frisätts på samma sätt som insulin och fungerar därför
som ett mått på insulinfrisättningen. Blodsocker och kreatinin kan påverka
C-peptidnivån och därför mätte vi alla tre för att få ett ordentligt mått på
transplantatets funktion. Med hjälp av den här mätningen har vi identifierat
fyra pretransplantfaktorer som påverkar transplantationsresultatet. En faktor
var relaterad till bukspottkörtelns transporttid, en till ö-cellsfunktion, och två
till den transplanterade vävnadsvolymen. När dessa fyra lättillgängliga fakto-
rer sattes in en prediktiv modell, förutspådde modellen ca 40% av variationen
i transplantationsresultaten. Modellen kommer att kunna användas för att
avgöra om öarna har tillräckligt hög kvalitet för att transplanteras.

Arbetet i denna avhandling identifierar och förbättrar ett antal kritiska de-
lar av ö-isoleringsproceduren. Med ännu mer standardisering kommer ö-
isolerering och transplantation att bli en mer kostnadseffektiv och tillgänglig
behandling för många typ 1-diabetiker.
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