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The Impact of Pancreatic Islet Vascular Heterogeneity on Beta Cell Function and Disease

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Abstract
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Diabetes Mellitus is a group of complex and heterogeneous metabolic disorders characterized by hyperglycemia. Even though the condition has been extensively studied, its causes and complex pathologies are still not fully understood. The occurring damage to the pancreatic islets is strikingly heterogeneous. In type 1 diabetes, the insulin producing beta cells are all destroyed within some islets, and similarly in type 2 diabetes, some islets may be severely affected by amyloid. At the same time other islets, in the near vicinity of the ones that are affected by disease, may appear fully normal in both diseases. Little is known about this heterogeneity in susceptibility to disease between pancreatic islets. This thesis examines the physiological and pathophysiological characteristics of islet subpopulations.

Two subpopulations of islets were studied; one constituting highly vascularized islets with superior beta cell functionality, and one of low-oxygenated islets with low metabolic activity. The highly functional islets were found to be more susceptible to cellular stress both *in vitro* and *in vivo*, and developed more islet amyloid when metabolically challenged. Highly functional islets preferentially had a direct venous drainage, facilitating the distribution of islet hormones to the peripheral tissues. Further, these islets had an increased capacity for insulin secretion at low glucose levels, a response that was observed abolished in patients with recent onset type 1 diabetes. The second investigated islet subpopulation, low-oxygenated islets, was found to be an over time stable subpopulation of islets with low vascular density and beta cell proliferation.

In summary, two subpopulations of islets can be identified in the pancreas based on dissimilarities in vascular support and blood flow. These subpopulations appear to have different physiological functions of importance for the maintenance of glucose homeostasis. However, they also seem to differ in vulnerability, and a preferential death of the highly functional islets may accelerate the progression of both type 1 and type 2 diabetes.

Keywords: Pancreatic islets, heterogeneity, islet vascularity, blood flow, islet transplantation, islet amyloid, insulitis, type 1 diabetes, beta cell proliferation

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List of Papers

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

I **Ullsten, S.,** Lau, J., Carlsson, P-O. (2015) Vascular heterogeneity between native rat pancreatic islets is responsible for differences in survival and revascularisation post transplantation. *Diabetologia*, 58(1):132–9

II **Ullsten, S.,** Bohman, S., Oskarsson, ME., Nilsson, KPR., Westermark GT., Carlsson, P-O. (2017) Islet amyloid deposits preferentially in the highly functional and most blood-perfused islets. *Endocrine Connection,* 6(7):458-68

III **Ullsten, S.,** Espes, D., Quach, M., Fex, M., Sandberg, M., Carlsson, P-O. (2017) Heterogeneities in vascularity and function between pancreatic islets may explain dissimilar propensities for disease development in type 1 diabetes. *Manuscript*

IV **Ullsten, S.,** Lau, J., Carlsson, P-O. (2017) Decreased beta cell proliferation and vascular density in a subpopulation of low-oxygenated rat islets. *Manuscript*

Contents

Abbreviations

AU	Arbitrary units
BB	BioBreeding
BS-1	Bandeiraea simplicifolia agglutinin-1
CGRP	Calcitonin-gene-related peptide
CPE	Carboxypeptidase E
ER	Endoplasmic reticulum
DAB	3,3'-Diaminobenzidine
DP	Diabetes prone
DR	Diabetes resistant
Fltp	Flattop
gDNA	Genomic DNA
HCL	Hydrochloride
HFD	High-fat diet
hIAPP	Human islet amyloid polypeptide
IAPP	Islet amyloid polypeptide
IBMIR	Instant blood-mediated inflammatory reaction
IDE	Insulin degrading enzyme
IGF1R	Insulin-like growth factor 1 receptor
KRBH	Krebs–Ringer bicarbonate buffer
LADA	Latent autoimmune diabetes in the adult
MHC-1	Major histocompatibility complex 1
mIAPP	Mouse islet amyloid polypeptide
MMP	Matrix metalloproteinase
MODY	Maturity onset diabetes in the young
NADPH	Nicotinamide adenine dinucleotide phosphate
PC	Prohormone convertase
PI	Propidium iodide
PP	Pancreatic polypeptide
SEM	Standard error of the mean
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBT	TATA-box binding protein
TIMP	Tissue inhibitor of metalloproteinase
TPU	Tissue perfusion units
VEGF-A	Vascular endothelial growth factor A

Introduction

The pancreas

The human pancreas is an elongated organ of approximately 12-15 cm located posteriorly in the abdominal cavity. The gland is anatomically divided into three parts: the head (caput), body (corpus), and tail (cauda) with a total average weight of 80-100 grams [1]. The pancreas is mainly an exocrine organ composed of acinar cells responsible for producing digestive enzymes and bicarbonate, which is transported through the pancreatic ducts to the papilla of Vater where it, together with the bile, is secreted into the duodenum. A minor proportion of the pancreas, only 1-2%, constitutes the endocrine tissue, the islets of Langerhans.

Islets of Langerhans

The endocrine part of the pancreas consists of cell clusters, the islets of Langerhans. The islets of Langerhans are named after Paul Langerhans who, in his doctoral thesis in 1869, was the first to describe the organ. His description was, however, only morphological and the endocrine function of these cell clusters was first proposed by Edouard Laguesse in 1893 [2]. The islets, which are commonly described as micro-organs, are scattered throughout the exocrine pancreas, and are crucial for maintaining glucose homeostasis through their insulin production and secretion. There are at least five different endocrine cell types in the islets, interspersed with vascular cells and neural cells. In human islets, approximately 50-80% of the endocrine cells are insulin-producing beta cells. The second most common cell type is the glucagon-secreting alpha cell which constitutes 15-20% of the islets. Less frequent in the islets are the delta cells (5-10%) which secrete somatostatin, the pancreatic polypeptide (PP) cells secreting pancreatic polypeptide, and the epsilon cells secreting ghrelin; the latter two represent approximately 1% of the islets' cells each [3]. The secreted hormones are released into the islets vascular system, which drains into the portal vein for further hormone distribution to the rest of the body.

Pancreatic islet vasculature

Pancreatic islets are highly vascularized, and the vasculature takes up around 8-10% of the total islet volume [4]. The afferent microvasculature of the islets differs depending on islet size. Small islets are most commonly supplied with blood from one arteriole whereas intermediate to large islets may be supplied by up to three arterioles [5]. Inside the islet, the arterioles divide into capillaries shaped in a glomerular-like network with a specific phenotype differentiating intra-islet microvasculature greatly from the blood vessels found in the exocrine tissue. Islet capillaries are highly permeable and contain about 10 times more fenestrations than exocrine tissue capillaries, a phenotype believed to optimize hormone secretion to the circulation [6, 7].

The endothelial cells that build up the islet vasculature are uniquely adapted to facilitate transport of nutrients, metabolites, and hormones from the pancreatic islets to the circulating blood. Further, beyond the transportation of nutrients to peripheral tissues, endothelial cells may also contribute to the unique microenvironment of the islet and have been shown to be of high importance for the function of the cells in their nearest surrounding. A well-developed communication between islet endothelial cells and endocrine cells, especially the beta cells, has previously been described in the literature [8, 9]. Interestingly, both beta cells and endothelial cells seem to be equally dependent on the other cell type, highlighting the important cross-talk between cell types to facilitate the development of islet cells with specialized phenotypes. As an example, endothelial cells secrete mediators such as thrombospondin-1, endothelin-1, and hepatocyte growth factor, which promote beta cell survival, proliferation, and insulin secretion [8, 10-12]. In the other direction, beta cells have a high secretion of vascular endothelial growth factor A (VEGF-A) which stimulates a dense and highly fenestrated vascular network, facilitating rapid secretion of islet hormones to the bloodstream [13]. It is very important to maintain the microenvironment in the islets for proper cellular function. Dysfunctional islet endothelial cells with an altered phenotype are believed to contribute to impaired beta cell function in diabetes [14, 15].

The efferent vessels that are responsible for transporting the blood out of the islet are arranged in either of two different types of microvascular systems. Most commonly, the efferent capillaries empty into collecting venules situated in the interstitial space under the capsule and forming a basket-like network of vessels between the endocrine and exocrine tissue. These venules later empty into larger intralobular venules and finally reach the portal vein, thus providing the islet with a direct venous drainage [4, 5]. The second type of microvascular system drains the efferent blood from the islet directly to capillaries in the acinar tissue. These islets have an insulo-acinar portal system, meaning that the efferent vessels of the islets directly anastomose with capillaries in the acinar tissue forming an intrapancreatic portal system [4, 5]. The insulo-acinar portal system has been shown to appear differently frequent in

different species, and it is believed that this vascular system is more important in larger species, such as primates [16].

Pancreatic islet blood flow

Despite representing only 1-2% of the total pancreatic mass, the islets receive as much as 5-15% of the total pancreatic blood flow [17, 18]. The arrangement of blood flow and endocrine cells in the islets has been extensively debated, with different hypotheses for the direction of blood flow in the islets. In rodents, the most acknowledged theory states that blood flows in a B-A-D direction where the vessels penetrate the islets and reach the beta cell-rich core. Thereafter, the capillaries branch towards the alpha cells and finally reach the delta cells located in the periphery of the islets. However, other theories suggest the opposite direction of flow, or in a top-to-bottom direction where blood is entering the islets in the arteriolar pole and then is transported through the islet to a venous pole [19, 20]. Human islet cells are arranged differently compared to rat islets, with the beta, alpha and delta cells dispersed in the islet [21]. Nevertheless, a B-A-D direction of circulation has also been suggested for human islets [22].

Islet blood flow has been widely studied using different techniques but most commonly by microspheres [23-25]. Using microspheres, an estimate of single-islet blood flow of approximately 20 nl/min/islet or 5-6 ml/min/gram islet has been recorded in rats [4, 23]. This value is however calculated from blood flow measurements of the whole islet organ since the microsphere technique does not have the resolution to measure blood flow at a single-islet level. To enable proper individual islet flow measurements without the need for whole islet organ analysis, the use of hydrogen gas clearance has been evaluated [26]. This technique provided mean single-islet blood flow values of 2 ml/min/gram islet and did, interestingly, indicate wide differences in single-islet flow (up to 6-10 times) between islets of the same size.

Diabetes Mellitus

Diabetes mellitus is a condition characterized by chronic high blood glucose levels caused by decreased secretion or sensitivity to insulin, or a combination of both. According to the International Diabetes Federation, there were 415 million people suffering from diabetes in 2015 and the number is thought to increase to 642 million people by 2040 [27]. The two major types of diabetes are type 1- (T1D) and type 2 diabetes (T2D), but there are also other subtypes of the disorder that exist, for example, latent autoimmune diabetes in the adult (LADA), maturity onset diabetes in the young (MODY), gestational diabetes mellitus and mitochondrial diabetes.

Type 1 Diabetes

T1D was formerly termed juvenile-onset diabetes due to the common clinical presentation at a young age. It is a disease characterized by a selective destruction of the insulin-producing beta cells, and it is associated with an infiltration of immune cells into the pancreatic islets with surrounding tissue. The autoimmune attack on the insulin-producing beta cells leads to insufficient insulin production and subsequent hyperglycemia. At the time of diagnosis, it has been believed that a clear majority (80-95%) of the insulin-producing beta cells are destroyed. Lately, however, this statement has been considered to be only partly true [28]. In fact, factors such as age, insulin resistance, and level of beta cell dysfunction may play a major role in the time-point of clinical presentation [29]. Another common misunderstanding is that a total destruction of the insulin-producing cells finally limits all production and secretion of insulin in patients with T1D. This statement has been thoroughly challenged and circulating residual C-peptide may, in fact, be detected in numerous patients with long-standing disease and, perhaps most intriguing, after as long as 50 years of disease, insulin-positive cells may still be present in the pancreas [30, 31]. Despite residual insulin production, no cure is available for patients diagnosed with T1D. To maintain glucose homeostasis after clinical onset, the patients are dependent on life-long exogenous insulin treatment, most commonly administered through several subcutaneous injections daily. Even though insulin treatment has improved over the years, it is challenging to mimic normal blood glucose concentrations and long-term complications including retinopathy, nephropathy, and neuropathy, as well as decreased average quality of life and life expectancy are predicted in these patients [32-34].

Type 2 Diabetes

Decreased insulin sensitivity and relative insulin deficiency are characteristics of T2D, formerly known as adult-onset or non-insulin-dependent diabetes. Clinical onset in T2D is more diffuse than in T1D, and the number of unidentified cases is believed to be high. In early stages of the disease, treatment includes life-style changes. However, when the disease progresses, treatment with oral drugs or exogenous insulin is common. A hallmark of T2D is the development of insoluble peptide aggregates, islet amyloid, in the islets. These aggregations are found in most diagnosed patients and have been described as toxic to the beta cells [35, 36].

