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# Functional in vitro studies of immunosuppressive agents in human pancreatic islets.


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# 1 Acknowledgements

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"The only true wisdom is in knowing that you know nothing. " Socrates

# 2 List of Papers

**Paper 1:**

Sahraoui A, Kloster-Jensen K, Ueland T, Korsgren O, Foss A, Scholz H. **Anakinra and tocilizumab enhance survival and function of human islets during culture: implications for clinical islet transplantation.**

*Cell Transplant. 2014;23(10):1199-211.*

**Paper 2:**

Kloster-Jensen K, Sahraoui A, Vethe NT, Korsgren O, Bergan S, Foss A, and Scholz H. **Treatment with Tacrolimus and Sirolimus Reveals No Additional Adverse Effects on Human Islets In Vitro Compared to Each Drug Alone but They Are Reduced by Adding Glucocorticoids.**

*J Diabetes Res. 2016;2016:4196460 (epub 2016 Jan 18).*

**Paper 3:**

Kloster-Jensen K, Vethe NT, Bremer S, Abadpour S, Korsgren O, Bergan S, Foss A and Scholz H. **Intracellular sirolimus concentration decrease in combination with tacrolimus exposure of human islets in vitro.**

*Transpl Int. 2015 Oct;28(10):1152-61.*

# 3 Selected abbreviations

| | |
|---|---|
| **ABC** | **ATP-binding cassette** |
| **BMI** | **Body Mass Index** |
| **CNI** | **Calcineurin inhibitor** |
| **CYA** | **Cyclosporine A** |
| **ELISA** | **Enzyme-linked immunosorbent assay** |
| **DM** | **Diabetes Mellitus** |
| **IBMIR** | **Instant blood mediated inflammatory reaction** |
| **IEQ** | **Islet equivalent** |
| **IHC** | **Immunohistochemistry** |
| **IHF** | **Immunofluorescence** |
| **IL** | **Interleukin** |
| **HLA** | **Human Leukocyte Antigen** |
| **MCP-1** | **Monocyte chemotactic protein 1** |
| **MRD1** | **Multidrug resistance protein 1** |
| **mTOR** | **mammalian Target of Rapamycin** |
| **NFAT** | **Nuclear Factor of Activated T cells** |
| **NF-κB** | **Nuclear factor- kappa B** |
| **OATP** | **Organic anion transporting polypeptide** |
| **SLC** | **Solute Carriers** |
| **OGTT** | **Oral glucose tolerance test** |
| **P-gp** | **P-glycoprotein** |
| **PP-cells** | **Pancreatic polypeptide cells** |
| **PTDM** | **Post-Transplant Diabetes Mellitus** |
| **T1DM** | **Type 1 Diabetes Mellitus** |
| **T2DM** | **Type 2 diabetes Mellitus** |
| **TNF-α** | **Tissue necrosis factor-alfa** |
| **IL-1β** | **Interleukin 1 beta** |
| **Tx** | **Transplantation** |

# 4 Introduction

## 4.1 Diabetes Mellitus

### 4.1.1 History

Diabetes has been recognised as a chronic disease for more than three million years.
It was first mentioned by the Egyptian physician Hesy-Ra of the 3rd Dynasty (around1552 B.C) and characterised as 'too great emptying of urine'. The Greek Apollonius of Memphis used the term "diabetes" (dia – through, betes – to go) meaning, "to pass through" about the disease for the first time around 230 B.C. He and his contemporaries considered it a disease of the kidneys and recommended among other treatments, bloodletting and dehydration[1].
Historical documents show that doctors from all over the world has been aware of the condition where patients have excessive passing of urine, but none could determine its cause. In India the physicians called it *madhumeha* ('honey urine') because it attracted ants. Despite the descriptions of diabetes no one knew how to live with diabetes, let alone treat or cure it. Children often died within days of onset and older people dealt with devastating complications. Remedies ranged from herbs to bleeding. In 1775 Mathew Dobson discovery that diabetes involves excess sugar in the blood (hyperglycaemia) and argued that the disease was not located in the kidneys[2].

It is only in the past 150 years that we really have seen a development in understanding what diabetes is and what causes it. In the middle of 19$^{th}$ century, the French doctor Claude Bernard discovered how liver play a role in glycogenesis and established that diabetes is due to excess production of glucose[3]. The young German medical student, Paul Langerhans described in his thesis (1869) different cell types that includes small, irregularly shaped, polygonal cells without granula within pancreas. These celles wore in 1893 named islets of Langerhans by the French histologist GE Languesse[4]. More than 20 years after Langerhans dissertation, in 1892, Joseph von Merring and Minkowski at the University of Strasburg removed pancreas from dogs and discovered the role of pancreas in the pathogenesis of diabetes[5]. One year later Watson- Williams and his associate Mr

Harsant published the first xenotransplantation where 3 pieces of sheep pancreas was placed into a 15-year-old boy with diabetes[6]. Unfortunately, he died 3 days later. In 1897 the average life expectancy of a 10-year-old child with diabetes was about 1 year, as for 30-year-old 4 years, and a newly diagnosed 50-year-old might live another 8 years. The debate around pancreas having an "internal secretion" of a substrate that could relieve symptoms of diabetes went on for decades. Attempts to isolate the pancreatic extract failed until 1921 when Banting, Best and Collip discovered the pancreatic extract that they named insulin[7-9]. The first patient was treated with injection of insulin in 1922; it was a 14-year-old diabetic boy that was dying at the Toronto General Hospital. His condition improved dramatically after treatment. News about using insulin to treat diabetes quickly spread around the world. Subcutaneous injections of bolus doses of insulin became the life- sustaining therapy for T1DM patients. Still today the golden standard for the majority of T1DM patients is standard or intensive exogenous insulin therapy.

### 4.1.2 Classification and Epidemiology

#### 4.1.2.1 Classification

Diabetes is defined as a group of metabolic diseases where hyperglycaemia results from defects in insulin secretion, insulin action or both[10 11]. A classification of diabetes into two types, based on "insulin insensitivity" was made by Sir Himsworth in 1936, categorizing diabetes as an insulin-deficient form (type 1) and an insulin-resistant form (type 2)[12]. With the later understanding of autoimmunity there are still two mains categories: the autoimmune type 1 and the non-autoimmune type 2. But in addition, a third group due to other mechanisms or diseases that causes diabetes including genetic defects, gestational diabetes, diseases of the exocrine pancreas and damage induced by drugs, chemicals, or infections[11 13 14].

##### 4.1.2.1.1 Type 1 diabetes mellitus.

T1DM is defined as a gradual loss of the insulin producing beta cells located in the islet of

Langerhans. It is a lifelong chronic disease where the underlying cause still is unknown. But probably it is due to genetic factors, environmental triggers and presence of auto antibodies against several beta cell proteins including insulin (IAA), glutamic acid decarboxylase (GAA or GAD) and protein tyrosine phosphatase (IA2 or ICA512)[15]. This is a disease mainly diagnosed in children and adolescents but can also occur in adults. In high-income countries, the majority of diabetes cases in children and adolescents is due to type 1 diabetes[16-18]. The prevalence varies among ethnic and regional populations but overall the incidence is increasing yearly by 3-5 %[19]. Knowledge of this disease is mainly derived from analysis of pancreatic specimens post-mortem, serum and peripheral blood lymphocytes of type 1 diabetic patients[20]. Biopsies from patients with resent onset T1DM is hard to obtain since it is associated with complications such as bleeding and pancreatitis. The Diabetes Virus detection (DiViD) study showed presence of inflammation in the pancreas at the time of diagnosis and how most of the inflammatory T cells were located around the islets. This study was however interrupted because it would be unethical to continue due to its complication rate[21 22]. From a genetic perspective, so far nearly 40 loci is known to affect T1DM susceptibility[23]. Half of the genetic susceptibility is assumed to come from the HLA region on chromosome 6. HLA class II has the strongest association with T1DM and the greatest susceptibility is with DRB1*0401-DQB1 and DRB1*0301-DQB1*0201. Resistance to the disease has been found in DRB1*1501 and DQA1*0102-DQB1*0602[24 25].

#### 4.1.2.1.2 Type 2 diabetes Mellitus

The most prevalent form of diabetes is T2DM, and includes 90-95% of all diabetic patientients[10]. T2DM is an endocrine and metabolic disorder with a progressive dysfunction of pancreatic beta cells and insulin resistance. It is mainly diagnosed in middle and old age, but an increasing number of children have been registered with type 2 diabetes. Though data are sparse increasing levels of obesity and physical inactivity among children in many countries confirming the trend[26]. The decline in beta cell function involves chronic hyperglycaemia (glucotoxicity), chronic exposure to non-esterified fatty acids (lipotoxicity), oxidative stress, inflammation, and amyloid formation[27-30].

Reduced secretion of glucagon-like peptide 1 (GLP-1), hyperglucagonaemia, and other regulatory hormones are also known contributors.

### 4.1.2.2 Epidemiology

According to the international diabetes federation (IDF) an estimated 425 million people had diabetes in 2017[31]. And as shown in figure 1 the distribution of diabetes varies for each subcontinent but is assumed to continue to rise in years to come and 80% live in low- and middle-income countries. By the year 2035, it is estimated that one adult in 10, or 592 million adults, will have diabetes[32]. The true incidence of type 1 diabetes worldwide is unknown because the distinction between type 1 and type 2 diabetes becomes blurred in later life. Although type 1 diabetes represents 7-13% of all diabetes cases[33], the disease is associated with an increased risk of serious and chronic complications. The highest incidence of children with type 1 diabetes is found in Europe with an annual increase around 3% [17 18]. In 2015 approximately 5.0 million people aged 20-79 years died from diabetes[34]. In magnitude this represents more deaths than the worlds 3 major public health problems combined: HIV/AIDS (1.1 million deaths), malaria (438.000), and tuberculosis (1.1 million)[35].

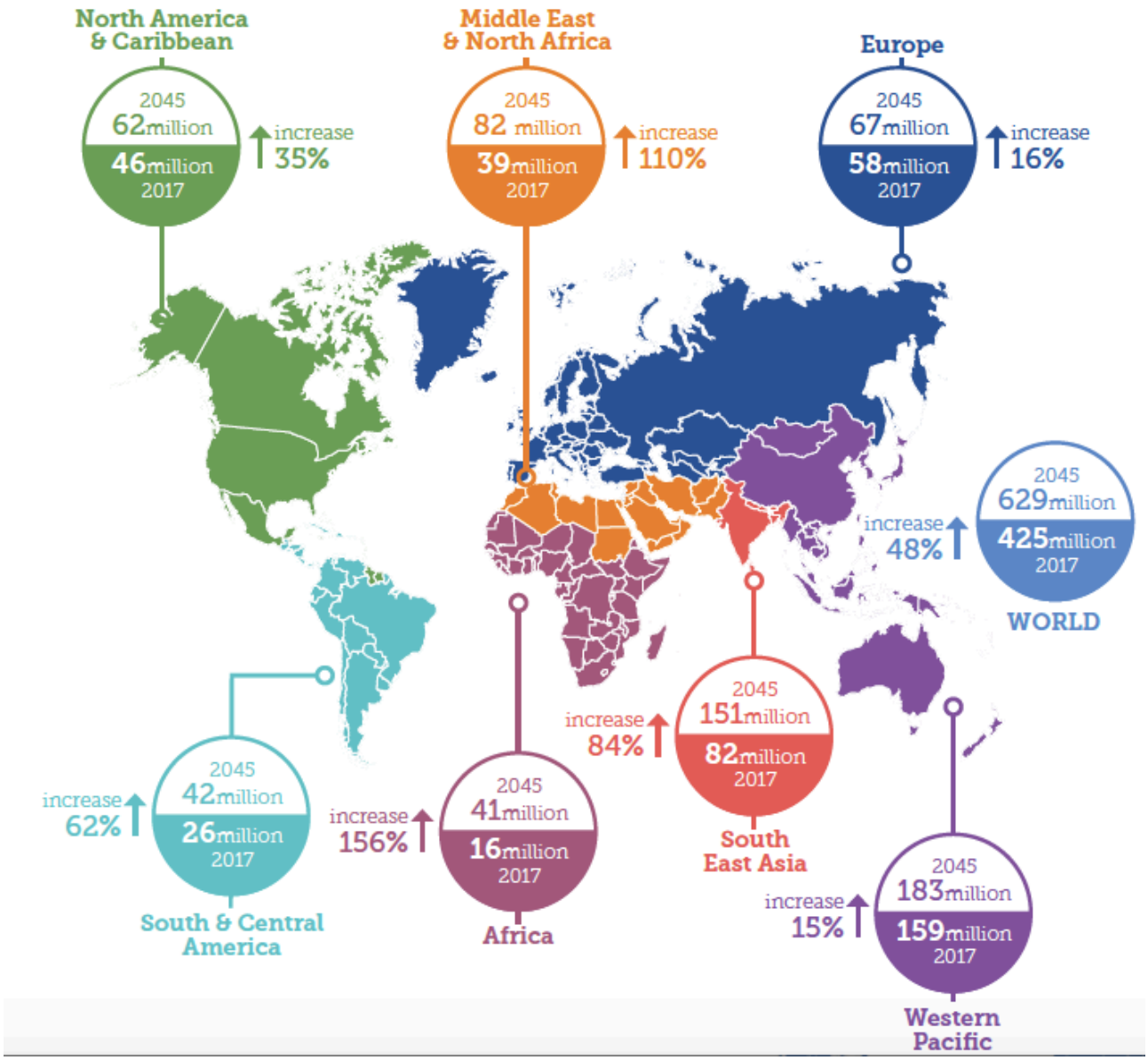


**Figure 1. The map show how many people between 20-79 years of age that live with diabetes, the prevalence in each subcontinent in millions and estimated change in 2045. Reproduced with permission from IDF (website [www.IDF.org)](http://www.IDF.org)**

## 4.2 Pancreas- the islets containing organ

The endocrine region of the pancreas only makes up 1-5 % of the total pancreas mass and consists of cell cluster known as the islet of Langerhans that are dispersed throughout the exocrine region. A normal pancreas contains roughly 1 million islets and each islet is comprised of an average of 1560 cells[36]. About 70% are insulin-producing beta cells, the remaining four cell types are alpha cells that produce glucagon, somatostatin-producing delta cells, ghrelin-producing epsilon cells and

pancreatic polypeptide-producing PP-cells (Figure 2). Islets are highly vascularized, receiving up to 15% of the pancreatic blood flow[37 38].

