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Transcriptional and morphological analysis of organ donor pancreata

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Abstract
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The diabetes disease panorama affects more than 400 million people world wide – a number projected to rise above 640 million in the next 20 years. Type 1 and type 2 diabetes are associated with adverse complications, resulting in severe morbidity and high healthcare costs exerting substantial strain on society. Type 1 diabetes results from the destruction of the insulin producing beta-cells, while type 2 diabetes is a multifactorial combination of a decreased ability of the beta cells to secrete sufficient insulin and a peripheral resistance to insulin-mediated glucose uptake. The cause of diabetes is unknown, as are the possibly sequential cellular events resulting in overt disease. In this thesis, organ-donor pancreata from donors with or without diabetes are analysed in order to deepen our understanding of the Islets of Langerhans, the beta cells and the pancreas.

In **Paper I**, islets from a donor that died at onset of type 1 diabetes, showing morphological signs of hydropic degeneration are analyzed on a gene-expression level, and the results are compared to islets from a donor without diabetes. We find no signs of ongoing inflammation, apoptosis or ER stress. In **Paper II**we compare the expression levels of genes related to cellular stress, in islets from donors with type 2 diabetes and from donors with high pre-mortal HbA1c levels but without a diabetes diagnosis, to the expression levels in islets from donors with normal HbA1c levels. We report that islets from donors with type 2 diabetes show signs of cellular stress on a transcriptional level compared with islets from donors without diabetes. In **Paper III** we performed a transcriptional analysis of the islets from organ donors aged between 1 and 81 years in order to elucidate whether age induces specific changes in the islet transcriptome. We found 20 genes that co-varied with increasing age. In **Paper IV,**the pancreatic expression of host-defense molecules is characterized, and we report the expression of several host-defense molecules not previously described in the human pancreas. Together, these results deepen our understanding of the effects of ageing and prolonged exposure to high blood sugar levels on the islets, as well as our knowledge about the innate immunity of the human pancreas.

Keywords: Pancreas, Diabetes, Islet of Langerhans, Beta-cell, Immunology, Inflammation, Ageing

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“I’m always looking, like a child, for the wonders I know I’m going to find – maybe net every time, but every once in a while.”

- Richard Feynman

List of Papers

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

I Hopfgarten, J., Stenwall, A., Wiberg, A., Anagandula, M., Ingvast, S., Rosenling, T., Korsgren, O., Skog, O. (2014) Gene expression analysis of human islets in a subject at onset of type 1 diabetes. *Acta Diabetologica*, 55:199-204

II Lundberg, M., Stenwall, A., Tegehall, A., Korsgren, O., Skog, O. (2018) Expression profiles of stress-related genes in islets from donors with progressively impaired glucose metabolism. *Islets.* 10:69-79.

III Stenwall, A., Seiron, P., Lundberg, M., Esguerra, J., Volkov, P., Renström, E., Korsgren, O., Skog, O. (2018) Transcriptional analysis of islets of Langerhans from organ donors of different ages. *Manuscript.*

IV Stenwall, A., Ingvast, S., Skog, O., Korsgren, O. (2018) Characterization of host defense molecules in the human pancreas. *Manuscript.*

Contents

Abbreviations

T1D = Type 1 diabetes
T2D = Type 2 diabetes
ER = endoplasmatic reticulum
GLUT1 = glucose transporter 1
HbA1c = glycosylated haemoglobin / haemoglobin A1c
HLA = human leucocyte antigen
GI = gastrointestinal
PRR = pattern recognition receptor
TLR = toll-like receptor
IdU = iododeoxyuridine
IAPP = islet amyloid polypeptie
LMD = laser micro dissection / laser-assisted micro dissection
IHC = immunohistochemistry
PCR = polymerase chain reaction
NGS = next generation sequencing
ICU = intensive care unit
GSEA = gene set enrichment analysis
FDR = false discovery rate
PCA = principal component analysis
UPR = unfolded protein response

Introduction

The diabetes disease panorama is a tremendous burden on society worldwide. A little over 400 million people are afflicted with some form of the disease, and the number is projected to reach above 640 million by the year 2040 [1]. Diabetes is associated with adverse short- and long-term complications and, as such, it is associated with staggering healthcare costs [2]. As of today, there exists no readily available cure for diabetes, and patients suffering from type 1 diabetes (T1D) usually have to rely on life-long treatment with exogenous insulin.