Islet transplantation and engraftment

An alternative to exogenous insulin injections for managing glucose homeostasis during TID is transplantation of pancreatic islets isolated from a brain-

dead donor. By infusion of the islets into the portal vein, which allows islet distribution within the vascular network of the liver, the transplantation may be performed by minimal-invasive surgery. This favors the treatment when comparing it to the alternative option, whole pancreas transplantation, which is associated with longer hospitalization and severe complications [37]. Islet transplantation is, however, not a treatment suitable for every patient since it is followed by life-long treatment with immune suppressive medication.

One of the major difficulties with islet transplantation is the excessive death of islet cells during and after transplantation. Only minutes after the injection of islets into the portal vein, about 50% of the islets may have died or been severely damaged [38]. This early cell death may be caused by acute effects mediated from the direct contact of the transplanted islets with the recipient's blood, an instant blood-mediated inflammatory reaction (IBMIR) [39]. The islets also suffer from hypoxia [40, 41] due to disruption of their vascular network during the isolation procedure. After transplantation, the islets are dependent on diffusion of oxygen and nutrients before a new vascular network has been established. It should be noted that the liver environment surrounding the islets after transplantation has an oxygen tension of only 5-10 mmHg, compared to the endogenous islet oxygen tension of around 40 mmHg, which limits the probability of successful oxygen diffusion [42]. Furthermore, an activation of the surrounding implantation environment resulting in a local inflammatory response, including nonspecific inflammation mediated by cytokines, will trigger post-transplant inflammation which severely affects the function of the graft [43, 44].

Following a high rate of cell death during the first days post-transplantation, major obstacles remain which need to be overcome to enable long-term graft survival. One such limiting factor for long-term graft survival is the low vascularization accompanied by low blood flow and oxygen tension identified in grafts even a long time after transplantation [45, 46]. Five-years after transplantation, up to 60% of transplanted patients may still be independent on exogenous insulin administration. However, this result may be achieved only by strict immunosuppressive treatment and a large number of donated islets, sometimes from up to three donors, representing the remaining obstacles to proper islet engraftment [47].

The understanding of graft failure is incomplete, and the need for many transplanted islets, as well as the overall lack of organ donors are clearly limiting factors. This emphasizes the need for efforts to improve the outcome of islet transplantation in order to provide this therapy for more patients.

Islet amyloid and IAPP

Islet amyloid was first described in 1901 by Opie as "islet hyalinization" present in the pancreatic islets of a patient with diabetes [48]. Later, postmortem examinations of T2D patients have revealed that islet amyloid deposits are present in most of the patients, and it is now considered a histological hallmark of the disease [35, 49]. The main component of islet amyloid is polymerized islet amyloid polypeptide (IAPP). IAPP is a 37-amino-acid residue peptide hormone characterized and named in 1986 after its ability to form islet amyloid [50]. It resembles the neuropeptide calcitonin-gene-related peptide (CGRP) with which it shares more than 40% sequence homology [50]. The sequence of the peptide is highly preserved between mammalian species. However, some variations occur in the region corresponding to human IAPP (hIAPP) 20-29 resulting in variable amyloidogenic properties between species. IAPP is only amyloidogenic, that is, it can produce amyloid deposits in humans, non-human primates, and cats, species which, interestingly, also spontaneously develop T2D [51-53]. Rodents, on the other hand, produce a non-amyloidogenic IAPP and do not develop spontaneous T2D. The loss of amyloidogenic properties of rodent IAPP is due to three proline substitutions in the variable region (residue 25, 28, and 29) [54, 55]. Proline is a beta-sheet breaker and will thus inhibit fibril formation when present. The non-amyloidogenic properties of rodent IAPP prevent studies of amyloid formation in rats and mice. Instead, transgenic animal models expressing hIAPP and isolated or transplanted human islets are examples of alternative methods for this purpose [56-58].

IAPP is initially produced as the 89-amino-acid long precursor protein preproIAPP [59]. The precursor contains a signal peptide which is cleaved off when processed into proIAPP upon entering the endoplasmic reticulum (ER) [60]. Further processing of proIAPP to mature IAPP is performed after transportation through the ER and Golgi apparatus and finally takes place in the secretory vesicles by the prohormone convertase (PC)1/3 and PC2 [61, 62]. IAPP is, in the secretory granule, co-stored with insulin in a 1-2:50 ratio and the two hormones are generally secreted in parallel [60, 63, 64]. Insulin has been found to be a strong inhibitor of IAPP aggregation, indicating the importance of balanced co-storage and secretion of the two hormones [63]. IAPP requires further amino acid removal from the COOH-terminal by Carboxypeptidase E (CPE) to allow amidation and the formation of a disulfide bond at positions 2 and 7, corresponding to the NH_2-terminal, before being considered as fully mature and biologically active [60, 65].

The physiological function of IAPP is still not fully understood, but the peptide is believed to be involved in the regulation of glucose metabolism. IAPP receptors have been found on beta cells, suggesting an autocrine and paracrine function of the peptide [66]. The peptide is thought to be a modulator of insulin secretion and has been shown to suppress glucose-stimulated insulin

secretion [67-69] and to stimulate basal insulin secretion via positive feedback [70]. The peptide also has peripheral action, mediated by receptors in the brain [71], and reduces food intake [72] and slows gastric emptying [73].

Heterogeneity among beta cells

Functional heterogeneity between individual beta cells was first described in 1987 indicating intrinsic differences between beta cells facilitating a complex response in insulin secretion to different glucose concentration levels [74]. At that time, islet beta cells were categorized into subpopulations based on glucose responsiveness, with high responders being more sensitive to glucose indicated by their entering into a metabolically active state at low-glucose concentrations, and low responders which need higher glucose concentrations to become activated [75, 76]. When studying these two subpopulations, a range of differencing parameters have been found, such as, the amount of newly or preformed insulin, protein biosynthesis, beta cell granulation, hormone content, and activity of glucokinase [74, 77, 78].

Differences between beta cells are not restricted only to metabolic activity. Differences originating from pancreatic topography and nuclear DNA content and synthesis including differences in proliferative capacity have also been described [79]. During the last years, the field of beta cell heterogeneity has regained new interest. With modern techniques and new methods, markers of beta cell heterogeneity have been developed and used to further understand the importance of subpopulations of beta cells. Examples of such markers include the flattop (Fltp) marker indicating functional maturation and proliferative capacity in islets [80]. Also, markers of aging beta cells, such as, insulin-like growth factor 1 receptor (IGF1R) facilitate studies of beta cell subpopulations regarding particular maturation stages during the life cycle [81].

How beta cell heterogeneity may affect the single islets or the endocrine function is still unknown but it has been speculated that the subpopulations provide possible adaptation to varying metabolic needs. It has been suggested that distinct beta cell populations may be increased during certain disorders, for instance, conditions with increased insulin secretion may be the result of an expansion of a high responding beta cell subpopulation [76, 82].

Heterogeneity among pancreatic islets

Pancreatic islet heterogeneity has recently gained more attention even though variations in both native islet biology and pathology have been recognized for many years. The potential physiological relevance of islet heterogeneity is however still debated. Most differences identified between islets today are based on islet morphology, function, vascularity, and maturation.

The morphology of pancreatic islets has been thoroughly investigated and described to be very diverse when comparing islets both between and within different species, and human islets appears to be more diverse than rodent islets [22]. Significant variations between single islets may also be detected when comparing islets in the same pancreas. One morphological parameter, perhaps the most obvious, differing between islets is the size, which displays large variations. Human islets have a mean diameter of 109 μm, but range from consisting of only a few cells to clusters of several thousands of cells with diameters larger than 500μm [83]. In addition to the islet size, differences in cellular composition, and perhaps most importantly, proportions of the different endocrine cells, describes further variability between the islets. Clear examples of such heterogeneities involve the significant variation in PP and alpha cell fraction between islets [22]. Islets with these variations are located in different parts of the pancreas and the variations are thought to be features inherited from the different embryonic pancreatic anlagen, for example, the PP-rich islets are preferably found in the ventral pancreas, [84, 85].

In rodents, wide variations in functionality between islets from the same pancreas have previously been described. Such differences have been coupled to, and may be identified by, differences in vascular support, pancreatic topography, or beta cell age [81, 86, 87]. Vascularity and blood flow have previously been described to vary substantially between islets [26], and this variation was in 2012 correlated to islet function in a rat model. In this model, islets with higher blood flow displayed augmented functions including higher proliferation and glucose-stimulated insulin release than other islets [86]. Regarding regional differences in islet function, islets in a mouse model were observed to have a different ability to adjust to a high-fat diet depending on islet location. In this model, islets located in the splenic region of the pancreas could adapt better to the metabolically challenging diet by increasing the beta cell proliferation rate and glucose-stimulated insulin release capacity [88].

It is still not fully understood how islet heterogeneity is induced, but one suggestion is that beta cells with similar function form clusters within islets. Even though large variations are still found between beta cells within the same islet, clustering effects of aging markers in beta cells have been described in islets which may indicate that cells with age-related dysfunction may be clustered within the same islet [81].

Islet heterogeneity in disease

In both T1D and T2D, wide heterogeneities in islet pathology have been detected indicating that some islets are more sensitive or exposed during disease development. Postmortem analysis of T1D pancreases has indeed revealed uneven distribution of insulitis and that some islets are more severely affected

during the disease progress, with increased beta cell loss and immune cell infiltration than other islets [89, 90]. In T2D, the pathological hallmark of the disease, islet amyloid, has also been described to affect islets in a heterogeneous pattern [91].

The background for this heterogeneity is still not fully understood, but differences between islets in the prediabetic phase have been identified. One possible influencing parameter was identified when studying a deceased non-diabetic donor with a high risk of developing T1D due to the presence of islet autoantibodies. In this donor, inter-islet differences in major histocompatibility complex 1 (MHC-I) were found, and further studies of these islets indicated that islets with increased expression of MHC-1 also had more infiltrating immune cells [92].

The regional distribution of severely affected islets during both T1D and T2D has also been studied. Interestingly, a lobular pattern of severely affected islets has been described in TID indicating that the affected islets are grouped in the same pancreatic lobules [92, 93]. In T2D pancreases, regional differences in amyloid deposits have also been described, but the pattern differs from T1D pancreases. In T2D, the most severely affected islets are instead preferentially located in the peripheral parts of the pancreas [94]. Furthermore, a preferential loss of beta cells has in T2D been described to occur in the head region of the pancreas [95]. In a population of Korean T2D patients, a selective destruction of beta cells in large islets has also been reported [90].

Aims

The overall aims of this thesis were to study and characterize subpopulations of pancreatic islets identified by their vascular support, to investigate the functional importance of these subpopulations in the healthy organ, and their potential involvement in disease development both in rodent models and in humans. The specific aims of the studies included in this thesis were:

I. To study the susceptibility of highly vascular and functional islets regarding cellular stress induced by cytokines and hypoxia, and to investigate their capacity to engraft after transplantation.

II. To study the propensity of the highly vascular and functional islets to develop high-fat diet induced amyloid deposits and to study the mechanisms behind amyloid formation in these islets.

III. To study if heterogeneities in islet vascular support reflect anatomical and functional differences that may underlie the variable susceptibility to an immune attack in the prediabetic BioBreeding-rat and to translate these findings to human type 1 diabetes.

IV. To study whether low-oxygenated islets, when not functionally challenged, rapidly cycle between activation and inactivation, or whether they represent a stable population that remains low-oxygenated. When confirmed to be a stable population, we aimed to characterize these islets regarding cell composition, vascular density and endocrine cell proliferation.