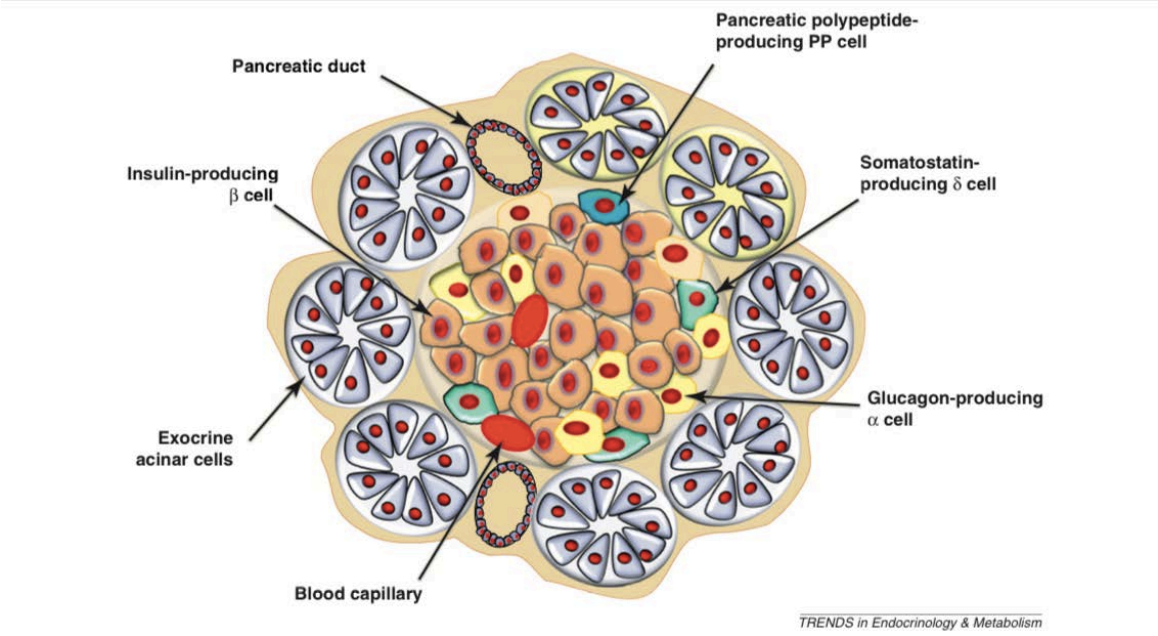


**Figure 2. The Human islet is composed of α, β, δ and PP cells and surrounded by exocrine acinar cells. Blood capillaries are dispersed throughout the islet. Image is reproduced with permission from Trends in Endocrinology & Metabolism[39].**

The architecture of an islet in rodents differs from that of humans (Figure 3). In rodents, the beta-cells are located predominately in the central core with alpha- and delta-cells localized in the periphery of the islet forming a mantle whereas in an human islets, the alpha- beta- and delta- cells are all dispersed throughout the islet[40 41]. Human islets have been found to have better intracellular communication due to structural composition and therefor are assumed to be more sensitive to insulin than rodent islets[42]. Another important differences is that rodent islets proliferate much more efficient than human islets[43]. Therefore, a direct comparison of human data with that of laboratory animals may be misleading.

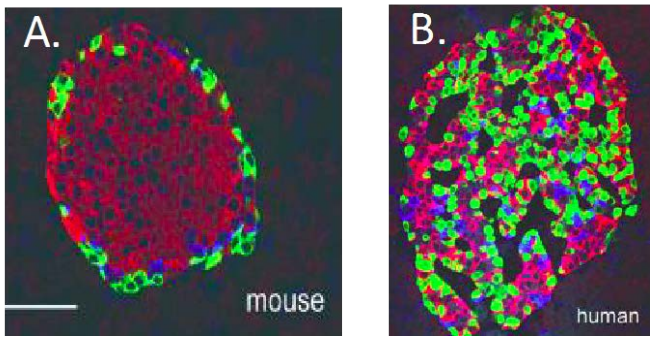


**Figure 3 Immunofluorescent images of a mouse (A) and human (B) islet. Insulin (red), glucagon (green), and somatostatin (blue). Scale bar 50 µm. Reproduced with permission from PNAS[44]. Copyright 2006 National Academy of Sciences, U.S.A**

### 4.2.1 Treatment options and secondary complications of T1DM

Patients suffering from T1DM needs to constantly manage their insulin levels. Initially bovine insulin was used for injection to diabetic patients, but in 1975 a synthetic human insulin was synthesized[45]. The gold standard treatment of T1DM is daily multiple injections, with a long acting insulin analogue for basal insulin and rapid-acting insulin to administer before meals, based on grams of carbohydrate consumed. The use of continuous subcutaneous insulin infusions (CSII; insulin pumps) has increased substantially over the past decade. Whether CSII is overall better than multiple daily injections for management of type T1DM is debated. In a recent study the CSII therapy was compared with daily insulin injection in a systemic review and meta-analysis of 24 studies involving a total of 585 patients. Main results showed lower HbA1C concentration with sensor-augmented pump therapy compared to injection therapy with similar rates of hypoglycemia[46].

Mono- or bihormonal artificial pancreas is defined as man-made technology to match the way pancreas works. It currently exists in 3 different categories: i) Closed-loop artificial pancreas (an insulin pump with a GCM, like the MiniMed 640G), ii) Bionic pancreas (a glucagon and insulin pump, so far not approved, but Dr Edward Damiano's Beta Bionics will hopefully be approved in 2018), iii) implanted artificial pancreas. These devices try to mimic pancreas to give an optimal

blood sugar level, however they have so far not been effective enough other than in steady state and not when people are physically active. Another disadvantage is that the devices are fairly expensive (Table 1).

| Year | Diabetes management |
|---|---|
| 1955 | Oral drugs to help lowering blood glucose levels |
| 1970 | Portable glucometer and insulin pumps |
| 1976 | HbA1c, measure of long term blood glucose levels in the body |
| 1983 | First biosynthetic human insulin |
| 1986 | The insulin pen system |
| 1996 | The first recombinant DNA human insulin analogue lispro (Humalog) |
| 2006 | First inhaled powder insulin product, Exubera |
| 2016 | "Artificial pancreas," MiniMed 670G hybrid closed-looped system |

**Table 1 Overview of development in diabetes management**

T1DM patients suffer from secondary micro- and macro vascular complications. Cardiovascular events are 10 times more common in T1DM patients compared to the general population, however hypoglycaemia can reduce the risk by 42% [47]. Micro vascular complications such as retinopathy, nephropathy, and neuropathy are also reduced by intensive diabetes treatment[48 49]. A recent published Norwegian cohort study shows that patients diagnosed with childhood T1DM had a 3 fold higher risk of deaths due to acute diabetic complications; including diabetic ketoacidosis and hypoglycaemia than the general population[50 51]. Intensive insulin therapy is limited by the risks of hypoglycaemia[52-54], hence, effort to resolve this by developing beta cell therapies has emerged.

### 4.2.2 Beta cell replacement therapy

**Pancreas transplantation**

The first pancreas transplantation was performed in 1966 by Dr. Kelly at the University of Minnesota [55]. Morbidity and mortality was initially extremely high in these transplants but this has

decreased greatly over the years due to better surgical technique, immunosuppressive treatment, donor/recipient selections and has now become a routine transplant procedure[56]. According to the International Pancreas Transplant Registry (IPTR) more than 48,000 pancreas have been transplanted worldwide[57].Traditionally the indications for pancreas transplantation are patients with chronic renal failure secondary to diabetes and non-uremic T1DM with significant hypoglycemic unawareness. There are currently 3 major categories of pancreas transplantation. 1) Pancreas transplant alone (PTA) (13%)[58 59], 2) Simultaneous pancreas kidney transplant (SPK) (76%). Both organs usually come from the same deceased donor however even though it is rare the organs can also come from separate deceased donors or one or both organs come from a living donor[60 61], and 3) Pancreas-after-kidney transplant (PAK) (11%). Deceased donor pancreas transplantation is performed after a previous living or deceased donor kidney transplant. Five and ten-year pancreas graft survival are 73 and 56% for SPK, 64 and 38% for PAK, and 53 and 36% for PA[62]. Especially SPK transplantation improve quality of life and life expectancy[63 64] compared to kidney transplantation alone[65]. Immunosuppressive treatment normally consists of induction treatment with either basiliximab or thymoglobulin or alemtuzumab[66-68]. Maintenance therapy in pancreas transplantation is tacrolimus-based immunosuppression; cyclosporine is used as secondary therapy in cases of tacrolimus intolerance.[69] Avoiding continuous steroid treatment after pancreas transplantation is important since steroids hamper islet function and inhibit islet insulin secretion[70 71]. A regime might include the use of belatacept and/or sirolimus[72].

**Islet transplantation**

The first attempt to use parts of pancreas to treat T1DM in a human was performed in 1893 when Watson-Williams and Harsant transplanted three pancreatic fragments from sheep, the size of "Brazilian nuts", into the subcutaneous tissue of a 13-year-old boy with ketoacidosis. The boy showed signs of improved glycosuria but died 3 days after the operation[6]. After the discovery of insulin in 1922[7 73], for some time the interest for islet transplantation remained low. The first human

bone marrow transplantation in 1956 by Dr. E. Donnall Thomas proved that transplantation of cells was possible[74], and in the 1960ies, the interests and attempts of minimally invasive method to transplant isolated islets was developed. In 1972 Lacy and co-workers cured diabetes in rats through intra-abdominal and intraportal transplantation of isolated islets[75]. To adapt these results to humans it was necessary to isolate a larger number of islets from the human pancreas, which proved to be more difficult than expected. The first islet transplantation was performed in 1977 but the inability to transplant enough viable islets hampered the possibilities to perform clinical trials[76]. In 1988 doctor Camillo Ricordi introduced the automated methods for islet isolation which included the use of the "Ricordi" chamber as shown in Figure 4 that combined enzymatic and mechanically digestion to liberate the endocrine islets from the exocrine tissue with intact integrity[77 78]. This method is still considering the gold standard for all centres working on isolation of human islets (Figure 4). For clinical outcome after islet transplantation, the first insulin independent recipient was reported in 1990 by the St. Louis group[79]. Two years later, Warnock and colleagues published the first case where a patient remaining insulin independent more than a year after transplantation[80]. The same year Pyzdrowski reported that 265,000 islets were sufficient to establish insulin independence[81]. Throughout the 90ties, reports of successful allogeneic islet transplantation continued to appear, but achievement of long-term insulin independence remained elusive in most recipients [82 83]. Results slowly improved but were clearly inferior to those of pancreas transplantation[84].