Despite enormous scientific effort during much of the 20th and the 21st century, what causes T1D and type 2 diabetes (T2D) remains largely unknown. Diabetes in an inherently difficult disease to study for several reasons. The pancreas is not easily accessible for analysis, and surgical intervention directed at the pancreas is associated with great risk [3] making the prospect of studying pancreatic tissue from living human subjects impractical and as of now, unlikely. Further, the pathologic events resulting in overt diabetes is generally initiated before the onset of clinical symptoms, making the temporal factor complicated as there currently exists no way to accurately predict the development of diabetes in an individual. As a result of these factors, diabetes research has long relied in part on animal models and pancreatic tissue from deceased organ donors.

The general purpose of the research behind this thesis is to develop methods for studying and analyzing pancreatic tissue from deceased organ donors with and without diabetes. These methods are subsequently utilized to deepen our understanding of how different factors, such as age and exposure to different blood glucose levels, affect the insulin-producing cells, and to characterize the pancreatic defense against environmental factors such as bacterial and viral infections.

The history of diabetes

Diabetes has been known to man for centuries, with early records of the disease stemming from ancient Egypt and Asia. It was initially described as a

condition associated with excessive thirst, weight loss and abnormally high urine production [4].

The organic background of the disease remained largely unknown until the late 1880s, when von Mering and Minkowski were able to induce the symptomatology of diabetes mellitus in dogs by surgically removing the pancreas from the animal [5]. The physiological cause of the symptoms was elucidated in several important steps. In 1869, Paul Langerhans discovered minuscule cell clusters in the pancreas [6], and in 1894, Laguesse showed that these "islets" were in fact endocrine micro-organs [7], though their exact function and significance remained unclear. Another important discovery was made in the early 1900s when Opie found a connection between diabetes and the presence of damaged islets in cadaveric pancreata. [8]

However, it was not until 1921, when Banting and Best isolated the secretory product they termed "insulin" from the pancreatic cell clusters and showed that insufficient amounts of the hormone was the cause of diabetes mellitus [9], that the disease could be explained on a physiological level. The first human patient was injected with insulin the following year, setting the gold standard for clinical management of diabetes mellitus – a standard that remains undisputed to this day, almost a century later.

The pancreas and the Islets of Langerhans

The pancreas is one of the largest glands in the human body. It measures approximately 15 cm lengthwise in an adult and weighs on average around 70g [10]. It is situated in the upper-left quadrant of the abdominal cavity, posterior to the ventricle. The macro-anatomy of the organ is divided into three parts; the caput (head), the corpus (body) and the cauda (tail) [11]. The pancreatic tissue is further divided into smaller lobules measuring between 1-10 mm in general [12]. The pancreas is histologically and functionally constituted of two distinctly different exocrine and endocrine niches with the exocrine compartment, including acinar and centroacinar cells and the pancreatic duct system, constituting approximately 98-99% of the total pancreatic mass. [13, 14]. The exocrine compartment of the pancreas excretes its products into ducts of progressively increasing size, which terminate in the main pancreatic duct emptying into the duodenum through the papilla Vateri.

The endocrine compartment of the pancreas consists of multicellular clusters – the Islets of Langerhans. These cell clusters are spread throughout the entire pancreas and vary considerably in size with a mean diameter of ~0.1 mm. In total, the Islets of Langerhans generally constitute about 0.4-4 % of the entire pancreatic mass with a considerable individual variation [15-17]. The endocrine cells of the Islets of Langerhans empty their secretory prod-

ucts into efferent venules, which in turn empty into exocrine capillaries connecting the islets to the systemic circulation [12].