Materials and Methods

Experimental animals

The local animal ethics committee of Uppsala University, Sweden approved all experiments procedures involving experimental animals. The animals had free access to water and standard chow throughout the studies unless otherwise stated. In paper I, adult male inbred Lewis rats weighing ~350 grams were purchased from Charles River Laboratories (Charles River Laboratories, Sulzfeld, Germany). In paper II, adult male mice deficient in mouse IAPP (mIAPP), but expressing human IAPP (hIAPP) driven by the rat insulin I promotor ($hIAPP^{+/+}$, $mIAPP^{-/-}$) were obtained from a local colony breed at Linköping University, Sweden [96]. Experiments on $hIAPP^{+/+}$, $mIAPP^{-/-}$ mice were performed after short-term (3-months) or long-term (10-months) supplemented lard diet (free access to lard, high-fat diet; HFD) on animals weighing ~30 or ~40 grams, respectively. In paper III, adult male Sprague-Dawley rats weighing ~350 grams purchased from Taconic (Taconic M&B, Ry, Denmark) were used with the exception of the long-term microsphere studies followed by insulitis analysis where ~130-gram male and female BioBreeding (BB) rats from a local colony bred at Lund University, Sweden [97], were used. The congenic DR.lyp/lyp rats were obtained by intercross breeding of heterozygous BB DR.lyp/+ rats. The *lyp* region of diabetes prone (DP) BB rats was previously introgressed to the diabetes resistant (DR) BB rat. The heterozygote BB DR.Lyp/+ rats were then kept in sibling breeding for more than 50 generations to yield 25% DR.lyp/lyp, 25% DR.+/+ and 50% DR.lyp/+ [98, 99]. The animals were earmarked at about 20 days of age and were after weaning at 30 days of age, transferred to Uppsala University. In paper IV, adult male Wistar Furth rats weighing ~300 grams were purchased from Harland Laboratories (Harlan Laboratories, Indianapolis, IN, USA)

Human islets

Experiments involving isolated human islets from brain-dead donors were approved by the human regional ethical board in Uppsala, Sweden. Islets from four heat-beating female and male donors (age 60 ± 8 years) were kindly provided by the Nordic Network for Clinical Islet Transplantation. Islet isolations

were performed at the human islet isolation core facility for the Nordic countries located at Uppsala University, as previously described [100]. Isolated islets were cultured at a glucose concentration of 5.5 or 20 mmol/l in CMRL 1066 (Thermo Fischer, Waltham, MA, USA) supplemented with fetal calf serum (10% (vol/vol); Sigma-Aldrich, St Louis, MO, USA), L-glutamine (2 mmol/l; Sigma-Aldrich) and streptomycin (0.1 mg/ml; Roche Diagnostics Scandinavia, Bromma, Sweden).

Human subjects

Procedures including human subjects were approved by the human regional ethical board in Uppsala and were conducted according to the declaration of Helsinki. T1D subjects were recruited from the Uppsala University Hospital and healthy volunteers were recruited by advertising. Oral and written information was provided to the subjects and a written informed consent was obtained from all subjects before inclusion. The following inclusion criteria were used for T1D patients: disease debut within three years, age 18-35, BMI 20-25, fasting C-peptide concentration >0.07 nmol/l and normal renal function (P-creatinine <100µmol/l) and, for healthy volunteers, normal fasting plasma glucose concentration (<6.1 mmol/l), normal HbA1C (<6%; <43 mmol/mol), BMI 20-25, and normal renal function (P-creatinine <100µmol/l).

Microsphere administration (papers I, II, & III)

Microspheres were administered to the animals by two alternative techniques: anterograde injection (papers I-III) to identify islets with high blood flow and retrograde injection of microspheres (paper III) to investigate the anatomy of islet venous system. When both injections were given in the same animal, the anterograde injection was completed before further surgical preparation for retrograde injection. The two consecutive injections were possible to differentiate during analysis through the use of differently colored microspheres.

Anterograde injection

Rats and mice were anesthetized by an IP injection of 120 mg/kg body weight Inactin (Thiobutabarbital sodium; Sigma-Aldrich) or 0.02 mg/g body weight Avertin (2.5% (v/v) solution of 2,2,2-tribromoethanol; Sigma-Aldrich, in 2-methyl-2-butanol; Kemila, Stockholm, Sweden), respectively, and placed on a heating pad to maintain a body core temperature of ~38ºC. To secure free airways, the animals were tracheostomized by inserting a polyethylene catheter into the trachea. For continuous blood pressure measurement and subse-

quent microsphere injection, polyethylene catheters were inserted into the ascending aorta through the right carotid artery, and to enable reference blood sample collection, a catheter was inserted into the left femoral artery. After completion of the surgical procedure, the animals were allowed to stabilize their blood pressure for 10-15 minutes before injection of microspheres into the ascending aorta. In paper I, a bolus dose of 1 ml 30 % (wt/vol) D-glucose was given in the femoral artery 10 minutes before microsphere injection to increase the number of microspheres in each islet [101]. In experiments with histological analysis of pancreatic and islet tissue, colored microspheres (10 µm, E-Z Trac Ultraspheres®; IMT, Stason Lababoratories., Irvine, CA, USA) were used, and in experiments when isolated islets were studied, fluorescent microspheres (10 µm, FluoSpheres Polystyrene Microspheres; Molecular Probes, Eugene, OR, USA) were used to enable microspheres visualization. To enable determination of blood flow per microsphere, blood sampling from free flow arterial blood obtained from the femoral artery was collected for a total of 60 seconds, starting 5 seconds before microsphere injection. In each animal, the adrenal glands were examined to evaluate adequate mixing of microspheres with the arterial blood, and only in animals with equal amounts of microspheres in the adrenal glands (<10% difference) the pancreases were taken to subsequent analysis.

Retrograde injection

After anesthetization and surgical preparation, both described in the former section, ~10 µl of heparin (Heparin LEO; 5000 IE/ml; Leo Pharma A/S, Ballerup, Denmark) was injected into the femoral artery to avoid coagulation during the experiment. A suture was thereafter placed proximally of the junction of the celiac trunk to obstruct the abdominal aorta before free retrograde flow from the portal vein was obtained by an incision of the aorta distally of the suture. Retrograde microsphere injection was performed through a polyethylene catheter inserted into the hepatic portal vein in the opposite direction of normal blood flow. To avoid microsphere distribution into the vascular system of the lower gastrointestinal tract, the lower mesenteric artery was ligated by a suture before the injection. Retrograde injection was performed at a speed of 100µl/min during five minutes for a total injection volume of 500µl.

Calculation of blood flow

To calculate blood flow (ml/min/g tissue) the number of microspheres (after anterograde injection) was counted in the reference blood sample, the endocrine- and the exocrine tissue. After retrieval, the pancreas was weighed, cut into 20-25 pieces and placed between object glasses. Immediate freeze-thawing thereafter visualized the islets [102]. Blood flow was calculated by using the formula $Q_{org} = Q_{ref} \times N_{org} / N_{ref}$, where Q_{org} is the organ blood flow

(ml/min), Q_{ref} is withdrawal rate of the reference sample (ml/min), N_{org}, is the number of microspheres in the organ, and N_{ref} is the number of microspheres in the reference sample.

Evaluation of likelihood of venous drainage (paper III)

Pancreatic islets may be drained by two different vascular systems. The islet capillaries can either be emptied in venules directly connected to the portal vein or be drained into capillaries in the exocrine parenchyma creating an insulo-acinar portal system. To evaluate the likelihood of direct venous drainage in highly blood-perfused islets, combined anterograde and retrograde injections were performed. To separate the two injections, two types of differently colored microspheres were used: green (anterograde injection) and violet (retrograde injection) microspheres. Firstly, anterograde injection of green microspheres was performed, as described above, to identify highly blood-perfused islets, and was followed by preparation and administration of violet microspheres by retrograde injection. As microspheres are unable to pass through capillaries, islets drained into the exocrine parenchyma will not be reached by a retrograde injection; the microspheres will be trapped in the exocrine parenchyma (Fig. 1) before reaching the islets.

To estimate the likelihood of venous drainage, the presence or absence of green and violet microspheres in the pancreatic islets was counted. The occurrence of venous drainage of highly blood-perfused islets (islets with green microspheres) was calculated as fold increase in the presence of having violet microspheres when compared to all islets [fraction of islets with green and violet microspheres of all islets with violet microspheres/fraction of islets with green microspheres of all islets].

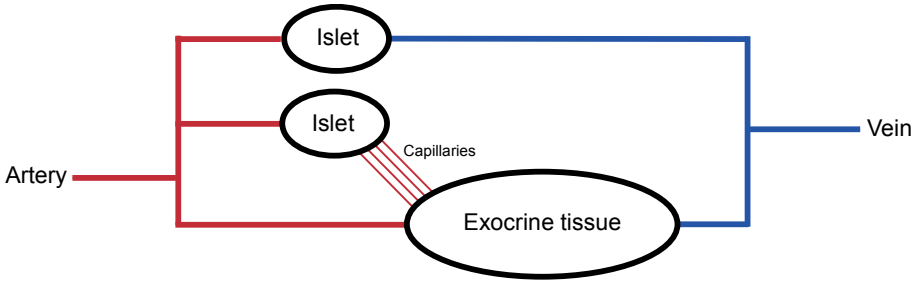

Figure 1. Anatomy of islet vascular system. Islets are supplied by arterial blood and drained either directly into the venous system or drained into the exocrine parenchyma via a portal system.

Isolation of rat and mouse pancreatic islets (papers I, II, & III)

In papers I and II pancreatic islets were isolated by collagenase digestion during shaking [103]. After retrieval, the pancreas was cut into small pieces and placed in collagenase solution (Collagenase from Clostridium Histolyticum, rat pancreas 1.5 mg/ml, mouse pancreas 1mg/ml; Sigma-Aldrich in Hanks balanced salt solution (HBSS, National Veterinary Institute, Sweden)) in a 37ºC water bath and underwent vigorous shaking followed by washing and hand-picking of the islets. When isolating islets after retrograde injection in paper III, collagenase digestion in combination with a density-gradient purification technique was used [104]. After anesthetization with 120 mg/kg body weight Inactin (Thiobutabarbital sodium; Sigma-Aldrich), the common bile duct was exposed and a clamp was placed over the entrance of the bile duct into the duodenum. Then the pancreas was inflated by injection of ice-cold collagenase solution (Collagenase P, 1 mg/ml, Roche Diagnostics in Hanks balanced salt solution) into the bile duct and transferred to a 37ºC water bath for 20 minutes. Islets were purified by separation from exocrine tissue using density gradient centrifugation using Histopaque-1077 and serum-free RPMI 1640 medium (Sigma-Aldrich) followed by hand-picking.

After isolation, islets were cultured free-floating in groups of 100 at 37ºC at a glucose concentration of 11.1 mmol/l in 5 ml RPMI 1640 medium supplemented with L-glutamine (2mmol/l; Sigma-Aldrich), fetal calf serum (10% (vol/vol); Sigma-Aldrich) and streptomycin (0.1 mg/ml; Roche Diagnostics). Before further experiments on isolated islets, microsphere detection was performed in a fluorescence microscope, and the islets were dichotomously sorted dependent on microsphere content.

Islet transplantation (paper I)

Rats were anesthetized by an IP injection of 60 mg/kg body weight of pentobarbital sodium (Apoteket) and placed on a thermostat-controlled heating plate to maintain body core temperature of ~38ºC. The left kidney was accessed by a left subcostal flank incision. After immobilization of the kidney, a small nick was applied in the renal capsule and a glass rod was inserted under the capsule on both sides of the nick, to create two small pouches. Approximately 100 islets with or without microspheres and packed in a braking pipette under visual inspection in a stereo microscope, could thereafter be transplanted separately into the preformed pouches. The kidney was kept moist by carefully applying saline throughout the transplantation procedure. Two days or one month post transplantation, the graft-bearing kidney was taken to vascular function analysis and/or removed and taken to histological analysis. To

evaluate hypoxia in two-day-old transplants, the hypoxia marker pimonidazole hydrochloride (HCL) (60 mg/kg body weight, Hypoxyprobe-1; HPI, Burlington, MA, USA) was administered by IV injection two hours before tissue removal.

Evaluation of graft vascular function (paper I)

Vascular function of 30-day-old grafts was evaluated by measuring oxygen tension and blood perfusion. Rats were anesthetized by an IP injection of Inactin (Sigma-Aldrich) and placed on a thermostat-controlled heating plate to maintain body core temperature of ~38ºC. Polyethylene catheters were inserted into the trachea to secure free airways, into the carotid artery for continuous blood pressure measurements (ADInstruments, Dunedin, New Zealand) and into the femoral vein for substitution of lost body fluid with Ringer's solution (Apoteket). The graft-bearing left kidney was exposed by a left subcostal flank incision and immobilized in a plastic cup covered in surgical cotton pads soaked in Ringer's solution (Apoteket) to maintain a humid environment. Multiple measurements (three or more) of both blood flow and oxygen tension were performed in each site, and a calculated mean from each location and animal was considered one experiment.

Islet graft blood flow

In vivo blood flow was measured in each of the two grafts of islets with or without microspheres and the internal control, adjacent kidney cortex, by laser Doppler flowmetry (Transonic BLF21 Series, probe 1.2mm; Transonic, Ithaca, NY, USA). By measuring a shift in wavelength, a Doppler shift, caused by the moving red blood cells impact on a transmitted laser-beam, an estimated blood flow in the grafts and kidney cortex could be expressed in arbitrary tissue perfusion unites (TPU).

Islet graft oxygen tension

In vivo oxygen tension was measured in each of the grafts and the adjacent kidney cortex by modified Clark microelectrodes [42, 105] (outer tip diameter <5 µm; Unisense, Aarhus, Denmark) with the aid of a micromanipulator. Before and after each experiment, the microelectrodes were calibrated in water saturated with $Na_2S_2O_5$ and air at 37 ºC. Briefly, the Clark microelectrodes measures oxygen partial pressure (pO_2) in solutions or tissues surrounding the oxygen reducing electrode. At a voltage range between -0.5 and -0.9 V, there is a linear response between the oxygen tension and the electrode current originating from the oxygen reduction at the cathode surface. A guard electrode ensures that oxygen is immediately reduced in the electrode, which results in

a current limited to the rate of oxygen diffusion from the surrounding tissue to the cathode, that is, the current of the electrode is directly proportional to the pO_2 of surrounding tissues [105].