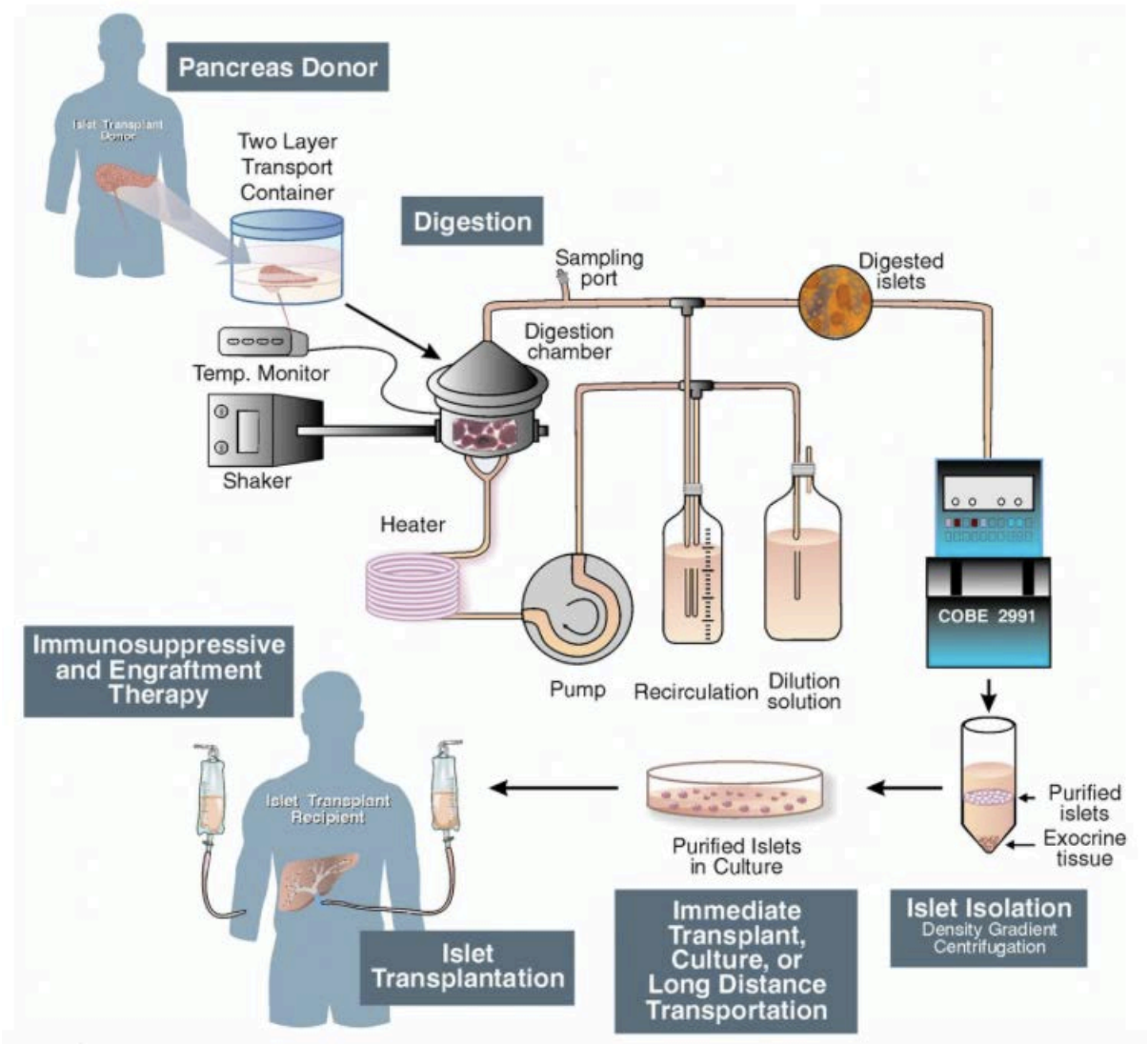


**Figure 4. The current method for isolating human islet. The pancreas is transported in a two-layer container and put into a digestion chamber. The digested islets are then separated from the exocrine tissue using the continuously gradient density purification, COBE®2991. Depending on whether islets are going to be transplanted immediately, transported to a distant centre or cultured the purified islet will be put into different kind of culture medium. Reprinted with permission from Shapiro and Clinical Science[85].**

In 2000 the Edmonton group presented a study where seven patients with brittle diabetes received islet transplantation and all remained insulin free 1 year after transplantation[86]. This article became a turning point for islet transplantation and the Edmonton Protocol became the new golden standard (Figure 5). It also triggered an increased optimism around using islet transplantation as a cure for T1DM. Compared to previous practise it contained several major changes as summarized in Figure 5, the most important being a steroid-free immunosuppressive protocol consisting of pre-treatment with daclizumab, and maintenance treatment with sirolimus and low-dose tacrolimus. The protocol

also introduced the use of a larger islet mass (~11,000 IEQ/kg recipient weight) for transplantation whereas previously the threshold had been as low as 600 IEQ/kg. Avoiding islet culture before transplantation was also introduced with the Edmonton protocol but now a days most centres leave the islets in culture for 24-48 hours[84 87]. This has been shown to reduce islet inflammatory stress and increase viability as well as facilitating recipient preparation.

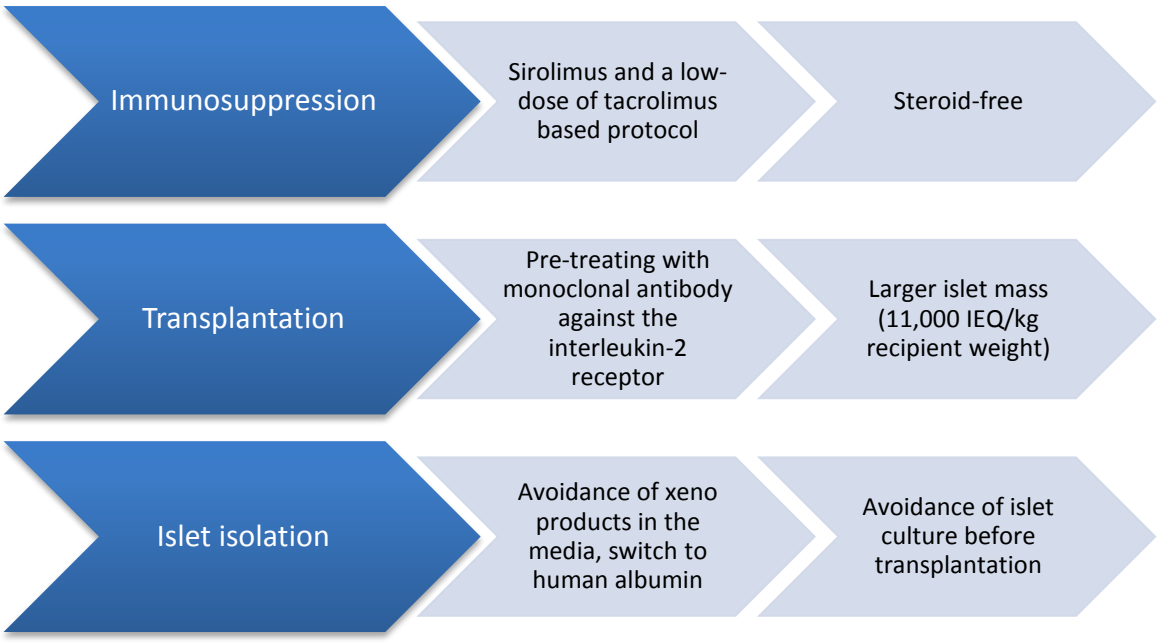


**Figure 5. The essential changes in the islet transplantation procedure, introduced by the Edmonton protocol.**

New optimism around islet transplantation was found after year 2000 and the number of transplantations increased. Collaborative Islet Transplant Registry (CITR) has recently published data from islet transplantations performed between 1999 and 2010[84]. It shows the overall improvement in clinical islet transplantation results both in terms of rate and duration of insulin independence yielded 47% at 3 years. This report also revealed how islet transplantation outcome is influenced by multiple factors such as donor harvesting, the islet isolation process, pre- and perioperative treatments of both islets and recipient as well as post transplantation immunosuppressive treatment. The immunosuppressive regimen has gradually ben changed from

mTOR inhibitor combined with calcineurin inhibitor (CNI) to inosine monophosphate dehydrogenase inhibitor (IMPDH) inhibitor. The Minnesota group report the first insulin independence using a single donor islet mass, which has given hope to the possibility of using only one isolated pancreas to treat a T1DM patient with islet transplantation. Essential for this result was the use of a potent induction treatment including rabbit antithymocyte globulin (ATG), methylprednisolone, daclizumab and etanercept[88]. Donor selection and pancreas procurement also play a crucial role in the overall outcomes of islet transplantation. Some centres report increased islet yield under certain donor specifications such as age and weight whereas others use BMI instead of absolute weight with improved results[12 89].

Improvement in results over the years have made islet transplantation not only a safe procedure but also the best therapeutic option for a selected group of diabetic patients with renal failure and patients suffering from life-threatening hypoglycemic-unawareness[90 91]. And it is also a good treatment option to prevent hypoglycaemia after donor pancreas failure[92]. Insulin independence rates at 5 years now exceed 50-70% in experienced centres. This is similar to the 5-year success rate of whole-pancreas-alone transplantation[93].

## 4.3 Pre- and post-transplantation loss of islet viability and function

### 4.3.1 Inflammatory responses in islet transplantation

Although the result of islet transplantation has improved, several hurdles limit a widespread use. It is generally accepted that 50%–70% of the transplanted islets are lost in the immediate post transplantation period in the liver[94]. Inflammation occurs from the procurement, isolation, and in the early post transplantation phases. From the very start of the procurement, soon after brain death occurs, an inflammatory reaction in the body start the decomposing process. Meticulous attention should be paid to keeping the organs cool at all times and minimize cold ischemic time[95]. The islet isolation process consists of several steps in which pancreas and the acinar tissue is digested and the islets are purified from the exocrine tissue. The enzymatic dissociation of islets from surrounding

exocrine tissue is considered one of the most important steps in the procedure. Some of the components of the enzyme blend are toxic to islets[96]. During the digestion phase of the isolation process islets are exposed to mechanical stress, anoxia, acidity and hyperosmolarity, which could cause apoptosis immediately after isolation. Once digested the islets must be isolated from the exocrine tissue using a gradient system. The preferred method today is the continuously gradient density purification using an COBE®2991 cell apheresis centrifuge system (Figure 4).

Beta cell dysfunction and death is also triggered by oxidative stress [97-99] and because of low levels of antioxidants islets are assumed to be particularly prone to damage during inflammatory conditions[100 101]. Islet response to isolation and purification procedures have detrimental effects, activating pro-inflammatory cytokines such as interleukin-1 beta (IL1-β), tumor necrosis factor-alfa (TNF-α)[102 103], Interleukin-6 (IL-6) and interleukin-8 (IL-8)[104]. IL-1β increase monocyte chemo attractant protein-1 (MCP-1) expression in islets[105] and MCP-1 attracts monocytes[106]. Another cytokine found in islet preparations is macrophage migration inhibitory factor (MIF) shown to inhibit macrophage migration and have an important part in T-cell activation[107].

Transplanting islets into the liver by way of ultrasonic guided percutaneous catheter infuse islets into the portal vein causing cell entrapment in the terminal venous branches of the liver. On the positive side, it provides islets with nutritional and physical support. But having already lost their dense vasculature and specialized extracellular matrix in the isolation and purification process, the microenvironment in the liver is very hostile[108] (Figure 6). In addition, the high concentrations of immunosuppressive drugs in the liver and hepatic steatosis induced by local insulin further contribute to loss of islet. During engraftment in the liver islets are exposed to a serious non-specific inflammatory response named IBMIR. It is mainly triggered by production and secretion of tissue factor (TF), MCP-1, IL-1β and IL-6 in the islet preparation[109-111]. Substitute islets with glass beads showed no inflammatory response in an experimental model of intraportal islet transplantation[112]. Thus, it is fair to assume that the triggered inflammatory process is a combination of the biological material islets represent and stresses islets have been exposed to prior

to transplantation. Efforts to promote anticoagulation through the use of systemic heparin, low-molecular-weight dextran sulphate or coating of islets with heparin or urokinase have been tried[113-115]. Another strategy has been to inhibit the inflammatory response by treating recipients with fusion proteins such as the TNF-α inhibitor, Etanercept[116], and IL-1 receptor antagonist (IL1ra), Anakinra which have found the way to the clinic[117 118]. Still, the best anti-inflammatory strategy for improving islet engraftment and reduce rejection are yet to be defined.