The Islets of Langerhans are complex multi-cellular micro-organs constituted of an intricate network of specialized cells, blood vessels and connective tissue. The islets consist of five major endocrine cell types. The insulin-producing beta cells and the glucagon-producing alpha cells are the major cell types constituting roughly 50-60% and 34-40% respectively of the total insular endocrine cell mass. The other main cell types of the islets are the pancreatic polypeptide secreting PP cells, the somatostatin-producing delta cells and the ghrelin-producing epsilon cells [18]. The cellular composition of the islets remains relatively constant throughout the entire pancreas except for at the uncinate process close to the pancreatic caput, where the PP cells are generally more numerous relative to the other insular cell types [19].

The Beta cells

The beta cells are the producers of insulin in the human body, making them a key component in the regulation of metabolism and energy homeostasis [20]. The beta-cell population of an individual is primarily generated in utero during fetal development with a post-natal burst of beta-cell proliferation peaking within the first two years of life [21]. Whether this post-natal proliferation burst results in the individual base-line beta-cell population, or if the beta-cell population is subject to change through proliferation and/or neogenesis during the course of an individual's life, has been greatly debated. It has been shown that beta-cell neo-genesis does take place through life and that existing beta cells can generate new cells by replication [22]. However, as beta-cell replication in adults is very limited [19], its potential relevance in the context of the total beta-cell population of an individual is not yet known.

The synthesis and release of Insulin

Insulin is originally synthesized as the precursor molecule preproinsulin, which is transported into the endoplasmatic reticulum (ER) following ribosomal assembly [23]. In the ER, a signal peptide is cleaved from preproinsulin yielding proinsulin [24]. Proinsulin then undergoes subsequent folding and is transported to the Golgi apparatus where it enters the secretory vesicles of the beta cell and is cleaved to insulin and c-peptide [25].

Insulin is released into the bloodstream in response to glucose stimulation of the beta cells. After oral ingestion of carbohydrates, the glucose molecule is transported from the circulating blood into the beta cell through the mem-

brane bound glucose transporter 1 (GLUT1) [26]. Inside the beta cell, glucose is phosphorylated to glucose-6-phosphate by glucokinase. Glucokinase has a low affinity for glucose, which makes this reaction the main rate-limiting step in insulin secretion. The phosphorylation of glucose and the subsequent steps in the glycolysis result in an intracellular increase in the ATP to ADP ratio, which activates ATP-dependent potassium-channels. This results in an increase in intracellular Ca2+ levels, which subsequently triggers the fusion of secretory vesicles to the plasma membrane and ultimately results in the release of insulin into the bloodstream. [27]

The definition and epidemiology of T1D

The classification of diabetes mellitus into type 1 and type 2 diabetes originates from 1979, when what was hitherto known as juvenile, or insulin-dependent, diabetes was classified as type 1, and adult, or insulin-independent, diabetes mellitus was consequently classified as type 2. [28] T1D is defined as an impaired insulin secretion resulting from loss of the insulin-producing beta cells of the endocrine niche of the pancreas. This in turn results in subsequent chronically elevated blood glucose levels and severe metabolic consequences for the individual. T1D is one of the most common chronic diseases in the pediatric population, with two peaks in incidence occurring between 5-7 years of age and prior to puberty [29]. T1D can debut at any age and roughly 50% of all individuals with T1D are at least 18 years old or older at the time of diagnosis [30]. The prevalence of T1D is 0.5% in Sweden, with no significant difference on a gender basis. There is a substantial variation in regional incidence and prevalence numbers across the globe, with Finland having the highest incidence (64.2/100 000 inhabitants/year) and China having one of the lowest numbers recorded (0.1/100 000 inhabitants/year) [29, 31]. The lifetime risk of developing and being diagnosed with T1D is 0.4% in the Caucasian western European, northern American and Australian population, and this risk is increased by a factor of 15 in individuals with a first-degree relative with a T1D diagnosis. Thus, the hereditary factor is one of the most important risk factors for developing the disease [32].