Evaluation of cellular stress *in vitro* (paper I)

Groups of 50 size-matched isolated islets with- or without microspheres after anterograde injection were assessed for susceptibility to cellular stress. 24 hours after isolation, the islets were incubated in culture media with or without a cytokine cocktail (50 U/ml IL-1β, 1,000 U/ml IFN-γ, and 1000 TNF-α; PeproTech, London, UK) for 24 h at 37ºC. In separate experiments, the islets were incubated in 20% (control) or 1.5% O2 (hypoxia; Air Liquid Gas, Stockholm, Sweden) in a humidified airtight chamber for four hours. To obtain correct oxygen levels during the experiment, an oxygen sensor (Dräger Pas III; Dräger Lübeck, Germany) was used. The viability was assessed after the experiments by staining cell nuclei with bisbenzimide (20 µ/ml, Hoechst 33342; Sigma-Aldrich) and propidium iodide (PI, 10 µg/ml; Sigma-Aldrich). Viability was assessed as the PI-to-bisbenzimide ratio and was measured and analyzed in a Kodak Image Station 4000 MM (Kodak, New Haven, CT, USA).

Glucose-stimulated hormone release (papers II & III)

Static incubation in low and high glucose

Size-matched isolated mouse (paper II) and rat (paper III) islets, with or without microspheres from anterograde- or retrograde injection, were analyzed for glucose-stimulated hormonal release in low (1.67 mmol/l glucose) and high glucose (16.7 mmol/l), as described previously [106]. Briefly, triplicates of ≤10 islets were consequently incubated in KRBH buffer (Krebs–Ringer bicarbonate buffer supplemented with 10 mmol/l HEPES and 2 mg/ml BSA) supplemented with low (1.67 mmol/l glucose) and high glucose (16.7 mmol/l) for one hour each. The supernatants were removed and stored at -20ºC until analyzed by ELISA kits detecting insulin (Mercodia, Uppsala, Sweden) and human amylin IAPP (EZHA-52K; Merck Millipore Corporation, Billerica, MA, USA). After glucose stimulation, the islet triplicates were pooled and homogenized by sonication in 200 µl redistilled water and analyzed for DNA content to ensure equal islet size between the groups (Quant-iT™ PicoGreen® dsDNA assay kit; Molecular Probes®, Eugene, OR, USA).

Single human islet hormonal release

As a measurement of islet function, single human islet glucose stimulation capacity was evaluated. Individual islets were pre-incubated in KRBH buffer supplemented with low glucose (1.67mml/l) for 30 minutes to obtain basal secretion before experiment the start. The islets were thereafter incubated in KRBH supplemented with low (1.67 mmol/l) and high glucose (16.7 mmol/l) for 60 minutes, respectively. The supernatants were analyzed by an Ultrasensitive Insulin ELISA (Mercodia).

Glucose responsiveness by perifusion

Groups of 50 size-matched rat islets, with or without microspheres after anterograde injection, were taken to graded glucose perifusion to investigate glucose responsiveness at different glucose levels. The islets were inserted into filter-covered perifusion chambers (Suprafusion 1000, 6-channel system; Brandel, Gaithersburg, MD, USA) and perifused (200µl/min) with KRBH buffer supplemented with stepwise increasing levels of glucose at 37ºC. To obtain baseline secretion, the islets were perifused at 2 mmol/l glucose for 30 minutes before the experiment start. Perifusion was then performed with the following glucose concentrations: 2, 4, 2, 6, 2, 8, 2, 10, 2, 12, 20, 2, and 2 mmol/l glucose for 14 minutes per glucose concentration (Fig. 2). Released insulin at each glucose level was analyzed by a Rat Insulin ELISA kit (Mercodia).

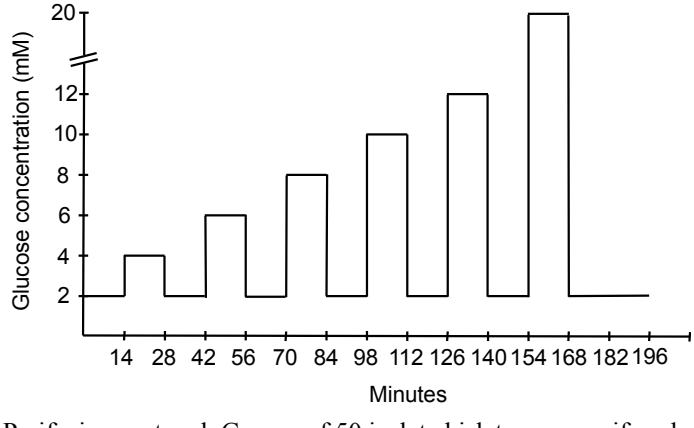

Figure 2. Perifusion protocol. Groups of 50 isolated islets were perifused at increasing glucose levels to estimate glucose responsiveness and threshold.

Gene expression of isolated mouse islets (paper II)

Tissue preparation

Groups of ~10 isolated size-matched mouse islets with- or without anterograde microspheres were washed in sterile PBS and lysed in 350µl RLT buffer followed by RNA extraction according to manufacturer's instructions (RNeasy Plus Mini Kit; Qiagen, Hilden, Germany). This kit contains a genomic DNA (gDNA) eliminator column which was used to remove unwanted gDNA from the samples. Lysed and extracted RNA samples eluted in nuclease-free water, were stored at -80ºC until cDNA synthesis.

cDNA synthesis

First strand c-DNA synthesis was performed by Superscript III First-Strand SuperMix (Thermo Fisher) according to the manufacturer's instructions. cDNA was stored in -20ºC until further use.

Primers

Primer pairs were either purchased from Tebu-Bio (All-in-One qPCR Primer; Roskilde, Denmark) or individually designed and purchased from Sigma-Aldrich (see supplementary table 1, paper II, for detailed primer information). Individually designed primers were selected to create short amplicons of 70-180 base pairs, with an annealing temperature of 60ºC and without predicted dimer formations. Selected primer pairs were identifying all transcript variants of the gene.

Quantitative real-time PCR analysis

qPCR analysis of cDNA samples was performed in duplicates with LightCycler FastStart DNA Master Plus SYBR Green 1 kit (Roche Diagnostics) in a LightCycler 480t (Roche Diagnostics). Expression of target genes were normalized against the expression of the reference gene Tata-box binding protein (TBT). Relative mRNA expression was calculated as $2^{-\Delta Ct}$.

Evaluation of islet susceptibility to immune cell infiltration (paper III)

Diabetes prone (DR.lyp/lyp) and diabetes resistant (DR.+/+ and DR.lyp/+) BB rats were before visible insulitis development, that is, at less than 40 days of age, with an IP injection of 60 mg/kg body weight of pentobarbital sodium (Apoteket) and placed on a heating plate to maintain a body core temperature of 38ºC. A polyethylene catheter was inserted into the carotid artery to facilitate injection of 1.2×10^5 colored microspheres into the ascending aorta (anterograde injection). This is a reduced amount of microspheres compared to

what is usually administered to rats [4], when considering the small size of the young BB-rats and to reduce the risk of side effects during long-term experiments. After injection, the carotid artery was ligated proximally and distally of the injection site to prevent bleeding and the incision site was closed with sutures. At 50 days of age, the pancreases were retrieved for histological analysis (Fig. 3). Blood glucose concentration was regularly monitored during the experiment.

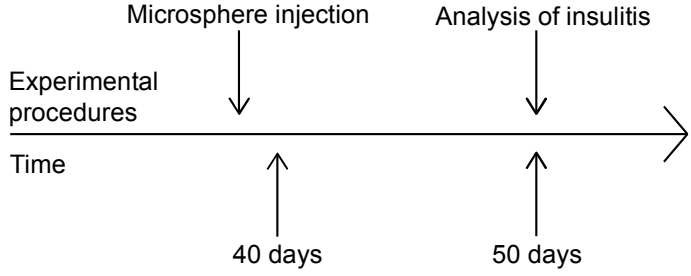

Figure 3. Timeline for microsphere injection and tissue retrieval in BB rats.

Modified glucose clamp technique (paper III)

To investigate glucose responsiveness in healthy individuals and T1D patients, a modified glucose clamp was performed. Briefly, the participants were clamped at increasing glucose levels, and the insulin release capacity at each level was measured as released C-peptide (Fig. 4). All participants were fasting overnight before glucose load, and participants with T1D withdrew from insulin treatment the morning of the glucose clamp. Insulin (Humalog 100 IE/ml, Eli Lilly Sweden AB, Solna, Sweden) and glucose (100mg/ml) was administered through a peripheral catheter in the left arm and blood glucose was continuously monitored every fifth minute through a peripheral catheter in the right arm (Contour XT, Bayer, Leverkusen, Germany). To obtain a starting plasma glucose level of 4 mmol/l, an initial infusion of 0.040 I/E/min x m^2 insulin was given. Body surface area was calculated by the DuBois method (body surface area = 0.20247 x height $(m)^{0.725}$ x weight $(kg)^{0.425}$). After achieving stable blood glucose of 4 mmol/l, insulin administration was withdrawn from healthy participants, whereas T1D participants continued periodically with insulin administration throughout the study to maintain steady blood glucose levels. Glucose infusion was thereafter individually adjusted to clamp the participants at each of the following blood glucose levels; 4, 6, 8, 10, and 12 mmol/l (±10%). At each glucose plateau, blood sampling was performed at 0 and 15-30 minutes. After the final blood sample was obtained, the diabetic subjects continued short-acting insulin treatment, and all subjects were monitored after the procedure until they maintained stable normal plasma glucose levels. Pulse and oxygen saturation were monitored throughout the study.

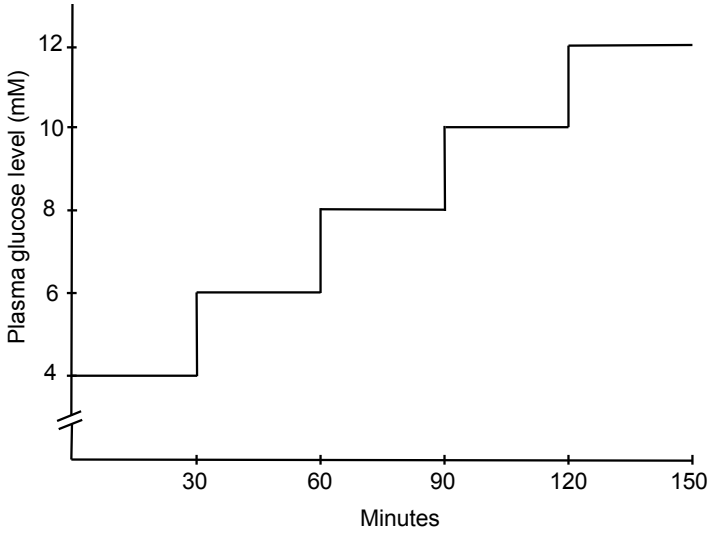

Figure 4. Experimental setup of modified glucose clamp. Each participant was clamped at increasing blood glucose levels for 15-30 minutes by individually adjusted insulin and glucose infusions. C-peptide was measured at each level to estimate glucose-responsiveness.

Detection of low-oxygenated islets by pimonidazole (paper IV)

The low oxygenation marker Pimonidazole [107] was used to identify low-oxygenated islets. Pimonidazole-HCL, a 2-nitroimidazole, binds to thiol-containing proteins and form stable adducts in hypoxic cells which may be identified by immunohistochemistry. The reaction is initiated by a reduction of the nitro-group, which is rapidly reversed and inhibited by oxygen, and as a result, the marker will form stable adducts only in environments with low oxygen [108]. According to manufacturer's instructions, rat tissue is recommended to be retrieved two hours after injection when only a low fraction of pimonidazole-HCL remaining in the circulatory system which limits the risk of unwanted background staining. To evaluate whether islets cycle between low-oxygenation and enhanced oxygenation by administrating the substance once or repeatedly within different time-intervals, we first needed to investigate adduct stability in our model. To investigate this, we injected pimonidazole-HCL intravenously and removed the tissue after two, four, eight, or 24 hours. When stability was defined, islet oxygenation at two time-points was studied by repeated pimonidazole-HCL injections with two or six hours between the first and second injection. Two hours after the last injection, the animals were killed and the pancreas immediately removed and taken to histological analysis.