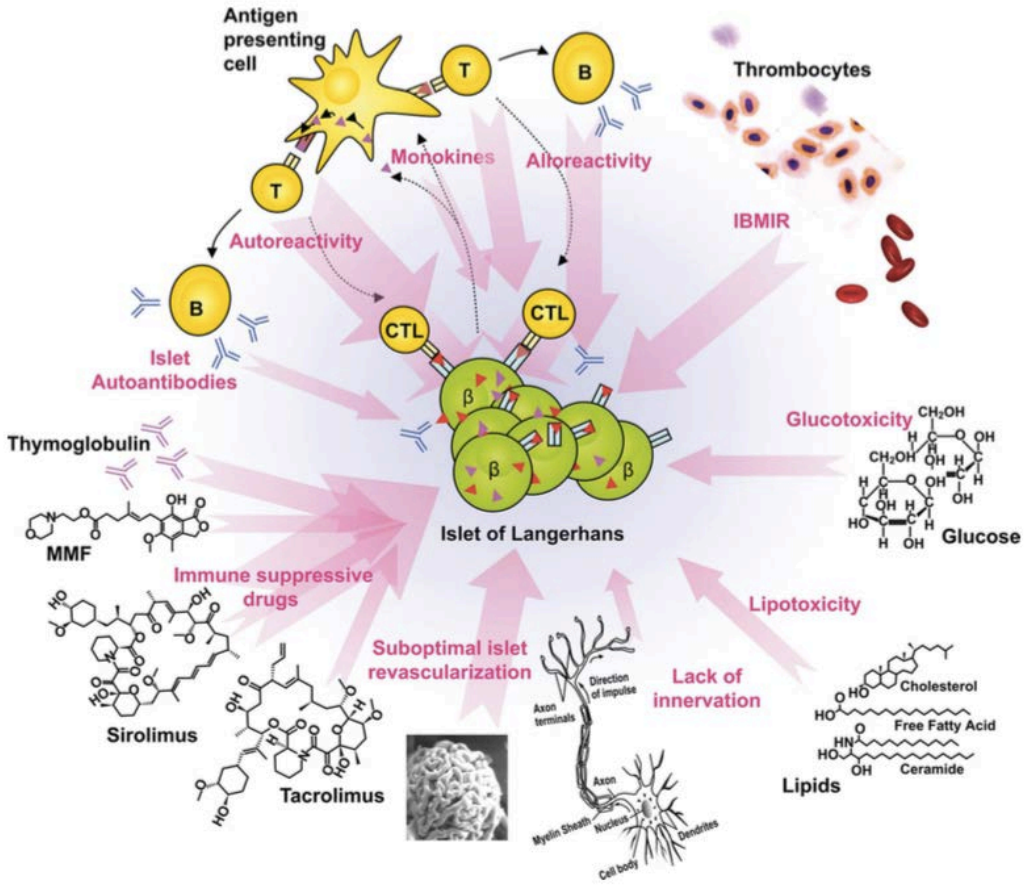


**Figure 6. Illustrating the multifactorial causes for islet loss following transplantation by inflammatory responses, metabolic stressors, pre-exciting and post-transplant auto- and alloimmunity. Printed with permission from Diabetes[119].**

### 4.3.2 Immunosuppressive protocols in islet transplantation

No guidelines or formal consensus on the "best" or "standard" immunosuppressive strategy for human islet transplantation are currently available. But it is safe to say that along its course immunosuppression for islet transplantation has followed the regimes used to treat pancreas transplant patients. At the first human pancreatic islet allotransplant reported in 1974, the immunosuppressive regimens depended heavily on steroids and azathioprine[76]. No patients achieved insulin independence with this treatment and very few showed transient graft function. The first report of insulin independence after islet transplantation came after using a cyclosporine based regimen[79].

The landmark protocol from the Edmonton is still the bases for most of the immunosuppression regimen worldwide but most active transplant centres have developed their own modified version in search of better long-term outcome results (Table 2). IS treatment is a necessary evil for transplanted patients. As a therapist, there is a fine line between treating patient with a to small dose, which give less side effects but with a potential risk of rejection and return of autoimmunity in a diabetic patient and on the other hand a too large dose, causing increased risk of infection and with a substantially increased risk of certain forms of cancer. The immunosuppressive therapy is responsible for 74% of post-transplantation diabetes mellitus (PTDM)[120 121] which emphasize the importance of correct immunosuppressive treatment and dose for each individual. Collaborative Islet Transplantation Registry (CITR) data, has reported changes in immunosuppression strategies during the past years[84]. A shift toward an induction treatment consisting of a T-cell depleting antibody (e.g., Thymoglobulin), with or without an inhibitor of TNF-α (e.g., etanercept) and an mTOR inhibitor (e.g, sirolimus) or an inosine monophosphate dehydrogenase inhibitor (e.g., mycophenolic acid) combined with a CNI (e.g., tacrolimus) for maintenance. Induction therapy with anti-CD52 antibody/alemtuzumab (e.g., Campath) has been reported with encouraging long-term function[122]. Biologic agents such as agents targeting co-stimulation pathways in immune cells and adhesion molecules (CTLA4-Ig, LFA-1 PD-1/PD-L1 CD40) and chemokine receptors

(CXCR1/2) that potentially lower islet cell and organ toxicity profiles are being evaluated and shows promising results[123-126].

Experiences from islet transplant centers have shown promising results adopting improved immunosuppressive regimen in clinical trials. As such, potent induction therapy with T cell depleting antibodies has been recognized to not only allow for successful single donor islet transplants but also improved long-term outcomes[127]. At the University of Minnesota, a combination treatment with both T-cell depleting anti-thymocyte globulin (ATG) and the TNF-α inhibitor, etanercept showed higher rates of insulin independence compared with patients treated with T-cell depleting antibodies alone or traditional standard IL-2 receptor antibody treatment[116 127]. In another clinical study performed by the University of California, San Francisco 8 out of 8 patients achieved insulin independence using an steroid and calcineurin free maintenance immunosuppression (sirolimus and MMF) including co-stimulation blockade (belatacept) or anti-LFA-1 antibody (efalizumab)[126]. And a multicenter study from Milan and Geneva recipients started pretreatment with rapamycin ≥30 days before transplantation, then gave one dose of ATG after first infusion and a CNI-free immunosuppressive maintenance treatment (rapamycin and MMF) together with short-term steroid treatment and IL-1Ra. The result was that only 10 out of 15 patients finished the pretreatment with rapamycin and out of those 10 patients only 1 completed the ATG treatment due to adverse effects. Four out of ten patients maintained insulin independence up to year 3 and it was considered a less efficient graft survival.[128]. The lack of induction therapy with ATG was considered an important reason for the graft loss.

| Center | Pub. Year | Induction | Maintenance therapy | Procedure | Nr. of tx | Ref-erence |
|---|---|---|---|---|---|---|
| **Washington** | 1990 | mALG | CsA<br>mPred | IAK | M | [79] |
| **Pittsburgh** | 1992 | | Tac<br>mPred | SIK/ SIL | S/M | [129] |
| **Miami** | 1992 | | Tac, Pred | SIK/ SIL | S/M | [130] |
| **Miami** | 1997 | OKT3 | CsA, Aza, mPred | IAK/ SIK | S/M | [131] |
| **Milan** | 1997 | ATG | CsA, Aza, Pred | IAK/ SIK | S/M | [132] |
| **Alberta** | 2000 | Dac | Sir, Tac | ITA | M | [86] |
| **Pennsylvania** | 2004 | Dac | Sir, Tac | ITA/IAK | M | [133] |
| **Minnesota** | 2004 | Anti-CD3, Sir | Sir,Tac | ITA | S | [134] |
| **Minnesota** | 2005 | ATG, Dac, Eta | Sir, Tac -> MMF 1mnth posttx | ITA | S | [88] |
| **Alberta** | 2006 | Dac | Sir, Tac | ITA | S/M | [135] |
| **Illinois** | 2008 | Dac,Eta | Sir, Tac | | | [136 137] |
| **Miami** | 2008 | Alem | Sir-Tac, 3mth followed by Sir-MPA | ITA | S/M | [122] |
| **Minnesota** | 2008 | Tep /ATG and Eta w/wo Dac | Low dose CNI mTOR inhibitor or MMF/MPA | ITA | | [116 127] |
| **Minneapolis** | 2008 | ATG; Eta | Everolimus -> MPA/MMF<br>CsA | IAK | S/M | [116] |
| **Lille** | 2009 | Dac | Sir, Tac | ITA | M | [138] |
| **GRAGIL consortium** | 2009 | Dac/ Bas | Sir, Tac | IAK | S/M | [139] |
| **UCSF** | 2010 | ATG, Bel/ Efa | Sir or MMF | ITA | S/M | [140] |
| **Baylor** | 2011 | ATG,Eta and Ana /Dac, | Sir and Tac or Tac and MMF, | ITA | S/M | [117] |
| **Milan** | 2014 | ATG,mPred, Pred, Ana | Sir and MMF | ITA | M | [128] |
| **NNCIT** | 2017 | ATG,Eta, Bel | Tac, MMF and Sir | ITA | S/M | [141] |

**Table 2 Immunosuppressive protocols published by selected Islet Transplant program Ana= Anakinra; Alem= Alemtuzumab; ATG= Antithymocyteglobulin; Aza= Azathioprin; CsA= Cyclosporine A; Dac= Daclizumab; Bas= Basiliximab; Bel= Belatacept; Camp =Campath-1H; Efa=Efalizumab; Eta= Etanercept; Infl= Infliximab; mALG= Minnesota antilymphoblast globulin; MMF= mycofenotil mofetil; MPA=Mycophenolic acide; mPre=methylprednisolone; NNCIT= Nordic Network Clinical Islet Transplantation; Pred= Prednisone; Sir=Sirolimus; Tac= Tacrolimus; Tep=Teplizumab; -> = changed to; S=single; M=multiple; IAK= Islet after kidney; ITA=Islet transplantation alone SIK=simulataneous islet-kidney; SIL=simultaneous islet-liver**

## 4.4 Immunosuppressive drugs for islet allo-transplantation

### 4.4.1 Calsineurin Inhibitors (CNIs)

#### 4.4.1.1 Cyclosporin A

Cyclosporin A (CYA) was isolated from the fungus *Tolypocladium inflatum* (Beaveria nivea), found at Hardangervidda, Norway in 1969[142]. As an antifungal agent it had very limited effect but in 1972 Harmann F. Stähling discovered it was a highly effective immunosuppressive agent[143]. And a few years later, R.Y. Calne showed its successful us in prevention of organ rejection[144 145]. CYA blocks the calcium dependent signal transduction pathways distal to engagement of the T cell receptor, which interrupts the activation of T cells. The activation cascade includes calcium binding to calmodulin, which leads to binding calcineurin. The activated calcineurin may dephosphorylate the cytoplasmic unit of NF-AT that allows translocation of the NF-AT from the cytoplasm into the nucleus to form a competent transcriptional activator of the IL-2 gene. CYA is the cytosolic binding protein for cyclosporin highly abundant in the cytoplasma.

The most commonly reported inhibition or induction of CYA metabolism is by cytochrome p450 enzymes. CYA have deleterious effects on islets, causing cell damage[146] and impairing insulin secretion[147 148] and transcription[149]. Major clinical toxicities are nephrotoxicity and ultimately renal insufficiency and elevation of bilirubin levels.

#### 4.4.1.2 Tacrolimus

In a soil sample from the foothills of Mount Tsukuba, Tokyo a fungus named *Streptomyces Tsukubaensis*, producing Tacrolimus ("Tsukuba macrolide immunosuppressant") a macrolide antibiotic was discovered (1984)[150 151]. It was quickly acknowledged to possess immunosuppressive features that inactivate T-cells through suppressing IL-2 production.

Tacrolimus binds to immunophilins know as FK506-binding protein (FKBP), and the complex inhibits the $Ca^{2+}$ dependent phosphatase calcineurin. Normally, calcineurin cleaves a phosphate

group off nuclear factor of activated T cells (NFAT); this transcription factor then enters the nucleus and initiates the production of IL-2 as well as a host of other chemokine's, which eventually leads to T-cell replication. With the tacrolimus-FKBP complex present, calcineurin-dependent IL–2 transcriptions and T-cell proliferations are prevented[152 153]. Tacrolimus down-regulates the nuclear factor-κB (NF-κB) pathway and induce apoptosis of activated T cells by activating caspase 3[154 155]. It also seem to have immune regulatory effects on macrophages[156]. Tacrolimus directly influence islets by reducing insulin secretion through suppressing glucose induced insulin release[157 158], reduce mitochondrial density and function[159] and inhibits revascularisation of islets[160]. Most common adverse events can be categorized as nephrotoxicity, neurotoxicity, and hyperglycaemia[152 161].

### 4.4.2 mTOR inhibitor

#### 4.4.2.1 Sirolimus

Sirolimus/Rapamycin is a macrolide with antifungal properties, produced by the *Streptomyces hygroscopius* bacteria. It was first discovered in a soil sample from Easter Island, also known as Rapa Nuiis in 1975.[162]-It binds to the immunophilin FKBP-12 making the Sirolimus: FKBP-12 complex. This complex binds directly to mTOR and blocks its function which inhibits the mTOR mediated signal-transduction pathways, result in the arrest of cell cycle in G1 phase inhibiting the progression into the S phase in various cell types. FKBP-12 is ubiquitously expressed in human tissue, with the highest levels found in skeletal muscle and the testes[163 164].

Adverse effects are well described and include stomatitis, hyperlipidaemia, bone marrow toxicity and diarrhoea usually mild and dose dependent. Other adverse effects are more serious such as the antiproliferative properties causing anaemia, wound-healing problems and lymphocele. Sirolimus has severe impact on islets in terms of reduced GSIS and basal insulin secretion as well as increased apoptosis and decreased viability and proliferation[165 166]. It is not nephrotoxic however it may aggravate CNI-induced nephrotoxicity[167 168].

### 4.4.3 Methylprednisolone

Methylprednisolone is a synthetic glucocorticoid drug mimicking a steroid hormone naturally produced in the body in adaptive situations such as stress or fasting leading to energy store mobilization. Compared to prednisolone and prednisone, methylprednisolone has an additional 6-α-methyl group and blocks the specific binding to transcortin, the protein that transports steroids in plasma. In the 1950s glucocorticoid drug was used to treat rheumatoid arthritis[169 170]. Goodwin and Mims used glucocorticoid steroids to cure acute rejections in a kidney transplant patient in 1961[171] and Starzl and Marchioro confirmed the effect in 1963[172].