T1D usually presents itself through symptoms arising from chronic hyperglycaemia: polydipsia, polyuria, polyphagia and weight loss. The clinical diagnosis of T1D is based on elevated blood glucose levels (fasting plasma glucose concentration of 7.0 mmol/l or more, or a plasma glucose concentration of 11.1 mmol/l or more two hours after a standardized oral glucose-load test) [33]. The concentration of glycated haemoglobin (HbA1c), a product of non-enzymatic irreversible glycation of the haemoglobin molecules of erythrocytes, as measured in peripheral blood can also be used as a diagnostic criterium. The HbA1c levels roughly reflect the mean blood glucose levels

of the last 2-3 months, as opposed to plasma glucose levels, which reflect the immediately present concentrations. T1D accounts for approximately 5-10% of all diabetes mellitus cases. It is not always straightforward to differ T1D from T2D in a clinical setting and, in order to achieve correct diagnosis, several factors besides symptomatology and blood glucose levels are taken into consideration. The presence of diabetes-associated antibodies, insulin dependence in close proximity to time of diagnosis, normal BMI and an absence, or a significant reduction, of circulating C-peptide levels are all diagnostic indicators of T1D [34].

The pathology of T1D

Despite enormous resources invested in elucidating the pathophysiology and etiology of T1D, the cause of the disease remains unknown to great extent. Some of the first clues to the pathological background to the disease were published in the early 1900s, when researchers reported the presence of cellular edema in islets of patients with T1D. The edema was termed "hydropic degeneration" and was considered a hallmark of the disease at the time [35-37]. In 1965, Gepts published his seminal paper describing immune cells infiltrating the islets and the peri-insular area around the islets, in autopsy specimen from deceased patients with T1D.

Gepts also described the widespread lack of insulin-positive beta-cells in the pancreata of deceased T1D patients, arriving at the idea that the beta cells underwent progressive immune-mediated destruction [36]. This in turn fueled two ideas in diabetes research: that the inflammatory lesions present in the islets of patients with T1D were the result of an insular infection, or that the inflammation was a consequence of an autoimmune process affecting the islets. The concept of T1D as an autoimmune disease gained traction in 1974, when islet-specific autoantibodies were reported [38, 39]. The parallel discovery of an increased risk of T1D associated with specific human leucocyte antigen (HLA) genotypes further established the autoimmune hypothesis as one of the key elements of T1D pathogenesis research [40-42].

The theoretical temporal course of T1D is widely accepted according to the model presented by Eisenbarth in 1986. According to this standard model for T1D, each individual is born with a certain multi-factorial predisposition and subsequent susceptibility to developing T1D. An inflammatory event, triggered by an environmental factor, initiates a sequence of immunologic destruction of the beta cells and symptoms arise when the remaining beta-cell mass is unable to compensate for the loss of insulin secretion, and clinical diabetes is established [43, 44].

However, despite intense scientific effort, the autoimmune model for T1D has not been able to provide a verifiable explanation for T1D pathogenesis. Attempts have been made to classify and characterize the immune-cell infiltrate in insulitic lesions in islets from patients with T1D and several cell types have been identified, with CD8+ T cells being the most prevalent. Other cells such as CD4+ T cells, B cells and Macrophages have also been identified in insulitic lesions [45]. The insulitic CD8+ cell infiltrates are not present in all subjects with T1D however [46, 47], and the role of cytotoxic T-cell infiltrates in T1D has been greatly debated. Current evidence for a decisive role for T cells in the pathogenesis of T1D is not conclusive [35, 48-51].

The role of auto-antibodies in diabetes pathogenesis has been subject to debate as well. The presence of one auto-antibody directed towards an islet antigen in a subject with a fist-degree relative with T1D is associated with increased risk (12.7%) for developing diabetes whereas the presence of three islet auto-antibodies is associated with a markedly increased risk (79.1 %) of developing the disease over a 10-year period. As such, islet auto-antibodies are generally considered as biomarkers for T1D [52]. However, the islet-autoantibodies usually analyzed in clinical practice are directed towards intracellular antigens, and their significance in the pathogenesis of T1D is unclear.