Induction of pancreatic hypoxia *in vivo* (paper IV)

To obtain a positive control for pimonidazole experiments, hypoxia was induced *in vivo*. The rats were anesthetized with 120 mg/kg body weight Inactin (Sigma-Aldrich) and placed on a heating-plate to maintain body core temperature of 38ºC. Polyethylene catheters were inserted into the trachea to secure free airways and into the carotid artery to facilitate continuous blood pressure measurements (ADInstruments). A graded aortic vascular clamp was positioned superior of the celiac artery and adjusted to allow an arterial blood pressure of 20-30 mmHg, resulting in an intra-islet oxygen tension of 5-10 mmHg [107]. When the blood pressure was stable, pimonidazole-HCL was injected intravenously (60 mg/kg body weight). The animals were monitored for two hours; the pancreases were thereafter removed and taken to histological analysis.

Histology

Tissue preparation

Pancreatic tissue was fixed in 10% buffered formalin (vol/vol), dehydrated and embedded in paraffin before sectioned (5-10 µm thick) and mounted on Polysine slides (Thermo Fisher). Sections were deparaffinized with xylene and rehydrated in a series of graded ethanol (100% to 70%) followed by distilled water, or deparaffinized in Rodent/Diva Decloaker (Biocare Medical, Concord, CA, USA) in a pressure cooker (2100 Retriever; Prestige Medical, Blackburn, UK)

Apoptosis (paper I)

After pre-treatment with modified citrate buffer (pH 6.1; Target Retrieval Solution, Dako, Glostrup, Denmark) apoptosis was detected by a primary antibody anti-cleaved caspase-3 (1:100; Cell Signaling Technology, Danvers, MA, USA). The signal was amplified by TSA Biotin System Kit (Perkin Elmer, Waltham, MA, USA) and visualized by 3-3´-diaminobenzidine (DAB; Sigma-Aldrich). The number of positive cells was counted in Image J (NIH, Bethesda, MD, USA). Apoptosis rate was defined as the percentage of cells positively stained per total endocrine area.

Pimonidazole (paper I and IV)

After pre-treatment with Pronase (0.01%; Roche Diagnostics) pimonidazole adducts were identified by Hypoxiprobe-1 Mab1 (1.25-1:40; HPI). Biotinylated secondary antibody (goat $(F(ab)_2)$ anti-mouse, 1:500 (Southern Biotech, Birmingham, AL, USA)) The secondary antibody was detected by incubation with TrekAvidin-AP Label (Biocare Medical) or Vectastain Elite ABC AP

500-Kit (Vector Laboratories, Burlingame, CA, USA) and visualized by Vulcan Fast Red Chromogen Kit 2 (Biocare Medical). In paper I, the hypoxic area was quantified by measuring pimonidazole positively stained area using a laser microdissection microscope (Leica LMD6000, Leica Microsystems, Wetzlar, Germany) and was defined as the positive area per total endocrine area. In paper IV, islet oxygenation was determined by the presence or absence of pimonidazole positive staining.

Endothelial cells (paper I, and IV)

In paper I, endothelial cells were identified, after heat-induction and neuraminidase treatment (0.1 U/ml, Neuramidase Type V; Sigma-Aldrich), by biotinylated lectin from Bandeiraea simplicifolia agglutinin-1 (BS-1; 1:100; Sigma-Aldrich). In paper IV, Wistar Furth rats were used and no pretreatment for endothelial cells was needed. After incubation with TrekAvidin-AP Label (Biocare Medical) or Streptavidin AP Label (Biocare Medical), the lectin was visualized by Vulcan Fast Red (Biocare Medical). Vascular density was quantified by measuring BS-1 positive area and defined as the area of endothelial cells per total insulin-positive area of the graft (paper I) or within the insulin-positive area of the islet (paper IV). Area measurements were performed using a laser microdissection microscope (Leica LMD6000).

Insulin (Paper I, II, and IV)

In paper I, insulin staining was performed with polyclonal guinea pig anti-insulin (1:3000; Fitzgerald, Acton, MA, USA). Antibody detection was performed by MACH 3 Rabbit HRP-Polymer Detection (Biocare Medical) followed by visualization by DAB. In paper II, insulin (polyclonal guinea pig anti-insulin,1:3000; Fitzgerald) was visualized by fluorescent anti-guinea pig 555 (1:1000, Thermos Fisher). In paper IV, insulin antibody was detected by Donkey anti-guinea pig HRP (1:500; Jackson Laboratories) and visualized by DAB when co-stained with endothelial BS-1. For fluorescent visualization in paper IV, the primary antibody goat anti-insulin (Insulin A, 1:100; Santa Cruz Biotechnology, Dallas, TX, USA) in combination with the secondary antibody anti-goat AF 555 (Thermo Fischer) was used instead. Insulin-positive area (μm^2) in grafts (paper I), insulin intensity (paper II), and the number of insulin-positive cells per islet (paper IV) were quantified using a laser microdissection microscope (Leica LMD6000), Image J software, and in Pancreas Image Detection software (Stardots, Uppsala, Sweden), respectively.

Connective tissue (paper I)

Connective tissue was evaluated by morphometry. The area of connective tissue was quantified in a laser microdissection microscope (Leica LMD6000) and defined as non-endocrine tissue per total graft area.

Necrotic tissue (paper I)

Necrotic cells were defined as cells with structural disintegration (pyknotic nuclei and cellular debris) and the area of necrotic tissue was quantified in a laser microdissection microscope (Leica LMD6000). The necrotic graft area was expressed as the total area of necrotic cells per the total graft area.

Location of islets (paper II)

To evaluate the distance of an islet from the center of the pancreatic tissue, the pancreases were sectioned perpendicularly to the longitudinal axis of the tissue. Whole section imaging was performed by Aperio Scenscope AT (Leica Microsystems, Wetzlar, Germany), and the images were analyzed with Aperio ImageScope software (Leica). The pancreatic center was defined as the *origo* of an image fitting the whole pancreatic section and the distance to this center was calculated for each islet.

Amyloid detection (paper II)

Amyloid was detected in sectioned isolated mouse islets in paper II by Congo Red (Sigma-Aldrich) [109] and in human islets by incubation with the specific oligothiophene pFTAA (30 μmol/l) in culture media for one hour [110, 111]. Amyloid was evaluated in mouse and human islets after fluorescent image acquisition and expressed as a fraction of the total islet area by ImageJ.

Prohormone convertases (paper II)

The two prohormone convertases PC1/3 and PC2 were detected by monoclonal antibodies [112]. Pretreatment before PC2 staining was performed by heat-induction in 0.01 mol/l sodium citrate. Both antibodies were visualized by a fluorescent anti-mouse 488 secondary antibody (Jackson ImmunoReseach Laboratories, West Grove, PA, USA). Intensity of PC1/3 and PC2 within insulin-positive staining was analyzed after digital acquisition by ImageJ.

Heparan sulfate (paper II)

Heparan sulfate glycosaminoglycans were visualized with Alcian Blue CEC staining (0.65 mol/l $MgCl_2$, pH 5.8; Sigma-Aldrich) [113, 114]. Reciprocal staining intensity was analyzed by ImageJ.

Glucagon (paper IV)

Glucagon was identified by the primary antibody mouse anti-glucagon (1:300; Abcam, Cambridge, UK) and visualized by the secondary antibody anti-mouse AF 647 (1:1000, Thermo Fisher). The number of glucagon positive cells per islet was quantified by Pancreas Image Detection software after digital image acquisition.

Proliferation (paper IV)

The marker Ki67 was used to identify proliferation. Heat-induced epitope retrieval preceded the application of the primary antibody rabbit anti-Ki67 (SP6, 1:300; Abcam). Visualization was performed by the secondary antibody anti-rabbit AF 488 (1:1000; Thermo Fisher). The number of Ki67 positive cells was quantified after digital image acquisition in Pancreas Image Detection software, and endocrine cell proliferation was expressed as the fraction of the insulin- and glucagon-positive cells that were also positive for Ki67.

Statistics

Statistical analysis was performed by SigmaPlot 12 (Systat Software, San Jose, CA, USA) or GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). All values are given as means ± standard error of the mean (SEM). In papers I, II, and III, comparison between two groups was performed by unpaired or paired two-tailed Student's t-test. Multiple comparison test in paper I was performed with a two-way ANOVA with Bonferroni´s or Dunnett´s (comparison to control) post hoc test. In paper II, the distribution of amyloid between mouse islets were calculated by a chi-square test. In paper III, comparison of non-parametric data was performed by Mann-Whitney U test. In paper IV, comparison between two groups of paired non-parametric data was performed by Wilcoxon matched-pairs signed-rank test and between more than two groups of non-parametric data by Kruskal-Wallis test with Dunn´s multiple comparison post hoc test. For all comparisons, p values <0.05 were considered statistically significant.

Results and Discussion

Using the microsphere technique to identify islet subpopulations

The microsphere technique has been used since 1967 to measure intra-organ distribution of blood flow [115]. The technique is based on an injection of small polystyrene particles (i.e., microspheres) into the arterial blood stream. These microspheres are distributed to the different organs together with the arterial blood and become entrapped in the capillary beds of these tissues. The number of microspheres in the organs may be quantified by either direct calculating or extracting labels such as radioactivity or fluorescence and is directly proportional to the tissues blood perfusion [25]. There are several criteria that need to be fulfilled to ensure adequate usage of the technique: (1) adequate mixing of microspheres in the circulating blood, (2) the microspheres should be completely cleared from the blood within the first passage through the tissues, (3) rheological properties of microspheres should be similar to that of erythrocytes, (4) the microspheres should not disturb or cause artifacts of the normal circulation, and (5) the microspheres should remain in the tissue after injection [4].

In our experiments, we injected the microspheres into the ascending aorta through a catheter inserted into the carotid artery. The blood flow in the ascending artery is turbulent and locating the microsphere injection at this site will facilitate proper microsphere mixing with the arterial blood without interfering with the cardiac output or aortic flow [4]. To ensure that adequate mixture and distribution of microspheres has occurred, paired organs like the adrenal glands can be studied. In our experiments, we included only animals with less than 10% variation in the number of microspheres found in the two glands to ensure that the injection was successfully executed.

The microspheres must also be cleared from the blood-stream in the first passage since recirculation of microspheres would lead to an overestimation of blood flow. To achieve this, the microspheres should be larger than the smallest arterioles and capillaries in the tissues. To minimize shunting of microspheres through the tissues, larger microspheres are preferred, but the usage of smaller microspheres enables entrapment in smaller arterioles and capillaries which gives the method a higher resolution [4]. The size of 10μm has been shown to be the best size of microspheres given to rats [18], and when using this size, approximately 98% of the microspheres are captured during

the first circulation [4]. In studies in mice, a diameter of 11μm showed minimal microsphere shunting (less than 2% of the pancreatic microspheres were found in the portal blood). The actual recirculation of microspheres is thus thought to be minimal, both due to the low number of shunted microspheres into the portal blood but also due to the low shunting (<0.05%) through the pulmonary bed to the pancreas [24].

At the size of 10-15μm, the microspheres have been seen to have similar properties as red blood cells and have also shown to cause minimal rheological influence, which is crucial when to avoid non-proportional distribution of microspheres [25]. Mainly, issues with "skimming," a phenomenon in which large microspheres travel in the center of the vessel, and blocking of afferent arterioles due to the size of the microspheres are arguments for using microspheres in the smaller range[116].

The number of injected microspheres is also important when calculating the risk for circulatory artifacts. A too large number of microspheres may affect both local microcirculation causing local ischemia and the general circulation. In rats, often but not always, more than one million microspheres can be given without any major effects on the general circulation [117, 118]. The use of 300,000 microspheres in ~300-gram rats and 100,000 microspheres in mice, numbers similar to the number of microspheres used in our experiments, have previously been thoroughly validated without any hemodynamic side effects [4]. Even though a low number of microspheres is wanted to minimize circulation disturbances, it is also important to have enough microspheres in the reference sample and the studied tissues to obtain accurate measurements of blood flow and to minimize the importance of statistical variation. Statistical calculations have shown that blood flow measurements with 10% accuracy necessitate 384 microspheres in the reference sample and the studied organs [119]. This number has later been questioned due to empirical testing which indicated that numbers of 200 microspheres per sample down to 50 still result in low experimental variation [117, 120]. Also in pancreatic islets, as few as 30 microspheres have been demonstrated to result in reproducible blood flow values [121].

The last criterion which needs to be fulfilled for accurate blood flow measurements is that the microspheres must remain in the tissue after the injection. This has been studied with 10μm microspheres in rats by repeated microsphere injections [122]. To separate the different injections, microspheres with two different colors were used. These experiments showed that the basal blood flow calculated with microspheres from the first injection did not differ from calculated blood flow calculated with the microspheres from the second injection indicating that the microspheres stay in the tissue after injection. These results also indicate that injection of 10μm microspheres deso not disturb the microcirculation of the pancreas.

The microsphere technique has been thoroughly validated for its most common use, to estimate tissue blood flow reproducibly. The technique has, however, also been used for the identification of a subpopulation of highly blood-perfused islets by Lau et al. in 2012 [86]. This study identified a highly functional subpopulation of islets by the occurrence of microsphere content suggesting a correlation between blood flow and metabolic activity.