The immunosuppressive effect of glucocorticoids on the innate immune system is among others inhibition of annexin-1[173],suppression of phospholipase A2 and inhibition of various leukocyte inflammatory events. It also inhibits NF-κB, a critical transcription factor involved in synthesis of mediators and proteins promoting immune response. This causes a reduction in the capacity of the immune system to mount a response[174]. The cell-mediated immunity is suppressed by inhibiting genes coding for cytokines, most importantly IL-2, causing small cytokine production and reduced T cell proliferation[174]. Glucocorticoids also cause glucocorticoid-induced apoptosis. Even though peripheral T cells are affected, the more prominent effect is on immature T cells still inside the thymus. The exact regulating mechanism lies in the Bcl-2 gene. Reduced expression of IL-2 and IL-2 receptors diminish B cell clone expansion and antibody synthesis, and as a consequence fewer T lymphocyte cells are activated. Though glucocorticoids reduce the expression of inflammatory cytokines and hence meliorate the environment for islet transplantation they also impose detrimental effects of insulin inhibition and reduces insulin secretion[71 175 176].

### 4.4.4 Antibodies

**Antithymocyte globulins (ATG)** destroy lymphocytes within the thymus and spleen and are used both as induction immunosuppression and treatment of acute rejection in solid organ and islet transplantation. **Muromonab-CD3 (OKT3)** was the first monoclonal antibody used. It acts by

blocking the function of CD3 in the membrane of T cell resulting in T lymphocyte activation and cytokine release, followed by blocking of T cell functions. **Daclizumab (Zenapax)** and **basiliximab (Simulect)** are monoclonal antibodies directed against the low-affinity IL-2 receptor α-chain, anti-CD25 antibodies. **Alemtuzumab (Campath-1)** is a monoclonal antibody binding the protein CD52 on the surface of mature lymphocytes causing these lymphocytes to be targeted for distruction[122]. **Belatacept (Nulojix)**, selectively inhibits co-stimulatory ligands CD80/CD86 of antigen-presenting cell surface to inhibit their interaction with the CD28 T-cell receptor needed to activate T cells[177]. **Efalizumab (Raptiva)** binds to human CD11 inhibiting anti-leukocyte functional antigen-1, causing inhibition of leukocyte adhesion to other cells and ultimately effecting T cell migration and activation. Efalizumab showed very promising results but due to concerns about the development of progressive multifocal myeloencephalopathy the drug was withdrawn in 2009[126 140 178].

Excess amounts of pro-inflammatory cytokines, particularly IL-1β, can compromise islet function and lead to beta cell death. **Anakinra (Kineret)**, is a recombinant human IL-1 receptor antagonist which has shown improved glycaemia in diabetic patients[179 180]. The humanized IL-6 receptor-specific monoclonal antibody, **Tocilizumab (RoActemra),** was originally designed as a therapy for the inflammatory disease, rheumatoid arthritis (RA), but the drug also decreased glycosylated haemoglobin (HbA1c) in diabetic patients with RA[181]. Tumour necrosis factor-α (TNF-α) is an important component of the inflammatory cascade know to be toxic to islets[182]. **Etanercept (Enbrel)** is a recombinant human soluble TNF-α receptor fusion protein that competes for TNF-α binding and renders it inactive[183]. Experiences with use of Etanercept has shown promising results in islet transplantation [118 137 184].

## 4.5 Drug metabolisms and transporter

Islets are particularly vulnerable to toxicity in the intraportal environment after transplantation, as they are exposed to high drug concentrations[185 186] and already have a reduced basement membrane due to the islet isolation process[108 187]. Variability in drug response between subjects and within a

person over time are two problems that need further investigation in order to understand the total effect of drugs.

To understand and accurately predict drug interactions as well as grasping the true meaning of variability in drug response between subjects, it is important to know the drug efficacy and toxicity. In later years intracellular drug uptake and its place in drug therapy has become a hot topic. To quantify intracellular drug concentration directly is difficult. The time-relationship between the administered drug dose and the doses found at different sites in the body including the effective drug concentration at the target site is a definition of pharmacokinetics. Drug effect depends on drugs reaching its site of action. Under the hypotheses that unbound drug on either side of a membrane are in thermodynamic equilibrium the measure of blood and plasma drug concentrations are our only means of getting information about drug uptake today [188]. Knowledge of drug concentration where it elicits its effects would be much more informative but unfortunately it is not possible in clinical work today. Immunosuppressive drugs are mostly entering the cells through passive diffusion over the cell membranes due to their non-ionized forms but also by active transport.

### 4.5.1 CYP

To facilitate excretion most drugs are first chemically altered to more hydrophilic compounds. The most important drug-metabolising enzyme family is Cytochrome P450, CYP. The highest abundance of CYP is found in the liver and the small intestine[189]. Drug metabolism carried out by enzymes in these two organs is especially important to pharmacokinetic interactions. Based on amino acids, CYP divides into families and subfamilies. The CYP3A enzymes are quantitatively the most abundant enzyme in the liver and intestines[190]. This subfamily plays an important role in the metabolism of xenobiotics, is highly inducible and can be inhibited by numerous drugs[191]. Often CYP3A is considered the most important human drug-metabolising enzyme because it involves the biotransformation of approximately 50% of therapeutic drugs presently on the market[192]. The association between islets and CYP3A has not yet been investigated thoroughly but its presence in

pancreas of both human and other species has been confirmed[193]. Also it is responsible for biotransformation of tacrolimus and sirolimus in humans[194] and for this we found it natural to include CYP3A in our investigations.

### 4.5.2 Drug transporters

Drug transporters are membrane proteins that facilitate transport of compounds into and out of cells. They control the uptake and efflux of crucial compounds such as amino acids, sugars and drugs and are divided based on passive or active mechanisms of function[195]. Passive or facilitated transporters allow diffusion of solutes across membranes down their electrochemical gradient whereas active transporters utilize energy coupling to create solute gradients across membranes. Two transporter superfamilies are essential; SLC[196] and ATP-binding cassette (ABC) transporters[197]. It include the organic cation transporters (OCTs/SLC22A), the multidrug and toxin extrusion transporters (MATE transporters/SLC47A), the organic anion transporters (OATs/SLC22A), the organic anion transporting polypeptides (OATPs/SLCO), P-glycoprotein (MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2) and transporters of the multidrug resistance-associated protein (MRP/ABCC) family. All present in different cell types and at different aspects of the cell membrane as demonstrated in Figure 7[198].

Depending on their coupling to cellular energy active transporters are classified as primary and include members of the ABC (ATP-binding cassette) transporter family and ion pumps (ATPases) or secondary which are ion-coupled and use ion gradients when transporting nutrient across biological membranes. The wide distribution of transporters threatens the hypotheses that all drugs are distributed by passive diffusion across cell membranes. Since membrane transporters effect elimination, distribution and drug bioavailability it seemed an important consideration when evaluating immunosuppressive drugs impact on human islets.

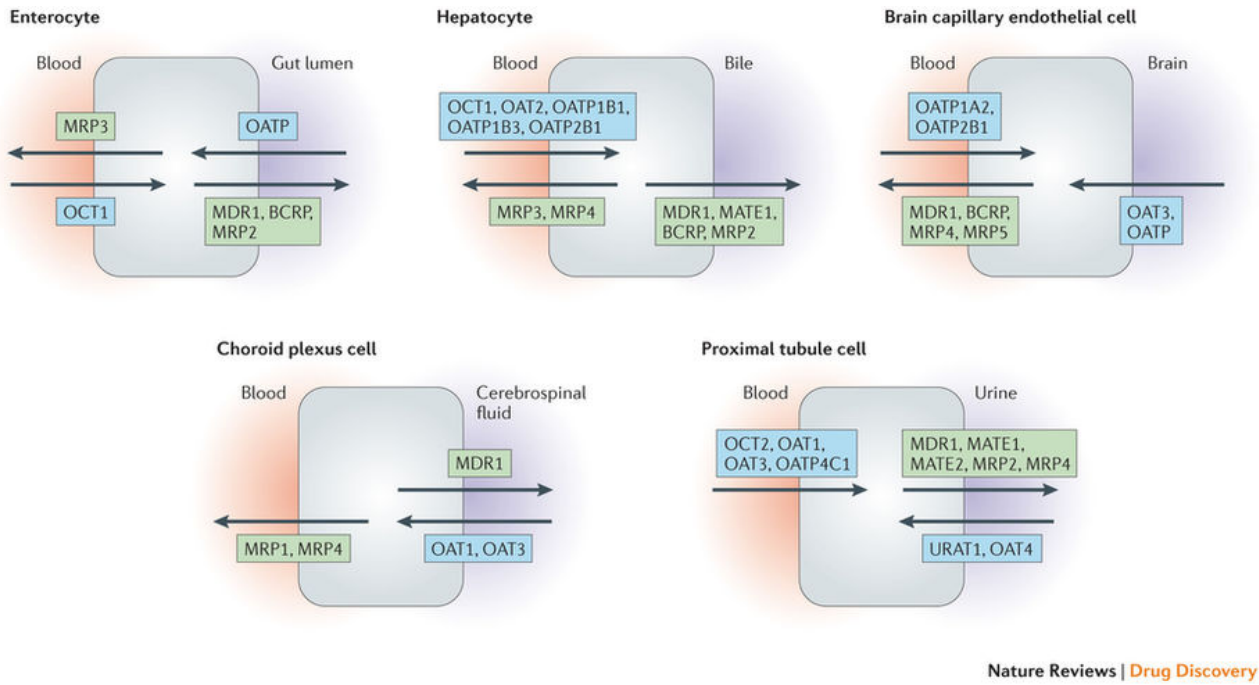


**Figure 7 Members of the solute carrier (SLC) and ATP-binding cassette (ABC) transporter families have been found in a variety of barrier epithelial cells (and other cell types), where they regulate the movement of small-molecule xenobiotics (such as drugs and toxins) and endogenous metabolites into and out of the various tissues and fluid compartments. Selected members of the SLC family (namely, organic anion transporters (OATs), organic anion-transporting polypeptides (OATPs), organic cation transporters (OCTs), organic cation/carnitine transporters (OCTNs) and multidrug and toxin extrusion proteins (MATEs)). Many other SLC and ABC drug transporters and their relatives are also expressed in these tissues but are not shown here. Reprint with permission from Nature Reviews Drug Discovery[198].**

### 4.5.2.1 ABCB1/ Pgp

ABCB1/P-glycoprotein (Pgp)/ multidrug resistance protein 1 (MRD1) is the most studied member of the ABC transporter family and is considered the most important efflux transporter. It is expressed in high amounts in epithelial cells and has been found to be present in cells of several normal human organs and tissues such as liver, exocrine pancreas and endothelial cells of capillary blood vessels. P-gp has been found around human islets and epithelial tissue of pancreas[199] and a mini-pgp has even been found to regulate phasic insulin secretion in rat islets[200]. Another member of the ABC transporter family, ABCA1 has been shown to regulate exocytosis of insulin granules in islets[201]. Its role in immunosuppressive therapy is still to be investigated.

### 4.5.2.2 OATP1B

Uptake transporters mostly belong to the superfamily of solute carriers (SLC). Among the most investigated influx transporters are the organic anion-transporting polypeptides (OATPs). OATPs are expressed in tissues such as the liver, kidneys, intestinal wall and the blood-brain barrier, OATPs translocate substrate through a rocker-switch type of mechanism [202]. Transport mediated OATP is independent of sodium, chloride and potassium gradients membrane potential and ATP levels[203]. OATP1B1 and OATP1B3, are highly expressed in hepatocytes and mediate cellular influx of various exogenous and endogenous substrates[203]. OATP1B1is mainly present in sinusoidal membrane of human hepatocytes where they mediated uptake of substrates from blood to liver. CyA inhibit OATP1B, OATP2B1 and OATP1B3 mediated uptake[204]. And as a curiosity reduction of OATP1B1 activity last for at least 18 hours after removal of CyA[205].

## 4.6 Future strategies to control post transplantation immune responses

Due to their immunosuppressive properties use of hemopoietic stem cells (CD34+ cells) in islet transplantation has been proposed for the potential of inducing chimerism. Following transplanting donor-derived haematopoietic stem cells in the bone marrow, donor-descendant cells can migrate to the thymus and promote central tolerance to donor-derived antigens (Figure 8a). The level of chimerism considered sufficient to protect a graft differs[206], but in islet transplanted NOD mice a mixed chimerism of > 30% was needed to induce tolerance[207]. Only aggressive induction treatment will allow for that kind of bone marrow engraftment, and it is therefore not an option in islet transplantation. In a NOD mice model high level of chimerism without myeloblative regimen and long term functioning islets was reached through protecting the transplanted bone marrow cells from rejections using T cell depleting agents and costimulation blockade molecules[208].