Environmental factors in T1D

The regional differences in T1D have yet to be fully explained. The increased risk in some parts of the world appears to be somewhat tied to the geographical area rather than to a genetically heterogenous population inhabiting the area, as demonstrated by the increase in incidence in populations moving from a low-risk region to a high-risk region [53] and by the fact that incidence can differ by as much as six-fold in a genetically homogenous group depending on which country a subject is living in, as exemplified by the difference in incidence between eastern Finland and the neighboring Russian Karelia [54]. This indicates that high incidence cannot be explained by genetics alone. There is also a significant temporal increase in the incidence of T1D over the last decades [1], which again cannot be explained by genetic factors. One must also take into account the relatively low concordance in monozygotic twins - only about 40% [55], which further suggests other factors than genetics being pivotal to T1D development. Thus, the idea of one or several, possibly synergistic, environmental factors is highly relevant in the context of T1D.

There has been much speculation regarding possible environmental factors acting as triggers in the development of diabetes, some of the most common

being infectious agents and dietary components. The connection between viral infections and T1D was made almost a century ago, when a link between viral infection and T1D was suggested [56]. There is evidence for the ability of enterovirus to infect human islets [57], and enterovirus has been detected in human islets in vivo [58]. However, causality between viral infections and T1D has not been demonstrated.

The intestinal bacterial flora has been linked to T1D [59], and so has leakage of gastrointestinal (GI) content in the intestinal epithelium [60]. The anatomic connection between the pancreas and the duodenal lumen has been suggested to allow for the translocation of bacteria, viruses and bile from the GI tract to the pancreas. This in turn has been hypothesized to result in subsequent inflammatory response, which could contribute to the development of T1D [35, 61]. The presence of bacteria in pancreata procured for organ donation is indeed high and varies between donors [62-64], suggesting the translocation of bacteria sometime during the life of the donor possibly due to the anatomic connection between the pancreas and GI tract. However, the mechanistic connection between the translocation of contents from the GI tract and the possible development of T1D in humans has not been shown, and thus, the role of GI reflux and a possible subsequent inflammatory event is currently unknown.

Innate immunity in T1D

The human immune system serves as a defense mechanism against harmful events such as microbe infections and is partly responsible for proper recovery after tissue damage. It is divided into the innate and the adaptive immune system, with the innate immune system serving as the primary response and the adaptive immune system serving as the secondary, specific defense [65].

The innate immune system is highly evolutionary conserved and serves as the first barrier against infections and harmful environmental factors. It consists of physiological barriers like epithelial cells and mucous membranes, and cells and molecules specialized in stereotypical response to pathogens and inflammatory stimuli. Its response is immediate and non-specific, and it is unable to develop an immunologic memory [65, 66]. The cells of the innate immune system exert their effects through contact-mediated interaction, phagocytosis and through the synthesis of antimicrobial and immunoactive molecules [65]. The complement system and the expression of antimicrobial molecules, such as the defensin peptide family, from various other cell types are also part of the immediate generic response of the innate immune system [65, 67, 68].

One way for the innate immune system to recognize non-host organisms is through a system of genetically preserved pattern-recognition receptors (PRR) with specificity towards a broad range of evolutionary conserved microbial antigens. The PRRs include toll-like receptors (TLRs), which detect various microbial structures, and RIG-I and MDA-5, which detect viral nucleic acids [69]. The TLRs are expressed in numerous kinds of tissue throughout the human body [70] and, interestingly, in beta cells [71]. Human beta cells express TLR2 and TLR4, which recognize peptidoglycan molecules on gram-positive bacteriae and lipo-polysaccharide on gram-negative bacteriae respectively [70]. The triggering of TLR2 and TLR4 by bacterial components initiates the release of pro-inflammatory cytokines and chemokines, and the activation of beta-cell TLRs has been speculated to initiate an inflammatory response and subsequent recruitment of immune cells to the islet [70]. The instillation of bacteriae in the pancreatic duct has been utilized in a rat model for pancreatitis, where it results in a potent activation of the innate immune system [61], and it has been speculated that similar mechanisms could result in a subsequent activation and recruitment of effector cells from the innate and adaptive immune system in the human pancreas, resulting in islet and beta cell damage [51].