When using the microsphere method for purposes other than blood flow measurement, certain cautions need to be taken to minimize the risk of experimental error. Especially, when using the technique for identification of an islet subpopulation, the requirement of minimal number of microspheres in the studied organ should be carefully addressed. When blood flow is measured in islets, all islets are considered one organ, and the number of microspheres in all islets is summarized and reaches wanted threshold levels for statistically significance [4]. The total number of microspheres in all islets is consequently stable, but the specific content in a single islet may be considered random. However, this hypothesis is not confirmed by repeated microsphere injections. If the microsphere content in each islet is random and does not reflect the perfusion of the islet, the microspheres would be randomly distributed in the islets after the two injections. Instead, the second injection of microspheres is shown to be, to a large extent, captured in the same islets as the microspheres after the first injection, indicating that islets with a high blood flow have an increased likelihood of capturing a microsphere [122]. Another study also indicating non-random microsphere distribution shows that the increase in blood flow seen in islets after glucose administration is preferentially in the already well-perfused islets marked by microspheres shown by a further increase in the number of microspheres in these islets. The fraction of microsphere-containing islets is, however, unchanged, indicating that the increased blood flow is not achieved by recruiting more islets to a well-perfused state [101].

Microspheres do not induce cell death or islet inflammation

It is unlikely that the presence of microspheres would elicit cell death or inflammation in islets. Islets with microspheres have previously been shown to have a better glucose-stimulated insulin release and a higher beta cell proliferation [86] than other islets which contradict any harmful effects from the microspheres.

When studying cell death of the islets in paper I, there was also no significant difference between the two control groups when cultured in standard media (PI/bisbenzimide ratio 0.7 ±0.1 AU vs. 0.6 ± 0.1 AU in islets with- or without microspheres, respectively, n=17 animals). To further evaluate the effect of microspheres on the viability of the islet cells, we measured the cell-

death ratio in the near vicinity (radius 50μm) of the microspheres compared to the whole islet. Islets exposed to cytokines or hypoxia showed a similar degree of cell death near the microspheres when compared of that in the whole islet (109 ± 19% and 100 ±11%, respectively, n=6 animals).

In paper III, the long-term effects of microsphere content were studied in control experiments with diabetes resistant BB-rats. Microspheres in these animals were injected before day 40 of age and at day 50 of age, the pancreases were removed and taken to histological analysis. This resulted in microsphere presence in the tissues for at least 10 days before analysis. Interestingly, no inflammation or cell death was observed in any islets of these animals, despite microsphere content, further indicating no harmful side effects of microspheres on the islets.

Highly blood-perfused islets are more sensitive to cellular stress *in vitro*

Two major environmental factors affecting the islets during, and early after, transplantation are cytokines [43, 123] and the loss of the vascular system causing limited oxygen supply before engraftment [124]. When challenging the islets by IL-1β, IFN-γ and TNF-α exposure in paper I, we observed an increase in cell death, measured as a PI-to-bisbenzimide ratio, compared to size-matched islets incubated under equal conditions but without cytokines. Also, hypoxia (1.5% oxygen for 4 hours) increased the cell death among islets. Interestingly, when comparing islets with- or without microspheres, we observed an approximately 50% higher ratio of dying cells in the islets containing microspheres after exposure to cytokines (PI/bisbenzimide ratio 1.9 ± 0.2 AU vs. 1.3 ± 0.1 AU in islets with or without microspheres, respectively, n=8 animals). Likewise, hypoxia augmented the cell death by 40% in islets with microspheres (PI/Bisbenzimide ratio 1.4 ± 0.2 AU) compared to non-microsphere-containing islets (PI/Bisbenzimide ratio 1.0 ± 0.1 AU, n=9 animals) indicating that islets with microspheres are more susceptible to cellular stress than other islets. When the two groups of islets were incubated in normal culture media, no difference in cell death could be observed between the groups. It should be noted that the tested cytokines play an role not only after transplantation; they are also known to be involved in beta cell death during the development of T1D [125-127]. This opens the question of how microsphere-containing islets are affected *in vivo*, for example during transplantation and the development of T1D when compared to other islets.

Highly blood-perfused islets are more sensitive to cellular stress after transplantation

To further evaluate the susceptibility of cells in the subpopulation of islets containing microspheres, we syngenically transplanted the islets under the renal capsule in paper I. This enables islet exposure to hypoxia [45, 124] and cytokines [43] in an *in vivo* model.

Two days after transplantation, the grafts were strongly hypoxic, as identified by pimonidazole staining. On average, 61 ± 3% of the grafts stained positive for the marker. In the center of the hypoxic area, necrotic regions containing disintegrated cells or cellular debris were visible. The area of those regions was almost doubled in the grafts of islets with microspheres when compared to grafts of islets without microspheres (32 ± 2% vs. 17 ± 1%, respectively, n=6 grafts). Similarly, apoptotic cell death, as identified by positive cleaved caspase-3, was augmented with ~20% in grafts with microsphere-containing islets (48 ± 2% vs. 39 ± 2% in grafts of islets with or without microspheres, respectively, n=6 grafts).

Pancreatic islets are vulnerable early after transplantation and a high cell death may contribute significantly to subsequent graft failure [128]. To evaluate the severity of the damage the graft had suffered after transplantation, the amount of connective tissue was evaluated 30 days post transplantation. The more substantial cell death early after transplantation in the microsphere-containing islets resulted in an ~30% increase in connective tissue one month after transplantation (50 ± 2% vs. 29 ± 2% in grafts of islets with or without microspheres, respectively, n=6 grafts). Since the microsphere-containing islets have a superior functionality and insulin release compared to islets without microspheres [86], preferential death of cells in these islets may lead to a further decrease in graft function after transplantation.

Highly blood-perfused islets become better engrafted

The ability of the transplanted tissue to form new blood vessels and rebuild a vascular network after transplantation is crucial for the survival of the graft. Even though islets are revascularized after transplantation, the vascular density and oxygen tension is not recovered to the extent of native islets [45, 129, 130]. Islets with microspheres have in the native pancreas a higher vascular density and wider vessel diameter [86], and when these islets were transplanted in paper I, they regained a denser vascular network (4.2 ±0.3% vs. 2.7 ± 0.1% in grafts of islets with or without microspheres, respectively, n=6 grafts) with more vessels penetrating into the endocrine tissue when compared to transplanted islets without microspheres. This indicates the presence of an intrinsic difference between these groups of islets. Indeed, differences in gene expression of several genes including increased VEGF-A expression in islets

with microspheres have previously been described [86] which may explain the vascular properties of these islets both in their native state and after transplantation.

To evaluate the functional capacity of the new vessels in the grafts, blood flow and oxygen tension measurements were performed. The grafts of islets with microspheres had a 50% higher blood flow (14.1 ± 0.6 TPU) when compared to grafts of islets without microspheres (9.3 ± 0.8 TPU, n=6 grafts). An even more prominent effect on oxygen tension was seen in grafts of islets with microspheres (30.3 ± 2.2 mmHg) when compared to grafts of islets without microspheres (8.7 ± 1.3 mmHg, n=6 grafts) with values similar to the surrounding kidney cortex (30.2 ± 4.5 mmHg). Increased revascularization has previously been shown to improve the restoration of islet function [131, 132], which highlights the importance of successful engraftment. Unfortunately, as seen in the previously described experiments, a large fraction of these islets does not survive the transplantation process. This highlights the importance of improving islet isolation and transplantation to minimize the loss of the most highly functional islets.

Insulin but not IAPP release is higher in highly blood-perfused mIAPP$^{-/-}$/hIAPP$^{+/+}$ mouse islets

After three months of HFD, islets were isolated and investigated for glucose-stimulated insulin release (paper II). Islets with microspheres were size-matched to other islets, and no difference in DNA content could be detected between the two groups. At low glucose concentrations (1.67 mmol/l), no differences regarding insulin or IAPP release was seen between islets with microspheres (289 ± 26 fmol insulin/islet/h; 2.1 ± 0.4 fmol IAPP/islet/h) when compared to islets without microspheres (302 ± 32 fmol insulin/islet/h; 2.0 ± 0.3 fmol IAPP/islet/h, n=6 animals). However, when exposed to high glucose (16.7 mmol/l), insulin release was augmented with ~30% in islets containing microspheres (1283 ± 188 fmol insulin/islet/h) when compared to other islets (987 ± 156 fmol insulin/islet/h, n=6 animals). This feature has previously also been described in rat islets [86]. Interestingly, the better response to glucose in microsphere-containing islets was not seen regarding IAPP release, where no difference could be detected between the groups (9.3 ± 1.1 vs. 10.3 ±1.9 fmol IAPP/islet/h from islets with or without microspheres, respectively, n=6 animals). This resulted in an ~30% decrease in the released IAPP to insulin ratio (ratio 4.7 ± 0.7 vs. 3.2 ± 0.3 in islets with or without microspheres, respectively, n=6 animals). IAPP and insulin are produced and co-secreted from the beta cells, and an increase in insulin release is therefore also expected to be in combination with an increase in IAPP secretion [64]. However, if an

increased amount of proIAPP or partly processed IAPP was formed in the microsphere-containing islets, the ELISA which binds only to fully processed mature IAPP may result in imprecise low values, particularly when compared to the insulin ELISA which, according to the manufacturer, cross-react (14%) with proinsulin. Another explanation could be that IAPP is partly bound to the islets and therefore not assessable during ELISA measurements of the supernatant. IAPP is known to bind heparan sulfate located in the basal membrane of the beta cells [133, 134], but the measured intensity of the glycosaminoglycan in islets with or without microspheres did not differ (reciprocal intensity 131 ± 6 AU vs. 137 ± 3 AU, respectively, n=4 animals). However, the N-terminal of non- or partly processed IAPP has a specific affinity for heparan sulfate which may, if present, facilitate retention of IAPP in the islets [135].

Augmented amyloid formation in highly blood-perfused and highly functional islets

Isolated islets from mIAPP$^{-/-}$/hIAPP$^{+/+}$ mice on HFD were size-matched to islets without microspheres and analyzed for amyloid by Congo Red staining (paper II). Amyloid was identified by green birefringence seen under polarized light and was found more frequently in islets with microspheres (67%) than in those without microspheres (42%). The area of amyloid was also twice as extensive in islets with microspheres (0.2 ± 0.06%) when compared to islets without microspheres (0.1 ± 0.03%, n=7 animals).

The propensity of human islets to develop amyloid deposits was also studied in paper II. As the microsphere method is restricted to experimental animal models, we used the glucose-stimulation index at day 0 as a surrogate marker for the highly functional islets. After 18 days of high glucose culture [136, 137], amyloid deposits were formed in the islets (range 0 - 30% of islet area), and the correlation between initial release capacity and amyloid development was investigated. Interestingly, islets prone to amyloid development (>1% of islet area) had an initial higher insulin release capacity (stimulation index of 13.2 ± 1.1, n=59 islets from four different donors) when compared to islets with little or no amyloid (<1%; stimulation index of 8.8 ± 1.0, n= 58 islets from four donors).

Amyloid has in previous studies been identified in post-mortem material from patients with T2D and after transplantation and is associated with loss of beta cell mass and function in both humans and rodents [36, 138-140]. It is thus probable that the preferential amyloid development in highly blood-perfused islets will affect these islets negatively. Such selective impairment may further decrease islet function during both T2D and transplantation, which will affect the outcomes of these two conditions negatively.

Prohormone convertase 1/3 is increased in highly blood-perfused islets

The mechanism behind amyloid formation has been studied comprehensively with results indicating that the IAPP molecule itself is an important factor for aggregation. A hypersecretion of IAPP is most likely a contributor to the development of amyloid but is not sufficient as a single cause [141]. The unprocessed or partly processed ProIAPP has, on the other, hand been shown to be more prone to develop amyloid and a hypersecretion of proIAPP due, for example, to a processing impairment may initiate aggregation [112, 142]. ProIAPP is processed to mature IAPP by the two prohormone convertases PC 1/3 and PC 2 [61, 62]. Glucose has been described as increasing the biosynthesis of PC 1/3 but not PC 2 in culture [143]. To evaluate changes in the processing of proIAPP, gene expression and the amount of PC 1/3 and PC 2 was evaluated. No differences in either PC 1/3 or PC 2 expression could be detected between islets with or without microspheres (fold change 0.90 ± 0.17 (PC 1/3) and 1.02 ± 0.22 (PC2)), in islets with microspheres relative to islets without microspheres, n=6 animals). However, studies have indicated that the regulation of the enzymes may be on a post-transcriptional level [144]. Therefore, the amount of the two convertases was instead evaluated by immunohistochemistry which revealed a higher PC 1/3 intensity by ~17% in islets with microspheres (relative intensity 1.17 ± 0.02 AU when compared to islets without microspheres, n=4 animals). Amount of PC 2 was, on the other hand, similar between the two groups (relative intensity 0.99 ± 0.05 AU when compared to islets without microspheres, n=4 animals).