Hematopoietic stem cells have potent immunosuppressive properties and can in theory protect islets against cytotoxic T cells[209 210]. Inducing tolerance represent an interesting tool for islet transplantation but it remains to be seen whether this is feasible. **ATG** is already part of the

standard immunosuppressive treatment but a new mechanism of action in which ATG has shown ability to expand antigen-specific regulatory T-cells. Combined with **CTLA4-Ig** it can prevent allo and autoimmune activation of T cells, which makes it a very strong immunosuppressive combination[211] (Figure 8 (h)). **Co-stimulatory pathways** to activate naïve T cells are under development. Antigen presenting cells (APCs) are required to activate naïve T cells into effector T cells (T effs), MHC-peptide complex presentation without costimulating signals results in apoptosis of T effs or in generation of regulatory T cells (T-regs). The most important pathways are B7.1/2-CD 28 and CD40-CD40L, and short-term blockage of these cause transplantation tolerance. Unfortunately long-term engraftment of islets has not been found. Other co-stimulator molecules that have been tested showed no results in tolerance (ICOS and OX40)[212] or played a role in auto and alloimmune response (PD-1 and PDL-1)[213]. **T-regs** are essential to prevent activation of auto reactive cells and maintain homeostasis in the immune system. CD4+CD25+Foxp3+ and CD4+CD25-IL10+ T cells both have regulatory characteristics[214]. In combination with rapamycin and IL-10 a selective expansion of T-regs was seen both in vitro and in models of diabetes or transplantation[215], so far it has only been confined to a non-autoimmune setting. CD49b and LAG-3 in coexpression can identify type 1 T-regs[216]. **Dendritic cells (DC)** play a major part in rejection through activation of naïve T cells. They have ability to prime immune response through direct and indirect presentation of alloantigen. Targeting donor DCs still needs more clinical studies but prolonged islet survival where seen when donor DCs in islet transplantation where targeted[217]. **Alemtuzumab** (Campath-1) is a well-known monoclonal antibody to CD52 used in organ transplantation. It is used as an induction treatment at the time of transplantation to provide a steroid and CNI free maintenance immunosuppression[122]. It also has a limited use in treating steroid resistant rejection. Compared to other induction treatments Alemtuzumab improves islet engraftment and the rate of insulin independence in conjugation with high dose TAC and MMF. Adverse effects are profound and long lasting T cell lymphopenia but without increase in infections or post transplantation lymphoprolifeative disorders[218]. Long-term islet graft tolerance is yet

unknown. **Anti -CD3 specific antibody** deplete peripheral T cells and expand T-regs compartment, inducing T effs. apoptosis[219]. This is an explanation for long-term effect on transplantation tolerance. Deplete T-effs. and drive remaining T cells to a Th2 response. Not reported long term engraftment in allogenic islets. OKT3 (HuOKT3γ1 Ala-Ala) is a new version that has promising results and does not promote cytokine release[220]. It induces tolerance in non-autoimmune models, reverses autoimmunity and slows down progression of diabetes in humans with recent onset diabetes. Unfortunately very strong adverse effects have been reported. **Anti TNF-α** is a know part of islet transplantation treatment[84]. TNF-α is detrimental to islets. Entanercept is soluble receptor and Infliximab is a blocking antibody of TNF-α. They have both been tested with standard immunosuppression and Infliximab shows no positive impact on the islet graft compared to control[221]. **IL-8/CXCR1-CXCR2** is involved in post transplantation inflammatory events and improves glycaemic control, decrease insulin requirements and enhance the c-peptide levels. Targeting this pathway has been a great success. IL-8 induces migration of T cells and NK cells to sites of inflammation through activation of CXCR1 and CXCR1. Blocking the IL-8 involvement in post-transplantation inflammation has also been shown to improve islet survival[123], block insulitis and might even be successful in preserving residual β-cells in patients with new-onset T1D[222]. Studies combine Reparixin and CTLA-4Ig show no difference in islet survival or insulin secretion between the group using the drug combination and the one using only CTLA-4Ig[123 125].However one study found that by inhibiting CXCR1/2 chemokine receptors could prevent and reverse diabetes in mice[222]. **Encapsulation** of islets (Fig. 8 g) is a strategy that potentially can free the patient of immunosuppressive drugs without risk of losing the islet graft. The primary idea behind this is to avoid antigen recognition and protect against immune response. It consists of a polymeric device with a semipermeable membrane. The semipermeable membrane allows a selective permeation of small molecules while isolating the islets from larger cells, antibodies and immune cells from the host. It inhibits humoral and T-cell mediated immunity from damaging the islet graft. Membranes can be divided into 3 systems, a perfusion intravascular chamber, diffusion chamber

and a small glomerular membrane. The success of encapsulated islets is limited by the ability to get a good biocompatible membrane, optimal oxygen diffusion and sufficient immunoprotection.

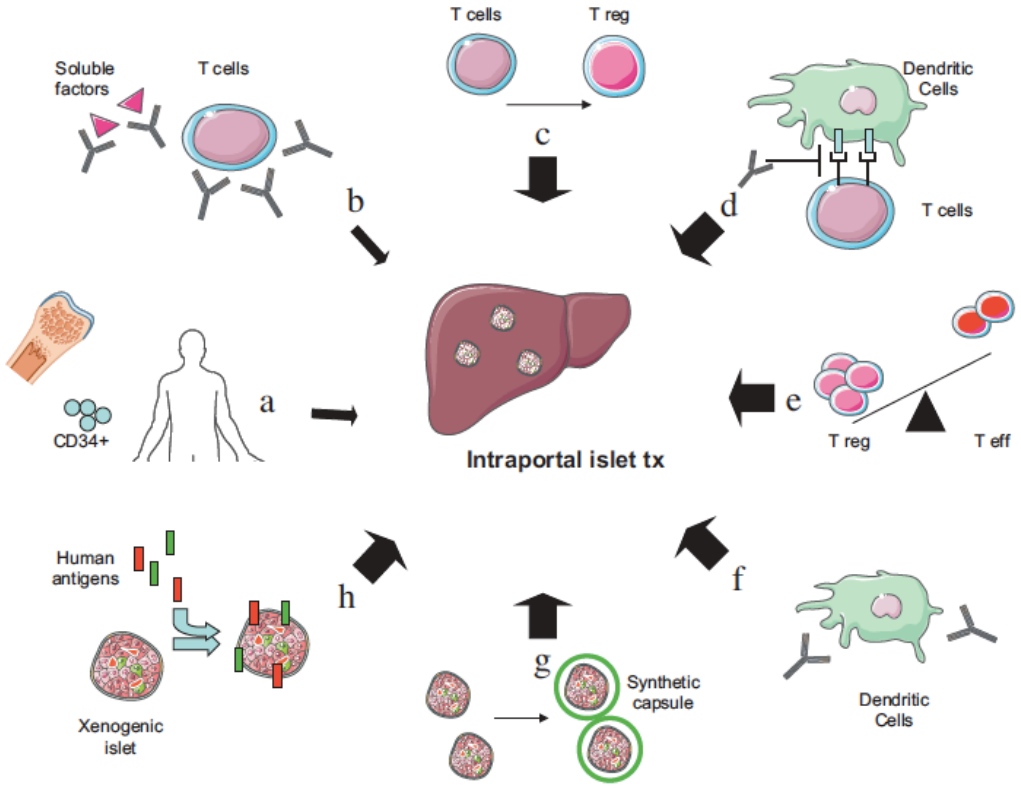


**Figure 8. Different immunological strategies to control immune responses after islet transplantation. (a) Donor CD34+ cells and islet infusion. (b) New monoclonal antibodies to target specific donor T cells or soluble factors. (c) T-regs induction. (d) Targeting of co-stimulation pathways to inhibit dendritic cells–T cells interaction. (e) Tipping the balance between regulatory and effector cells. (f) Targeting of donor dendritic cells. (g) Synthetic encapsulation of the islet. (h) Generation of humanized animals with introduction of strategic immune relevant antigens of islet cells. Abbreviations: Regulatory T cells (T-regs). Printed with permission from Pharmacological Research and Copyright Clearance Center[223].**

# 5 Aims of the thesis

Islet transplantation is a safe and efficient treatment alternative to restore glycaemic control and to avoid sever hypoglycaemia in type 1 diabetic patients. In the past decade improvements in both the procurement of the donor organ, the islet isolation process, and the immunosuppressive protocols have enhanced the patient outcome resulting in transition from an experimental procedure to a clinical reality. However, islet transplantation is still hampered by a significant and progressive loss of islet function over time. Efforts to reduce this loss have generated focuses in several directions such as i) pre-treatment of the islets with bioactive substances to reduce apoptosis and inflammatory responses, and ii) optimising the immunosuppressive drug regimen to improve the overall outcome of islet transplantation. Lifelong uses of maintenance immunosuppressive drugs are on one hand mandatory for transplant survival but on the other hand their toxicity influence the recuperation of islets after transplantation. The combination of drugs and drug doses used for islet transplantation has changed over the years to include increased level of immunosuppressive at the time of islet administration and minimize the use of steroids and CNIs for maintenance. To measure the drug concentration at its intracellular target site is probably more relevant than whole blood concentrations for predicting drug effect in vivo. However, there is a lack of knowledge on how to accurately measure the intracellular concentration and more importantly how to interpret the results of intracellular concentrations.

The specific aims were as followed:

1. Anti-inflammatory mediators have been introduced as part of the treatment regimen for clinical islet transplantation. We wanted to elaborate how anti-inflammatory agents blocking IL-1β, anakinra and IL-6, tocilizumab improve the human islets function and survival using both an in vitro and an in vivo approach.

2. Investigate whether the combinations of Tacrolimus and Sirolimus have a different impact on islet function and viability compared to each of the drugs alone. Establish how glucocorticoids affect islets when it was included to the drug combination in vitro.
3. Investigate the intracellular concentration of tacrolimus and sirolimus in human islets both alone and in combination as well as explore the presence of some transporters that potentially might influence the pharmacokinetics of islets in vitro.

# 6 Methods

## 6.1 Ethics

All work involving human tissue was conducted according to the principles expressed in the Declaration of Helsinki and in the European Council's Convention on Human Rights and Biomedicine. Human pancreata (for clinical transplantation and for use in research) were obtained from brain-dead donors after verbal informed consent from relatives. Written consent is not sought, nor required according to the Health Authorities and Ethics Committees. The consent to donate was documented in the hospital record of the donor. The Regional Committee for Medical and Health Research Ethics Central in Norway approved the verbal consent procedure and the procedure of human islets and use of the tissue for research. We had free access to donor characteristics, i.e. age, gender, height, weight, blood type etc., while donor anonymity was maintained. All animal experiments were approved by the Norwegian National Animal Research Authority project license no FOTS ID 2164/09 (paper 1). The animal experiments were performed in accordance with the European Directive 2010/63/EU and The Guide for the Care and Use of Laboratory Animals, 8th edition (NRC 2011, National Academic Press).

## 6.2 Human islet isolation and culture

Pancreata from brain dead, heart-beating donors were sent to the Central Laboratory of the Nordic Network for Clinical Islet Transplantation in Uppsala, Sweden for isolation. The organs were obtained under standard organ procurement procedure[224]. Centres in the Nordic Network for Clinical Islet Transplantation include the University Hospital in Gothenburg, Malmö, Stockholm, and Uppsala in Sweden, Helsinki University in Finland, Oslo University Hospital in Norway, and Rikshospitalet Copenhagen in Denmark. Islets were isolated according to the automated Ricordi method, refined by the Nordic Network for Islet Transplantation [225] after appropriate consent for multiorgan donation and to be used in research. Quality testing using dynamic insulin secretion was performed on hand-picked islets, only using islets preparations with insulin stimulation index >2

and purity of >50% based upon Dithizone staining in the experiments. The islet preparations were qualitatively approved for human transplantation, however, the low quantity of islets made these preparations available for research. Islets were maintained free floating in cell culture media CMRL 1066 supplemented with 10 % ABO-compatible serum, 2 mM L-glutamine, 50 μg/mL gentamicin, 5 mM sodium pyruvate, 20 μg/mL ciprofloxacin and 10 mM HEPES in a humidified 5% $CO_2$ atmosphere at 37 °C overnight. Thereafter the islets were kept at 25 °C changing the media at day 1, and every second day thereafter until shipment to Oslo. At arrival in Oslo (within 5 days after isolation), the islets were seeded in Petri dishes in supplemented CMRL 1066 media containing 10% ABO-compatible human serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin and kept in an standard incubator at 37 °C (5 % $CO_2$) until the experiments were performed.

## 6.3 Glucose-stimulated static insulin secretion assay

Assessment of islet functional potency was performed by static glucose-stimulated insulin secretion (GISIS). Following treatment, groups of 20 handpicked islets were transferred into 12 Transwell trays and pre-incubated in Krebs–Ringer bicarbonate buffer containing 1.67 mM glucose at 37°C (5% $CO_2$) for 30 min before the islets were incubated for 40 min in fresh Krebs–Ringer bicarbonate buffer containing 1.67 mM glucose (basal insulin secretion). Finally, the islets were incubated for 40 min in fresh Krebs–Ringer bicarbonate buffer containing 20.0 mM glucose (stimulated insulin secretion). The supernatants were subsequently collected and immediately ice-chilled, stored at -70°C until analysed. Stimulatory index was determined as ratio of insulin secretion measured using a commercial insulin enzyme immunoassay (EIA) at 1.67 mM to 20.0 mM glucose/40 min.