Another key trigger for innate immune-system activation is the presence of viral antigens, and viral infections in Islets of Langerhans induce an inflammatory environment [72, 73], which may promote the subsequent infiltration of effector cells from the adaptive immune system to the site [74]. Indeed, the presence of cytokines related to innate immune system activity has been detected in islets from organ donors recently diagnosed with T1D [75] and in peripheral blood from patients before and after the time of T1D diagnosis [76] suggesting a potentially important role for innate immunity in T1D development, possibly related to environmental factors. However, the pancreatic expression of innate immune factors is not well characterized, and further research is needed in order to link bacterial or viral infections to pancreatic inflammation and immune cell activation.

The definition and epidemiology of T2D

T2D is defined as a state of chronic hyperglycaemia arising from the inability of the beta cells to secrete sufficient amounts of insulin into the blood or impaired peripheral insulin action, or both [77]. This is the end result of a complex interplay between a progressively increasing resistance to insulin signaling in peripheral tissue and the inability of the beta cells to cope with the rising demand of insulin secretion.

The disease is associated with several environmental and lifestyle factors such as diet [78] and obesity [79], and co-morbidity is relatively common

[80]. There is also a genetic predisposition factor with more than 100 susceptibility loci being identified through large-scale genome-wide studies [81]. The reported heritability of T2D is estimated between 20-80% depending on material and study design. The lifetime risk of developing T2D is approximately 40% for people with one parent diagnosed with T2D and roughly 70% for individuals with both parents diagnosed with the disease. Furthermore, individuals with a first-degree relative with T2D are three times more likely to develop T2D compared to individuals without a family member with T2D. [82]

The diagnostic procedure of T2D is identical to that of T1D, and the clinical symptomatology is often similar with early symptoms involving polyuria, polydipsia and polyphagia [83]. Besides fasting plasma-glucose levels above 7.0 mmol/l being a diagnostic criteria, HbA1c levels above 6.5% are considered diagnostic for T2D. Individuals showing HbA1c levels between 6.0% and 6.5% are generally considered as being at high risk for developing the disease [84]. The treatment for T2D ranges from lifestyle adjustments such as weight loss and specific diet changes, to pharmacological agents affecting endogenous insulin secretion, agents increasing peripheral insulin sensitivity and agents affecting the rate at which glucose is absorbed in the GI tract. Different regimes of insulin substitution are utilized when other treatment modalities are deemed inefficient or inappropriate [79].

T2D is a multifactorial disease

T2D is the end result of a complex interplay between impaired ability to secrete adequate amounts of insulin and decreased action of insulin in peripheral tissue. As in T1D, the sequence of pathologic events ultimately resulting in overt disease is generally initiated before the onset of clinically significant symptoms, and the disease is developed in a gradual manner where the acceleration of glucose intolerance eventually results in chronically elevated blood glucose levels and manifest disease [79]. The chain of adverse events resulting in fulminant T2D is complex and probably heterogeneous. Despite intense scientific efforts, the pathogenesis of T2D remains unknown.

Several factors have been linked to the development of T2D, though none of them have been hitherto proved to be of decisive importance. The decreased function of the beta cells is probably of key importance in developing T2D, and has been attributed to several factors such as an altered beta-cell metabolism tentatively related to mitochondrial dysfunction [85, 86] or a progressively dysfunctional ER, hampering the secretory abilities [85, 87]. The harmful effects of prolonged exposure to excess fatty acids, described as lipotoxicity, has been proposed as another cause for beta-cell dysfunction

through factors such as defective pro-insulin processing, reduced insulin gene expression and ER-stress [88, 89]. The toxic effects of prolonged exposure to high glucose levels, termed glucotoxicity, has been proposed as a contributing factor as well [90].

Reduced beta-cell mass in patients with T2D has been reported in several papers [91, 92], though the importance of these findings in a diabetes setting is somewhat unclear as the proposed magnitude of the loss is probably insufficient to explain the subsequent development of T2D [79]. The proposed loss of beta-cell mass has been appointed to factors such as beta-cell apoptosis due to toxic harm [93] or loss of beta-cell phenotype due to de-differentiation [94, 95].