To further evaluate the involvement of the two prohormone convertases for the increased amyloid development seen in microsphere-containing islets, the expression of their regulators proSAAS and 7B2 was studied. Interestingly, the expression of proSAAS, which regulates PC 1/3 activity, was lower in microsphere-containing islets (fold change 0.54 ± 0.13 relative to islets without microspheres, n=6 animals), indicating a potential increase in the activity of the enzyme [145]. Expression of 7B2, the endogenous regulator of PC 2, was, however, similar between the two groups (fold change 1.22 ± 0.46 in islets with microspheres relative to islets without microspheres, n=6 animals). The higher PC 1/3 amount, together with the lower expression of its regulator proSAAS, indicates an increase in the processing performed by PC 1/3. PC1/3 can process both the N- and C-terminal of insulin, but only the C-terminal of IAPP [61, 62, 146]. A higher activity of PC 1/3 may, therefore, lead to an increased processing of insulin but also to a misbalanced processing of IAPP, resulting in an accumulation of unprocessed N-terminal, which has been identified in human amyloid deposits [147]. This proform has been described as the most detrimental form of proIAPP for amyloid formation due to its high affinity for heparan sulfate and amyloidogenic features [58, 62, 135]. Unfortunately, in our studies, we were unable to measure the N-terminal.

Highly blood-perfused islets have a higher expression of matrix metalloproteinases

To investigate if the degradation of amyloid was impaired or affected in the microsphere-containing islets the expression of insulin-degrading enzyme (IDE) and neprilysin, both known for their ability to degrade amyloid, were studied. No difference in enzyme expression could be detected between the two groups of islets (fold change 1.5 ± 0.5 (IDE) and 1.2 ± 0.7 (neprilysin) in islets with microspheres relative to islets without microspheres, n=4-6 animals) [148, 149]. However, another amyloidogenic peptide, Aβ, has been described as increasing the transcription of vascular matrix metalloproteinase-9 (MMP9), which in turns degrades the peptide to more soluble and less amyloidogenic fragments [150, 151]. To investigate if islet amyloid also may affect the expression of matrix metalloproteinases, the expression of MMP9 and MMP2 was studied. Interestingly, the expression of MMP2 and MMP9 was higher in the microsphere-containing islets (fold change 7.6 ± 1.3 (MMP2) and 4.3 ± 2.1 (MMP9) relative to islets without microspheres, n=5-6 animals). Whereas no difference could be detected in the expression of their endogenous inhibitors, tissue inhibitor of matrix metalloproteinase (TIMP) 1, 2, and 3 (fold change 1.8 ± 0.8 (TIMP1), 1.1 ± 0.2 (TIMP2), and 1.1 ± 0.3 (TIMP3) relative to islets without microspheres, n=6-7 animals). Thus, one can argue that higher MMP 2 and MMP 9 expression may be mediated through stimulation by the fibrillation process or formed amyloid as a rescue mechanism aiming to reduce the amyloid load. However, MMP9 has also been shown to be important for islet function and angiogenesis [152, 153], which are two characteristics of the microsphere-containing islets and the increase in expression may thus simply reflect the phenotype of these islets [86].

Using retrograde injection to identify islets with venous drainage

While one to three arterioles are entering the islets and supplying the tissue with blood rich in oxygen, the vascular anatomy defining how the blood exits the islet may differ between islets[4]. The efferent vessels of the islet may either empty into venules which are directly transporting the blood to the portal vein, or the islet capillaries may be connected to an insulo-acinar portal system. The latter means that two serially connected capillary systems incorporate the efferent vessels of the islets with the exocrine capillary network [16, 154]. The vascular network is formed differently in different species. In larger animals, like primates, the portal system seems to be more common [16]. These previous studies have been investigating vascular anatomical differ-

ences mainly with corrosion casts or perfusion with ink, but also with retrograde injection of microspheres [155]. Identification of venous drainage by retrograde injection is based on the anatomical properties of the two vascular systems. Microspheres will be able to reach the islets with venous outflow but will be trapped in the exocrine capillaries before reaching the islets with insulo-acinar outflow.

An alternative explanation of why retrograde microspheres get trapped in the vasculature of the islet, could be that the microspheres are forced into the capillaries by high pressure from the injection which destroys and enlarges the capillaries. To minimize the risk of destroying the vasculature during retrograde injection, a very low injection rate (100µl/min) was used in our experiments. To our knowledge, this injection rate does not affect the islet vasculature significantly. To control this, we calculated blood flow in the pancreas by anterograde injection alone or in combination with retrograde injection. If the infusion rate were demising the vasculature, the retrograde injection would most probably flush out the previously injected anterograde microspheres and thus result in erroneous low blood flow values. There was however no difference in calculated blood flow between animals subjected to an anterograde injection alone or in combination with a subsequent retrograde injection (pancreatic blood flow 1.63 ± 0.73 ml x min^{-1} x g $pancreas^{-1}$ vs. 1.97 ± 0.18 ml x min^{-1} x g $pancreas^{-1}$; islet blood flow 136.3 ± 50.1 µl x min^{-1} x g $pancreas^{-1}$ vs. 165.3 ± 7.4 µl x min^{-1} x g $pancreas^{-1}$, n=3 animals in both groups).

To further control vascular and tissue damage, we also isolated the islets after retrograde injection and evaluated their function. In low glucose, no leakage of insulin was detected when we compared islets with or without retrograde microspheres (0.38 ± 0.05 ng/islet/h vs. 0.30 ± 0.07 ng/islet/h, n=7 animals) which would be expected if the islets were damaged.

Preferential venous drainage and lower glucose threshold in highly blood-perfused islets

To investigate whether vascular anatomical differences distinguish the subpopulation of highly blood-perfused islets from other islets, combined anterograde and retrograde injection was performed. After anterograde and retrograde injection, 81.0 ± 1.8% of the islets contained no microspheres, 14.8 ± 1.8% of the islets contained anterograde microspheres, and 4.5 ± 0.6% contained retrograde microspheres. Both anterograde and retrograde microspheres were found in 2.0 ± 0.2% of the islets (n=8 animals). When calculating the likelihood of venous drainage, it was found that highly blood-perfused islets have a 3.2 ± 0.3 times higher probability for venous drainage than other islets.

The release capacity of islets with retrograde microspheres after isolation was studied to characterize this subpopulation of islets. Interestingly, the insulin release was increased in high glucose (2.40 ± 0.35 ng/islet/h) when compared to islets without retrograde microspheres (1.8 ± 0.33 ng/islet/h, n=7 animals), a feature also seen in islets with anterograde microspheres [86]. The presence of this characteristic release pattern seen in islets after both anterograde and retrograde injection further indicates that the two injections identify the same subpopulation of islets, that is, that the highly blood-perfused islets have a venous drainage.

When studying the glucose threshold for insulin release by perifusion we found that highly blood-perfused islets, marked by anterograde microspheres, had a lower threshold when compared to other islets. Already at 2 mmol/l glucose, the islets with microspheres had an increased release (0.21 ± 0.04 ng/min/50 islets) when compared to islets without microspheres (0.12 ± 0.04 ng/min/50 islets). This increase in release capacity was also seen in islets with microspheres at glucose levels of 4 mmol/l (0.34 ± 0.05 ng/min/50 islets with microspheres vs. 0.22 ± 0.04 ng/min/50 islets without microspheres) and 6 mmol/l (0.71 ± 0.08 ng/min/50 islets with microspheres vs. 0.52 ± 0.07 ng/min/50 islets without microspheres, n=7 animals) but not at higher glucose levels.

The hormones released by islets with a venous drainage will reach the portal blood and quickly be distributed to the liver and other peripheral tissues. Islets with an insulo-acinar portal system may, on the other hand, effect the surrounding exocrine tissue [16]. The difference in glucose threshold may indicate that the different types of islets not only disperse their released hormones to different target tissues, they may also have a different purpose in glucose homeostasis. If a subpopulation of islets, like the highly blood-perfused islets, increases their insulin secretion already at low glucose levels and have a quick release pathway to the peripheral tissue, these islets may be of high importance for the fine-tuning of the glucose homeostasis. To have the ability to respond to a small increase in blood glucose by increasing the secretion in only a fraction of the islets may be favorable as it decreases the risk of subsequent hypoglycemia. However, when the blood glucose concentration increases significantly and is above the level of immediate risk of hypoglycemia, more islets may be activated to decrease the glucose levels more efficiently. This dynamic response to glucose obtained by subpopulations of islets may indicate the importance of having the endocrine tissue divided into smaller micro-organs with independent regulation.

Higher susceptibility to immune cell infiltration in the highly blood-perfused islets of the prediabetic BB-rat

The previously shown susceptibility to cellular stress in the highly blood-perfused islets may also predispose the islets for immune cell infiltration during T1D development. To study the potential involvement of the highly blood-perfused islets in disease development, these islets were studied during the prediabetic phase in an animal disease model of T1D, the BB-rat. Microspheres were injected at an age of 40 days or younger to mark highly blood-perfused islets before the presence of insulitis [156]. At this time-point, all animals were normoglycemic (diabetes prone 5.7 ± 0.2 mmol/l, n=6 animals; diabetes resistant 4.6 ± 0.3 mmol/l, n=3 animals). At day 50, all animals remained normoglycemic (diabetes prone 4.3 ± 0.2 mmol/l; diabetes resistant 4.5 ± 0.3 mmol/l) although 43 ± 0.04 % of the islets of the diabetes prone animals showed signs of insulitis. Interestingly, islets with microspheres preferentially developed insulitis (72 ± 0.06 %, n= 6 animals) indicating that highly blood perfused islets may be affected more severely or earlier during disease development. No signs of insulitis were observed in any islets in the diabetes resistant animals, despite microsphere content, demonstrating no harmful effect of the microspheres themselves.

T1D patients have higher glucose threshold for insulin release when compared to healthy individuals

Results from our studies indicating an increased susceptibility of highly blood-perfused and functional islets in a T1D model urged us to investigate whether it was possible to identify such susceptibility also in humans. The microsphere technique is, however, restricted to experimental animals due to the invasive properties of the technique and may not be used in humans. For human studies, we instead used the previously identified lower threshold for glucose-stimulated insulin release (measured as released C-peptide) to estimate activity of islet subpopulations at different blood glucose concentrations. For this, we recruited T1D patients with remaining C-peptide secretion and age- and gender-matched healthy volunteers. As expected, T1D patients had higher HbA1c (6.5 ± 0.3% (48 ± 4 mmol/mol), n=8 patients) when compared to healthy controls (5.0 ± 0.1% (30 ±1 mmol/mol), n=10 patients). Also, fasting blood glucose levels were increased in these patients (7.4 ± 0.6 mmol/l relative to 5.3 ± 0.1 mmol/l in controls). The maximum C-peptide release in the T1D patients was 12 % of that in healthy subjects indicating a substantial loss of beta cell function. When investigating C-peptide release while stepwise increasing blood glucose levels, an increased response to glucose was seen in all steps in healthy individuals. However, in T1D patients, no increase from

basal insulin secretion level could be detected when blood glucose was increased from four to six mmol/l. In these patients, an increase could first be detected at eight mmol/l. A diminished response at low glucose levels is consistent with our previous findings showing that the highly functional islets which are more responsive at low glucose levels are preferentially targeted early in disease development. A preferential death of this subpopulation of islets could, therefore, be expressed as decreased insulin release activity in low glucose. It should however be noted that we cannot exclude the possibility that glucose desensitization may play an important role in the T1D patients. We did, however, identify the same pattern of release capacity in two patients with well controlled Hba1c within the normal range (5.4% (36 mmol/mol) and 5.7% (39 mmol/mol)), which indicates that glucose sensitivity is not a major contributor to this finding. A preferential death of the highly functional islets may increase the severity of the disease and speed up the disease progress. This highlights the importance of decreasing the immune attack and beta cell stress early in disease development to rescue these highly functional islets. Interestingly, such attempts have been performed by inducing beta cell rest in T1D patients, which resulted in temporarily preserved residual C-peptide secretion [157, 158].

Using pimonidazole to detect low-oxygenated islets

Pimonidazole is a 2-nitroimidazole which rarely occurs naturally and provides a method to identify low-oxygenated cells in living organisms. The molecule can be efficiently metabolized by most organisms by using nitroreductase enzymes and form stable adducts which may be identified by immunohistochemistry, ELISA, or Western Blot. The compound has been widely used in different forms of tumor research and accumulates in cells with an oxygen tension below 10 mmHg [108, 159].