## 6.4 Oxygen consumption assay

To measure islet survival we used oxygen consumption rate (OCR) in paper 3. OCR is a real-time, operator-independent method of assessing fractional cell viability[226]. It was determined by using the Seahorse extracellular flux analyzer XF24 [227]. Samples of up to 80 treated human islets were plated

into each wells of the manufactory islet plate preloaded with 400 mL unbuffered assay medium containing 3 mM glucose, 0.8 mM $Mg^{2+}$, 1.8 mM $Ca^{2+}$, 143 mM NaCl, 5.4 mM KCl, 0.91 mM NaH2PO4, phenol red 15 mg/mL for 1h at 37 ˚C in air. Four wells were kept empty as controls in every experiment. Screens were carefully put on top of the depression of all wells with tweezers, and the plate was pictured in order to normalize for the possible different islet numbers in each well. The OCR was measured at basal glucose levels (3 mM) as well as with high glucose (20 mM) and results expressed as percentage of baseline.

## 6.5 Luminex® Multiplex Assays

The multiplex assay enables simultaneous analysis of secreted proteins, this reduce time, and costs over traditional methods such as EIA and western blotting. We analysed islet secreted mediators in cell free supernatants or in islets lysate for selected cytokines and chemokines (paper 1-3) and phosphorylated proteins (paper 1 and 3) by multiplex-based assays pre made by Bio-Rad Laboratories, Hercules, CA following the manufacturer's instructions. Each sample was correlated to the protein content of the islet pellet measured by bicinchoninic acid assay.

## 6.6 Exposure to immunosuppressive drugs

Within 5 days of isolation, islet preparations were placed in cell culture media with a drug combination. In paper 2 islets were exposed to tacrolimus, sirolimus or combination thereof for 24 hours at 37°C (5 % $CO_2$) and to the combination of tacrolimus and sirolimus with or without methylprednisolone was also explored. In paper 3 islets were exposed to tacrolimus, sirolimus, or combination thereof for 24 and 48hours. To verify the unique findings in intracellular concentration islets was also exposed to a combination with another CNI, cyclosporine A and sirolimus. All drugs were dissolved in methanol and diluted in cell culture medium to reach their final concentrations. Control islets were cultured and added the same volume of methanol (<0.001% of total volume) as the 30 μg/L condition but in the absence of drugs. The drug concentrations were selected based on the target trough level for each drug used in clinical practice. Since portal vein peak concentrations

of immunosuppressant drugs generally reach four times that of systemic levels after oral administration we used high doses of drugs [228].

## 6.7 Quantification of intracellular immunosuppressant drug levels in islets

After incubation, approximately 1000 human islets were handpicked into columns, washed two times with ice-cold phosphate buffered saline, lysed in 150µL water and homogenized by sonication. A 25µL aliquot of the homogenate was transferred to a 1.5 mL microcentrifuge tube and 4 volumes of methanol containing ascomycin and $D_4$-everolimus reagent conc. 3.75 µg/L and $D_{12}$-cyclosporine A reagent. The tube was vortexed and centrifuged, and 50µL supernatant was mixed with 30µL water in a liquid chromatography injection vial. Drug quantification was performed with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using an Alliance HT 2795 HPLC-coupled to a Micromass Quattro micro API MS/MS-instrument with electrospray ionization. A Kinetex $C_{18}$ column (2.6 µm, 50 × 2.1 mm) was used for separation at 50 °C with a SecurityGuard ULTRA $C_{18}$ cartridge (2.1 mm) in front. Mobile phase A consisted of 35 % methanol in water, and mobile phase B was 100 % methanol. Both mobile phases contained 2.0 mM ammonium acetate and 0.03 % (v/v) formic acid. The liquid chromatography gradient was as follows: 100 % A until 5.5 min, then B was linearly increased to 40 % between 5.5 min and 9.0 min, thereafter 92 % B was pumped until 12.0 min, and the column was re-equilibrated with 100 % A until 16.0 min. The flow rate was 0.300 mL/min and the injection volume was 20µL. Precursor ammonium adducts were fragmented and monitored for tacrolimus (m/z 821 to 768), sirolimus (m/z 931 to 864) and cyclosporine A (m/z 1219.5 to 1202.5), and for their internal standards ascomycin (m/z 809 to 756), $D_4$-everolimus (m/z 979 to 912) and $D_{12}$-cyclosporine (m/z 1231.5 to 1214.5), respectively. Calibrators (tacrolimus, sirolimus and cyclosporine A) were processed in parallel with the samples and Waters MassLynx software was used to calculate the analyte concentrations based on linear responses of peak area ratios between analyte and internal standard signals.

## 6.8 RNA isolation and gene expression analysis

Total RNA was isolated from frozen handpicked human islets pellets using RNEasy spin columns. Total RNA was evaluated with a spectrophotometer and the concentration of all RNA samples was quantified using a NanoDrop ND-1000 UV/Vis spectrophotometer before reverse-transcribed into complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit. The cDNA-synthesis was followed by amplification of the target genes and selected reference genes such as aminolevulinate delta-synthase 1 (*ALAS1*), beta-2-microglobulin (*B2M*), ribosomal protein L13a (*RPL13A*), beta actin, and GAPDH using the standardized real-time quantitative polymerase chain reaction (RT-qPCR). In paper 3 all the RT-qPCR runs included analysis of cDNA from a liver biopsy as a positive control and calibration point between runs. We used the $2^{-\Delta\Delta Ct}$ method (paper 3) or the calibration curve of known standard method for quantification of results (paper 3).

Quantification of mRNA expression was performed using the following TaqMan assays: human IL-1$\beta$: Hs00174097m1 and IL-8: Hs00174103m1 with an ABI 7900HT Fast Real-Time PCR System. Results were normalized to the housekeeping gene beta-actin and data were analysed using the $2-\Delta\Delta Ct$ method (paper 2).

## 6.9 Immunofluorescence

Sixty to eighty human islets were dissociated into single cells with TripLE express, washed in PBS, and spun to microscope slides by centrifugation at 800 rpm for 8 min. The cytospin slides were fixed and permeabilized by 4% paraformaldehyde and 0.3% Triton X-100 in PBS, respectively. Double immunofluorescence staining was performed for insulin and ABCB1(Pgp) or glucagon and ABCB1(Pgp). Section was incubated with polyclonal guinea pig anti-insulin 1:500, polyclonal rabbit antiglucagon 1:50, monoclonal mouse anti-Mdr1(PgP) 1:25 overnight at 4 °C in humidified chamber. After washing with 1x Tris-buffered saline with tween (TBST), slides were incubated with Alexa-Fluor 488 Goat-Anti-Guinea pig 1:300 in combination with

AlexaFluor 488 Donkey-Anti-Rabbit 1:300, or AlexaFluor 594 Donkey-Anti-Mouse 1:300 for 1 h at room temperature and thereafter mounted with Slow Fade Gold Antifade Reagent with DAPI for nuclear staining. Images were taken by the Axio Observer Inverted Microscope with ZEN lite software.

## 6.10 In vivo islet transplantation

Diabetes was induced in male SCID mice by a single intravenous injection of 200 mg/kg streptozotocin. All mice that were used in the experiments had blood glucose levels above 20 mM two consecutive days before the islet transplantation. The mice were anesthetized by 1.5% isoflurane mixed with oxygen and a treated or not minimal human islets graft (600 IEQ) was loaded into a PE 50 tubing and then transplanted under the left kidney capsule[229]. We chose the subcapsular islet transplantation in murine models since this has shown to be significantly more efficient compared to intraportal transplantation and required fewer islets [230]. Monitoring blood glucose levels daily for the first 7 days, then twice weekly post transplantation assessed graft function. 21 days after transplantation intraperitoneal glucose tolerance tests were performed after a minimum 6-h fast. Islet graft function was proven by removal of the graft-bearing kidney to confirm the recurrence of diabetes.

## 6.11 Statistical analysis

Data are presented as means ± SEM (Paper 1 &2), and as mean ± SD (Paper 3). Comparison of results across groups was performed with Kruskal-Wallis one-way analysis of variance (ANOVA) (Paper 1-3). Bonferroni correction was used in paper 1. When significant differences were found, comparisons between groups were performed with two tailed unpaired Student's t-tests (Paper 2 &3). For data analysis the statistical software GraphPadPrism 4.0 (paper 1) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used (paper 2 & 3). Differences were considered significant at levels of $p < 0.05$.

# 7 Summary of results

**Paper 1:**

**Anakinra and tocilizumab enhance survival and function of human islets during culture: implications for clinical islet transplantation.**

In an attempt to improve the inflammatory reactions known to occur in relationship to islet transplantation, islets are being pre-treated with anti-inflammatory drugs. We used two known anti-inflammatory agents Anakinra (IL-1 receptor antagonist) and tocilizumab (monoclonal IL-6 receptor antibody) to evaluate whether islets pre-treated with these drugs could reduce the inflammatory reaction seen in pre-transplantable human islets. Both drugs are used in treatment of inflammatory conditions and also have positive effects on blood glucose levels and glycosylated haemoglobin in diabetic patients.

Islets were pre-cultured with anakinra or tocilizumab for 48 hours. We then evaluated viability and apoptosis of the islets in an in vitro model. Pre-treated islets wore also transplanted in a marginal mass model using human islets in immunodeficient mice. None of the drugs were toxic to human islets and the inflammation markers were significantly reduced. When islets where stimulated by proinflammatory cytokines, Anakinra significantly reduced the quantity in inflammatory cytokines and hence protected the islets. In vivo, pre-treated islets had a significantly improved engraftment compared to the vehicle.

In conclusion, we found that pre-treating islets with anakinra and tacolizumab prior to human islet transplantation had a positive effect on islet survival and function.

**Paper 2:**

**Combined treatment with tacrolimus and sirolimus reveals no additional adverse effects on human islets in vitro compared to each drug alone.**

A potent immunosuppressive protocol in islet transplantation is currently needed to avoid graft rejection, but they are proven detrimental to human islets. To what extent combination of immunosuppressive agents equals the toxic effect of each drug is not clear.

We dosed human islets with the immunosuppressive agents, tacrolimus and sirolimus separately and in combination in order to evaluate its influence on islet function, viability and inflammatory response. Also, we compared the drug combination with and without methylprednisolone to evaluate its ability to reduce inflammation. Human islets were treated with tacrolimus, and/or sirolimus for 24 hours. Methylprednisolone was added to the drug combination in parallel experiments. We found that islets dosed with combined tacrolimus and sirolimus had decreased viability and glucose-stimulated insulin secretion at the same magnitude as islets dosed with each drug separately. When methylprednisolone was added to the drug combination islet survival was improved and the expressions of inflammatory cytokines was reduced compare to the regimens without methylprednisolone. Insulin secretion was as expected reduced when methylprednisolone was added.

In conclusion, reduced islet function and viability after treatment with the drug combination did not exceed that we found in islets dosed with a single drug. Exposure to methylprednisolone significantly decreased the inflammatory response. We suggest a possible benefit of using a tapering steroid therapy in the initial post-transplantation week.

**Paper 3:**

**Intracellular sirolimus concentration decrease in combination with tacrolimus exposure of human islets in vitro.**

Knowledge of intracellular concentration of immunosuppressive drugs and membrane drug transporters can help predict the drugs impact on β-cells and understand the mechanisms of action when islets are exposed to regimens of immunosuppressive drugs.

We exposed human islets to therapeutic or toxic doses of tacrolimus, sirolimus or tacrolimus and sirolimus for 24 and 48hours. A toxic dose of CYA and sirolimus + CYA at 24hours was also investigated. Intracellular concentrations of drugs were evaluated, using a quantification technique established in our lab. The membrane drug influx and efflux transporters SLCO1B1 and ABC1B, as well as the enzyme CYP3A4 were quantified. We found that independent of dose and exposure time islets incubated with sirolimus and tacrolimus contained a reduced intracellular concentration of sirolimus compared to islets incubated with sirolimus alone. This reduction was not observed in sirolimus and CYA. When intracellular sirolimus concentration was decreased, p70s6k phosphorylation was increased which suggest preservation of the mTOR-signaling pathway. Expression of ABC1B, CYP3A4 and SLCO1B1 mRNA was found in human islets and presence of ABC1B protein was verified with immune staining.

In conclusion islets exposed to sirolimus + tacrolimus show a considerable reduction in intracellular concentration of sirolimus which may limit its toxicity to islets. SLCO1B1, ABC1B and CYP3A4 were all detected in human islets; but not involved in the interaction between sirolimus and tacrolimus. Intracellular drug concentrations in human islets have never previously been described and understanding its full potential will need further investigation. We believe that in the future this approach could help develop a better and more personalized immunosuppressive treatment regimen for patients undergo islet transplantation.

# 8 Discussion

Understanding how islets interact with immunosuppressive and anti-inflammatory drugs might help us finding the optimal treatment regimen for islet transplantation. Immunosuppressive drugs have an immense impact on transplanted islets compare to the effects on transplanted solid organs because islets are transplanted in cluster of cells (islets) directly into the circulation. Drug doses used in clinical transplant protocols as well as the selection of immunosuppressive drugs that are combined will influence islets in different ways. Whether the intracellular concentration of drugs and their transporters help in this process is yet to be clarified as we only are at the start of how this information will elucidate the overall impact immunosuppressive drugs have on cells.