The effects of ageing on Beta cell biology

Glucose tolerance is decreased in the ageing human, as a result of an impaired insulin secretion in response to glucose stimulation of the beta cells, and a peripheral resistance to insulin signaling [96-98], though it should be pointed out that most aging individuals do not develop diabetes, which suggests significant individual factors in diabetes susceptibility and implies careful interpretation of age-related effects on the beta cells. The progressive age-related decline in secretory capabilities of the beta cells has been tentatively appointed to several different factors, two of the most prominent being a deteriorating beta-cell physiology and an age-related decline in the number of physiologically active beta cells [99].

Proliferation of beta-cells in the adult human is generally considered to be low. Key studies investigating the post-natal beta-cell proliferation based on Ki67 as a marker for cell division find very little evidence for on-going proliferation in adults [19, 100]. Similar results are shown in studies investigating age-related accumulation of lipofuscin bodies in beta cells as an indicator of replication [101]. A study measuring the incorporation of iodo-deoxyuridine (IdU) in beta cells from subjects receiving thymidine analogues in clinical trials for oncologic therapy, found no signs of beta-cell replication in subjects older than 30 years [102]. Other studies investigating beta-cell biology in relation to ageing have shown an increased expression of negative cell-cycle regulators such as p16INK4a in mouse [103, 104] and human [105] islets, suggesting an age-dependent regulation of replication in islet cells, which could possibly affect beta cells numbers in the aging human.

Loss of beta-cell mass as a result of increased rate of apoptosis due to factors such as aggregation of Islet Amyloid Polypeptide (IAPP) and increased synthesis of reactive oxygen species have been suggested to affect beta-cell

numbers in the aging human [99]. Indeed, IAPP is secreted together with insulin and the state of insulin hypersecretion that is often present in early T2D has been suggested to result in an increased intra-cellular accumulation of IAPP, which in turn increases the rate of beta-cell apoptosis [106, 107]. The loss of beta-cell mass has also been speculated to arise from a progressive de-differentiation of the beta cells through which they lose their beta-cell specific phenotype [94].

The effects of aging on beta cell function and physiology have been tentatively attributed to factors such as inability to adequately respond to insulin signaling and disrupted ion dynamics. An age-related decline in appropriate coordination of Ca2+ signaling between beta cells in isolated human islets, affecting insulin dynamics has been reported [108], and the membrane-bound K+-transporter crucial to insulin secretion has been shown to be negatively affected by advancing age in rodents [109]. Further, beta cells from both rodents and humans have been suggested to suffer an age-related decline in mitochondrial function, resulting in disrupted Ca2+-dynamics and an impaired insulin secretion [110]. Indeed, mitochondrial function has been shown to be altered in islets from patients with T2D [85]. Taken together, aging may induce changes in beta-cell physiology, but the possible relevance of these changes is not well understood.

Aims

The overarching aim of this thesis is to develop and establish methods for investigating organ donor pancreata and to utilize these methods in order to learn more about the pancreas, the Islets of Langerhans and the beta cells in non-diabetic organ donors and in the context of diabetes mellitus. The human pancreas in T1D and T2D is not well characterized, and tissue from deceased organ donors provides a unique opportunity to gain novel insights in the biology and pathology of diabetes and other pancreatic diseases.

Paper I

The aim of paper I was to characterize the expression of genes related to cellular stress, apoptosis, ER stress and islet function in islets affected by hydropic degeneration in a donor that died at onset of T1D. The purpose of paper I was also to develop, optimize and utilize laser micro dissection protocols and to compare the expression data from islets obtained through laser micro dissection with the results from islets obtained through clinical isolation procedures.

Paper II

The purpose of Paper II was to utilize laser micro dissection to examine the cellular response to stress on a transcriptional level in Islets of Langerhans from organ donors with progressively increased HbA1c, including donors diagnosed with T2D, and to compare the expression data to non-diabetic control donors. Cellular stress was measured as the expression levels of genes related to unfolded protein response, mitochondria and oxidative stress. The aim of paper II was also to evaluate the feasibility of an array-based approach to the transcriptional analysis using multiple pre-made PCR arrays.