Pimonidazole metabolism has been discussed as potentially affected by intracellular levels of NADPH and thus inducing the reductive metabolism of 2-nitroimidazoles [160]. However, in liver studies, reduction of pimonidazole has been shown to not depend on NADPH levels [161]. This concern has, however, not been addressed in islets and, as hyperglycemia may induce NADPH levels, we chose to perform all experiments at normoglycemia, similar to previously used protocols on islets [107]. Interestingly, glucose has been suggested to induce hypoxia in islets *in vitro,* but increased blood glucose concentrations in otherwise healthy mice have been shown not to increase pimonidazole accumulation [162].

Pimonidazole has previously been evaluated in pancreatic islets and dispersed beta cells, the threshold for pimonidazole adduct accumulation was set to an oxygen tension around 10 mmHg [107].Pimonidazole adducts have also

been identified in the islet core after culture, preferentially in large islets, indicating the diffusion limitations in the islets [40, 107]. The oxygen tension in superficial islets of the pancreas have previously been shown to be around 40 mmHg [42], and a positive staining for pimonidazole thus indicates a substantial decrease in islet oxygen tension. However, the low staining intensity seen in a proportion of the native islets when compared to hypoxic regions of an islet transplant most likely indicates a state of low-oxygenation in these islets, and not true hypoxia.

Low-oxygenated islets are a stable subpopulation

Previously, low-oxygenated islets have been identified by pimonidazole administration two hours before tissue removal [107]. In our experiments, 29.9 ± 2.5% of the islets were positively marked after such administration (n=6 animals). Islet staining by pimonidazole gives a momentary view of the islet oxygenation due to the half-life in rodents of 30 minutes [163]. To investigate if islet oxygenation varies over time or if the low-oxygenated islets are a stable subpopulation, we administered pimonidazole by a single or by repeated injections over an eight-hour period. To control pimonidazole adduct stability, the substance was administered, and the pancreas was removed after four, eight, or 24 hours. Adducts were identified after four (32.0 ± 3.2% positive islets, n=5 animals) and eight (32.6 ± 2.8% positive islets, n=5 animals) hours, but after 24 hours, adducts were very faint or unidentifiable. To investigate if the same islets are low-oxygenated over time, repeated administrations were performed with two or six hours between the injections. When studying islet adducts after repeated pimonidazole injections, two outcomes were possible. If the oxygenation of the islets varied over time, different islets would accumulate adducts during the two injections and thus increase the number of positively stained islets. If instead, the oxygenation in islets was stable over the investigated period, a similar number of positive islets would be detected after both single and repeated administration of pimonidazole. In our study, the latter outcome was observed with 30.0 ± 1.8% positive islets after repeated administration with two hours between the injections and 31.3 ± 3.5% positive islets after repeated administration with six hours between injections (n=5-6 animals). These results indicate that the low-oxygenated islets also constitute a separate subpopulation of islets, however with the disadvantage of a restricted study length of eight hours.

Characterization of low-oxygenated islets

The indication of a stable subpopulation of low-oxygenated islets initiated further characterization of the group of islets. Firstly, islet cell composition was

studied. There was no difference in beta cell fraction in the islets positive (75.2 ± 1.8%) or negative (76.0 ± 1.5%, n=6 animals) for pimonidazole adducts. Likewise, no difference in alpha cell fraction was seen between the positive (16.8 ± 1.8%) and negative (17.0 ± 1.5%, n=6 animals) islets. The pimonidazole positive islets did, however, have a lower vascular density (5.3 ± 1.1%) when compared to islets negative for pimonidazole adducts (7.3 ± 1.2%, n=6 animals) in insulin-positive areas. This may explain the lower oxygenation of these islets and indicate that the lower blood perfusion in these islets is not strictly regulated at an arteriolar level. It is well known that islet endothelial cells are closely connected to beta cell function and proliferation [11, 86, 106, 164], and a decreased vascular density could, therefore, decrease such paracrine signaling between the cell types. To investigate such effect on the endocrine cells, endocrine cell proliferation was studied. No difference in alpha cell proliferation could be detected between pimonidazole positive (1.5 ± 0.4%) and negative (1.6 ± 0.4%, n=6 animals) islets. However, the beta cell proliferation was decreased in pimonidazole positive islets (1.8 ± 0.3%) when compared to negative islets (3.2 ± 0.4%, n=6 animals). This could be explained as a result of the detected decrease in islet vasculature. However, the possible effect of decreased metabolic activity on beta cell turnover could not be discarded. Interestingly, the pimonidazole positive islets have, in fact, been described to have a 50% lower leucine-dependent protein biosynthesis [107], and most probably also a consequently decreased glucose metabolism which is an important regulator of beta cell proliferation [165]. These previous findings suggesting the pimonidazole positive islets to be dormant [107] in combination with the present proliferation results may further strengthen the hypothesis of blood perfusion as an indicator of islet endocrine function as discussed in this thesis and previously [86].

Conclusions

Paper I

- Highly functional rat pancreatic islets are more sensitive to cellular stress *in vitro* than other islets, as indicated by more cell death when exposed to the cytokines IL-1β, IFN-γ, and TNF-α for 24 hours or 1.5% hypoxia for 4 hours.
- Highly functional rat pancreatic islets are more sensitive to cellular stress *in vivo* than other islets, as indicated by more apoptosis and necrosis two days after transplantation followed by extensive fibrosis 30 days after transplantation.
- Highly functional rat pancreatic islets become better engrafted than other islets, as indicated by superior vascular density, blood flow, and oxygen tension 30 days after transplantation.

Paper II

- Highly functional mouse pancreatic islets have a higher susceptibility for amyloid deposits than other islets after 10-months of HFD.
- Islet amyloid preferentially develops in human islets with a high function.
- Highly functional mouse islets have a higher MMP 2 and MMP 9 expression than other islets, either contributing to the islet phenotype or stimulated by an increased amyloid formation.
- Highly functional islets have, when compared to other islets, a higher amount of PC 1/3 enzyme and a lower expression of its endogenous inhibitor proSAAS while having a similar amount of PC 2 enzyme and expression of its corresponding endogenous inhibitor 7B2. These findings indicate an overall enhancement in PC 1/3 processing but not PC 2 processing, which may lead to a misbalance of pro-hormone processing and subsequent amyloid formation.

Paper III

- Highly functional islets have an increased likelihood of direct venous drainage when compared to other islets
- Highly functional islets have a lower glucose threshold for insulin release than other islets and, thus, secrete insulin to a greater extent at lower glucose levels.
- Highly functional islets are more severely affected by insulitis than other islets in the prediabetic BB-rat.
- Recent onset type 1 diabetic patients have a higher glucose threshold for insulin release when compared to healthy controls. This may be caused by a selective autoimmune attack with subsequent destruction of the most highly functional islets.

Paper IV

- Low-oxygenated islets are a stable subpopulation of islets.
- Low-oxygenated islets have a similar composition of endocrine cells and a lower vascular density when compared to other islets
- Low-oxygenated islets have a decrease in beta cell proliferation when compared to other islets.

Sammanfattning på svenska

Diabetes Mellitus är en grupp av endokrina sjukdomar som har gemensamt att kroppens behov av insulin inte kan tillgodoses. Detta leder till att kroppens vävnader inte kan ta upp glukos vilket in sin tur leder till att celler i dessa vävnader svälter samtidigt som sockerhalter i blodbanan stiger okontrollerat, ett tillstånd som i längden är livshotande. De två mest kända typerna av diabetes är typ 1 diabetes, tidigare känt som ungdomsdiabetes, och typ 2 diabetes, tidigare känt som åldersdiabetes. Vid typ 1 diabetes angrips de insulinproducerande cellerna av kroppens egna immunförsvar och förstörs, medan vid typ 2 diabetes är det i första hand en insulinresistens i kroppens vävnader som leder till en relativ insulinbrist som orsakar sjukdomen.

Insulin produceras av kroppens beta celler som ligger grupperade med andra endokrina celler i öar om ca 3000-4000 celler, de sk. Langerhanska öarna. Hos människor finns omkring 1-3 miljoner av dessa öar utspridda i bukspottkörteln. Öarnas huvuduppgift är att frisätta glukosreglerande hormoner i kroppen som gör att vi både kan ta upp samt lagra glukos i kroppen.

De Langerhanska öarna har länge ansetts vara ett endokrint organ där alla öar har samma funktion. Detta synsätt har dock blivit allt mer ifrågasatt och under de senaste åren har teorier om subpopulationer av öar med olika funktion presenterats. I denna avhandling studeras förekomsten av två subpopulationer av Langerhanska öar, deras funktion samt inblandning under sjukdomsutveckling.

I det första delarbetet studerades en grupp högt genomblödda öar som tidigare har visats ha en bättre insulinfrisättande funktion samt högre celldelning än andra öar. Dessa öar kan märkas ut med hjälp av mikrosfärer som injiceras i blodet och som följer blodets flöde ut till alla kroppens vävnader. Mikrosfärerna fastnar i kroppens minsta kärl, kapillärerna, och antalet sfärer i en vävnad korrelerar direkt med vävnadens blodflöde. I studie I studerades känsligheten av dessa öar för yttre stress i form av cytokiner och låg syresättning, hypoxi. Efter att öarna utsatts för cytokin- eller hypoxistress tog de högfunktionella öarna större skada i form av ökad celldöd än andra öar. För att ytterligare studera öarnas känslighet transplanterades även grupper av högfunktionella och övriga öar under njurkapseln på råttor. Vid transplantation utsätts den transplanterade vävnaden för stor yttre påverkan, till exempel så förstörs den ursprungliga blodtillförseln till öarna och den transplanterade vävnaden utsätts således för stor hypoxistress innan nya kärl har växt in i den. Transplantaten studerades två dagar och 30 dagar efter transplantation och även i denna

modell skadades de högfunktionella öarna i större utsträckning än andra öar. Transplantat av högfunktionella öar hade dock efter 30 dagar återfått fler kärl och ett högre blodflöde, två egenskaper som karakteriserar subpopulationen i dess normala miljö och som även visats vara väsentligt för den transplanterade vävnadens funktion.

I studie II studerades utvecklingen av amyloid i högfunktionella öar i en musmodell uttryckande det humana hormonet IAPP. IAPP frisätts tillsammans med insulin, men vid typ 2 diabetes sker en felveckling av IAPP som leder till att proteinet bildar ett aggregat, amyloid, som ansamlas i öarna och är toxiskt för cellerna i dess närhet. Högfunktionella musöar visade sig bilda mer amyloid än andra öar, samma sak observerades för humana öar med högre funktion. För att förstå varför amyloid bildas i större utsträckning i högfunktionella öar studerades spjälkningen av IAPP. Högfunktionella öar kunde man då se hade en ökad närvaro av enzymet PC1/3 som tillsammans med PC2 spjälkar IAPP till dess aktiva form. Då samma ökning inte kunde detekteras av enzymet PC2 är en möjlighet till den ökade amyloidbildningen i de högfunktionella öarna att IAPP spjälkas felaktigt.

I studie III studerades kärlens anatomi i de högfunktionella öarna. Dessa öar hade i en högre omfattning än andra öar ett direkt venöst blodflöde vilket främjar distribution av hormoner från öarna ut till kroppens perifera vävnader. De högfunktionella öarna visade även en ökad känslighet för glukos i låga koncentrationer vilket medför att dessa öar kan reglera blodsockret redan vid små förändringar. Samma öar visade sig även vara mer utsatta för autoimmunt angrepp i BB-råttor, en djurmodell för typ 1 diabetes. När insulinfrisättning studerades i typ 1 diabetes patienter var responsen vid låga glukoskoncentrationer obefintlig. Den uteblivna insulinfrisättningen vid låga blodsockerkoncentrationer i patienter med tidig typ 1 diabetes skulle följaktligen kunna bero på en omfattande skada på de högfunktionella öarna.

I studie IV studerades en annan subpopulation av öar, en grupp av lågt genomblödda öar, vilka kan identifieras med en markör för låg syresättning, pimonidazol. Dessa öar har tidigare visat sig ha en minskad metabol aktivitet och utgör troligtvis en vilande grupp av öar som aktiveras när den metabola belastningen ökar. För att stärka bevisen för att dessa öar utgör en egen population studerades syresättningen i öarna över tid. Dessa försök visade att syresättningen i öarna var stabil över åtminstone åtta timmar. Öarna karakteriserades därefter och visades ha en minskad kärldensitet samt celldelning vilket ytterliga stärker hypotesen att dessa öar är en population av vilande öar med låg metabol aktivitet.

Sammantaget beskriver de fyra delstudierna två subpopulationer av Langerhanska öar som kan identifieras med hjälp av deras blodflöde; en grupp av högt genomblödda öar med en hög funktionalitet samt en grupp av lågt syresatta öar med en låg metabol aktivitet. Närvaro av subpopulationer av öar med olika funktion kan vara fördelaktigt för kroppens förmåga att finreglera blodsockernivåer. Samtidigt har subpopulationen av högfunktionella öarna ökad

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