Immunosuppressive drugs have been an important and essential part of success in transplantation, Azathioprin revolutionised organ transplantation in the 1960st making it possible for organs to survive in a donor environment. In islet transplantation changes in the immunosuppressive drug treatment made patients insulin free 1-year after transplantation [86]. The preferred transplantation site for islets still is the intraportal vein despite early post-transplantation complications and islet loss and is due to low oxygen tension, an active innate immune system, provocation of an inflammatory response (IBMIR) and the toxic use of immunosuppressive drugs. Several alternative transplantation sites have been evaluated[231 232] and there is a continuous search for a more optimal site[233-235]. Until such a site has been found we have to continue to do our best for the islets to survive in an intrahepatic environment. Focusing on what could reduce islet graft rejection shortly after transplantation and investigate the impact of the immunosuppressive drugs on islets could be a good strategy. We first evaluated two anti-inflammatory agents as pre-treatment of islets in paper 1 in order to improve islets survival and function before transplantation. Next, in paper 2 we investigate how direct treatment with tacrolimus and sirolimus influence human islets function and survival. Finally,

in paper 3 we investigated whether intracellular concentration changes the immunosuppressive drug composition. All of these strategies focus on making a more optimal environment for islet to survive. Investigating how immunosuppressive and anti-inflammatory drugs influence islets offering a potentially new way to think about a optimal drug composition.

## 8.1 Effects of anti-inflammatory suppression in human islets

As a response to the stressing nature of the isolation process and cell culture, human islets produce several cytokines[104 236]. IL-8 and MCP-1 are key factors in selectively recruiting monocytes and T-lymphocytes to sites of inflammation[237 238]. Increased levels of MCP-1 and IL-8 in human islets have been linked to impaired graft function[123]. Other inflammatory cytokines such as IL-1β, TNF-α and IFN-γ can also potentially damage β-cells[239].
In contrary to the Edmonton protocol where islets wore transplanted without latency, many centres nowadays culture islets prior to transplantation. The advantages are many, such as securing islet quality[240], decreasing immune effects by removal of impurities and fragmented islets [117 241]and making it possible to ship islets to remote sites for transplantation[242].

We explored the use of two anti-inflammatory drugs to reduce the production of cytokines in the pre-transplantation process. Anakinra is a recombinant, non-glycosylated human IL-1 receptor antagonist (IL-1Ra) and Tocilizumab a recombinant, humanized, anti-human interleukin 6 receptor (IL-6) monoclonal antibody. These drugs are already used in treatment of rheumatoid arthritis to reduce inflammatory processes[181 243]. In our experiment we found that Anakinra attenuates the inflammatory effects of the cytokines. This is well in coherence with previous finding that islet viability and function wore protected by Anakinra[244]. Others have shown similar effects when pre-treating islets with other pro-inflammatory cytokine such as sulfhydryl protease, Papain, cleaving human leukocyte antigen class I from human lymphocytes[245] and an GLP-1 receptor agonist, exenatide[246].

We showed that both Tocilizumab and Anakinra reduced the inflammatory processes in post-isolated islets. Islets viability was not affected by 48 hours incubation with Tocilizumab or Anakinra compared to none treated islets. This we interpreted as an indication that islet survival is not directly affected by the presence of these two drugs. However handpicking viable cells might have caused them to be more resistant to drug exposure compared to normal cell culture incubation. Islet function is an important indicator of islet survival and no negative effect on function potency was observed with the use of the two drugs. This is consistent with others findings of IL-1Ra islet influence [244 247] whereas no other studies involving islets and IL-6 monoclonal antibody have been found. We also found that Anakinra and Tocilizumab prevent cell death, which we interpreted to be because of the increased levels of ATP we found compared to control. To further test the effect of the treated islets we used an in vivo marginal mass mouse model and found that islets treated with Tocilizumab or Anakinra faster achieved normoglycaemia compared to untreated islets. The transplanted mice had significantly lower non-fasting blood glucose levels and an improved islets engraftment when transplanted with islets treated with anti-inflammatory agents prior to transplantation compare to untreated islets. The drugs had no direct negative effect on islet survival and function but helped reduced the quantity of inflammatory cytokines. It has become more and more common to include at least Anakinra in induction treatment[117 118 247]. IL-6 is a two edged sword on one hand protecting islets from pro-inflammatory cytokines[248] and on the other hand might lead to the destruction of the pancreatic beta cells by induction of increased expression of inducible NO-synthase (iNOS) when combined with IL-1[249].The complex nature of IL-6 needs further studies to determine whether it has a place in treatment of islet transplantation.

## 8.2 Impact of common immunosuppressive agents on human islets

Focusing on establishing an optimal treatment alternative for islets during and immediately after transplantation has been a priority for quite some time. Early loss of transplanted islets is estimated to be 50-70 % [250 251]. Changing the induction therapy or switching immunosuppressive drugs after transplantation has so far given increased islet survival and 3-year insulin free patients in 44%[84].

There have been debates about whether tacrolimus and sirolimus interact or antagonize one another[252-254]. Despite showing an antagonistic effect of tacrolimus on sirolimus it has so far not been shown a disadvantage in using the two drugs in combination[255-257]. These drugs are part of the standard regiment for islet transplantation and though not optimal is seem to be one of the best alternatives we have at the moment. Some transplant centres have started to exchange sirolimus for MMF due to adverse events that become intolerable for the patients. And there have been clinical trials using CNI-free treatment[128].

Using glucocorticoids as part of immunosuppressive treatment was completely abandoned after the success of the Edmonton protocol[86]. In the latest CIT report however it was noted an increase in use of steroids as part of pre-treatment of recipients[84]. But it is still not generally believed that glucocorticoids help in the islet transplantation process. We have showed that the use of glucocorticoids in combination with tacrolimus and sirolimus does not decrease islet survival. However, we found that insulin secretion was reduced when glucocorticoids was added to the incubation of islets and immunosuppressive drugs. This is consistent with our previous findings where in vitro islets incubated with glucocorticoids showed increased intracellular insulin concentration and that in vivo islets perform a better insulin secretion after glucocorticoids are removed[175]. Corresponding with the hypotheses that glucocorticoids inhibit insulin secretion not transcription[175 258].

## 8.3 Intracellular drug concentration and interaction

It has been said “To use the drug concentration in blood to determine drug impact on cells is like measuring the water in a fish tank to establish the amount of water the fish consumed”. For the time being serum concentration is the most accurate we have to go by but we know that it does not bring us exact information about the drug concentration and little is known of the impact on cells. In later years the mapping of intracellular protein transporters and their presence in cells have made huge leaps into a new era of how to understand cell uptake and drug use. The active components in most drugs are activated within the cell and it is likely that membrane transporters play an active part. We wanted to investigate the intracellular uptake of immunosuppressive drugs such as tacrolimus and sirolimus in islets and whether there was a difference in uptake when both drugs were used. Our results showed that intracellular levels of sirolimus decreased significantly when combined with tacrolimus. To further investigate whether this was true for all CNI or just tacrolimus we tested the combination of sirolimus and CYA but did not find similar results.

We related the difference in intracellular levels of drugs to the common receptor shared by tacrolimus and sirolimus, the ligand FK506-binding protein 12 (FKBP12). Both drugs bind to this receptor and initially it was shown to be antagonistic[253] and have a competitive binding but later it was discovered that the two drugs attach to different parts of FKBP12[259]. FKBP12 is the mechanical target of a serine/threonine protein kinase known as the mammalian target of rapamycin (mTOR). It has multiple functions including cell growth, proliferation, survival, protein synthesis, and angiogenesis[164]. mTOR is exerted through two different multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Both use mTOR as a catalytic subunit. Where mTORC1 is sensitive to rapamycin is mTORC2 on the other hand insensitive to rapamycin. P70S6K is a mitogen-activated serine/threonine protein kinase that is required for cell growth and G1 cell cycle progression. Multiple phosphorylation events control

this kinase and subsequently phosphorylates ribosomal protein S6. mTORC1 activation via p70S6K is implicated in the control of islet proliferation[260]. We used the phospho-p70S6K as a verification of the mTOR involvement in the pharmacodynamics of intracellular sirolimus. It demonstrates how when tacrolimus and sirolimus are combined, tacrolimus antagonized the inhibitory effects on phosphorylation of p70S6K caused by sirolimus[261]. Islets exposed to the combination sirolimus and CYA showed no similar effect, since CYA does not bind to the FKBP12 receptor but makes a complex with cyclophilin a complex that inhibits calsineurin protein phosphatase activity. This confirm the link to p70S6K activation[262].

The presence of ion channels in β-cells are well established[263] but there are few investigations into protein membrane transporters in human. The presence of OATP1B3 in pancreatic tissue[264 265] have been documented and its insulinotropic effect evaluated. We investigated the drug transporters OATP1B1 and ABCB1 and the metabolizing enzyme CYP3A4 expression in human islets. Both at mRNA and protein levels ABCB1 was expressed in islets, but we found no involvement in the interaction between tacrolimus and sirolimus. Even though we found low quantities of OATP1B1 and CYP3A4, compared to the presence in liver tissue we did find their presence in islets, which has not been presented previously.

# 9 Limitations

This study was mainly based on in vitro experiments and question of whether the conditions are applicable to in vivo conditions arises. The most obvious concern would be with the relative high drug dose used in our experiments. Secondly the fact that the drug concentration in vitro studies remains fairly stable throughout the experiment. Intrahepatic studies on dogs have shown how intrahepatic islets are exposed to doses 10x higher than the initial dose given during transplantation[186]. Thus, our use of high dose in the in vitro setting of experiments is probably reflecting the in vivo situation for intraportal islet transplantation. However, no one have yet measured the intracellular concentration after islets transplantation and compared it with the systemic concentration, a study that would be of great interest.

Variation in donors parameters will influence the islets used in in vitro research[12 266]. However, all islets used have been approved for transplantation to humans and is thus considered in quality good enough. Importantly, we always use the same islets as control in one experiment and perform experiments with several donors reducing the donor heterogeneity and influence of the isolation process itself.

# 10 Future Perspective

Over the past decades several remarkable discoveries to permanently cure diabetes mellitus has been presented. Pancreatic stem cells to rejuvenate functional β cells, reprogramming cells through induced pluripotent stem (iPS) cell technology to generate insulin-producing cells as well as xenotransplantation using pig islets. Despite this search for an unlimited source of islets to be able to treat a greater number of patients, human islet transplantation, the way it is done today will continue to be a very good treatment alternative for sever type 1 diabetic patients[267]. Most of the mentioned treatment options will continue to require immunosuppressive treatment

in order for islet to survive. Finding an optimal immunosuppressive regiment will therefor still be of essence. New immunosuppressive strategies consist of combinations of drugs, a pre-treatment of islets, induction treatments and maintenance treatment that separately reduce inflammation or immune reaction prior to, during transplantation as well as post ´transplantation. Investigations are needed on the long-term effect these strategies have on islets.

Chimerism is defined as “the presence in an individual of cells of different origin”. Bone marrow transplantation is an example of this. Donor bone marrow and host cells exist together without signs of graft-versus-host rejection disease. Chimerism would be the ultimate treatment goal for transplantation in general and islet transplantation in particular, as it would relive patients from immunosuppressive drug treatment, leaving them without side effects of drugs such as islet death and PTDM. It has been possible to achieve permanent chimerism in animals[268-270]. So fare in humans obtaining permanent chimerism has been more difficult but in the past year some promising results has been presented[271 272].

As long has islet transplant patients are treated with immunosuppressive drugs, their impact on islets will continue to be a challenge. Investigating the islets membrane will enlighten what transporters islets consist of, where transporters are situated and their role in intracellular communication.

Intracellular communication in islets has been shown to play a special part in insulin secretion, where not all insulin inside each β-cells are emptied out at one time[273]. It would be of interest to further look into the distribution of drugs within the components of an islets and register how the intracellular communication is affected by drugs.

# 11 Conclusions

**The main conclusions of the present work are:**

1. Using the IL-1β receptor antagonist, Anakinra is an effective way to reduce inflammatory mediators in islets by blocking IL-1 signalling, causing decreased pro-inflammatory mediators, increased secretory capacity of islets and prevention of cell death. Tocilizumab, an IL-6 inhibitor, also prevent cell death and abolish suppressed glucose stimulated insulin secretion however it cannot counteract the negative effects of pro-inflammatory cytokines. Due to its complex nature IL-6 needs further investigation prior to including it into treatment of human islet transplantation.

2. No difference in islet function and survival was found when islets where exposed to tacrolimus and sirolimus combined compared to each drug alone. Nor did the drug combination increase islets pro-inflammatory cytokine secretion. Methylprednisolone significantly reduced pro-inflammatory response caused by the combination of tacrolimus and sirolimus but it also reduces insulin secretion.

3. Intracellular islets concentration of sirolimus was significantly reduced when human islets were exposed to a combination of tacrolimus and sirolimus. The p70S6K-signaling pathway was involved. Human islets express the membrane transporter ABCB1 but we found no direct connection to the drug-drug interaction of tacrolimus and sirolimus. Membrane transporter, OATP1B1 and the metabolic enzyme CYP3A4 was found in low quantities in islets. Their involvements in pharmacokinetic processes in islets are still uncertain.

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