Paper III

The purpose of paper III was to analyze the full transcriptomes in the islets of organ donors representing the entire human age span, in order to elucidate whether advancing age induces specific gene expression patterns. The purpose of paper III was also to evaluate the use of next generation sequencing (NGS) in the gene expression analysis of islets obtained through laser micro dissection.

Paper IV

The aim of paper IV was to characterize the expression of host defense molecules on a morphological level in the pancreas of 10 non-diabetic organ donors and of a donor that died at onset of T1D, and to investigate any correlations between the expression of host defense molecules in the pancreas and donor age, tissue infiltration of inflammatory cells and the intestinal expression of host defense molecules.

Materials, study design and methods

Considerations on some aspects of study design, methods and the material included in this thesis are described below. Full descriptions can be found in the respective papers.

Organ donor pancreata

The basis of this thesis is the access and availability of human pancreatic and intestinal tissue procured from deceased organ donors where consent is given, not only for clinical transplantation of tissue and/or whole organs, but also for scientific research.

The vast majority of research in the diabetes field is performed on animal models, which are accessible and well established in the scientific community. They are not without limitations however, and results from animal models in diabetes research are rarely directly transferrable to human biology and physiology [111]. In the context of diabetes, it is a somewhat unavoidable tradeoff however, as pancreatic tissue from living humans is inherently difficult to obtain. This is due in part to the anatomic location of the organ in question but first and foremost to the high risk of adverse complications related to surgical resections and/or biopsy procurement of the pancreas [3]. Well-preserved pancreata from organ donors is preferable to pancreata from autopsy specimens, as the pancreas is subject to intense autolysis post-mortem [112]. Thus, pancreata from deceased organ donors is an invaluable resource in diabetes research.

The tissue included in this thesis is procured from heart-beating organ donors where the process of tissue donation for clinical transplantation and research purposes is initiated after brain death and after the clamping of systemic circulation, minimizing cold ischemia time and thus minimizing post-mortem inflammation induced by tissue hypoxia. The tissue has been rapidly taken care of after being surgically removed, and prepared for clinical islet isolation and/or storage in -80°C or embedding in formalin according to well established standardized protocols. The tissue has been catalogued and stored in accordance with high standard biobank regulations and practices.

Pancreatic tissue from deceased organ donors constitutes a unique possibility for diabetes research, but several important factors need to be considered when analyzing the results. It is of vital importance to point out that pancreatic tissue from organ donors is neither entirely representative nor the biologic equivalent of pancreatic tissue from living healthy individuals. Organ donors have all suffered some kind of adverse medical event prior to death, and the actual procedure of organ harvest can be preceded by anything from less than 24 hours in an intensive care unit (ICU), to several weeks. Likewise, varying degrees of pharmaceutical interventions prior to death is almost always undertaken. This may affect the systemic and local homeostasis of the body and could thus influence tissue morphology, gene expression levels and overall physiology in organ donors. It has been shown that the length of hospital stay prior to death does not affect immune cell numbers in pancreatic biopsies [113], but the potential effect of many pre-mortal factors remains to be elucidated. These are all factors not always available to the researcher when designing a study, making many of the pre-mortal conditions of the donor an unknown variable. However, it is often possible to include and match study subjects on several known factors such as gender, age, pre-mortal HbA1c, BMI, chronic illnesses, the prevalence of autoantibodies, etc.

Despite important donor variables beyond the control of the researcher, pancreatic samples from organ donors constitute an exclusive and invaluable resource, and will remain the best equivalent of healthy human pancreata until methods are developed for safe and minimally invasive resections of pancreatic tissue from living donors.

Isolated pancreatic islets

Isolation of pancreatic islets from organ donor pancreata is a routine procedure at Akademiska Sjukhuset Uppsala. It is performed as part of the islet transplantation program according to standardized procedures previously described [114]. After enzymatic digestion of the pancreatic tissue, the islets are removed and processed for downstream application i.e clinical transplantation or research. The isolated islets can be studied ex vivo in a multitude of ways such as in cell cultures and in closed perfusion systems, which is not possible with islets obtained through laser micro dissection.

Isolated islets provide a unique opportunity to study living human islets for a variety of purposes. However, when analyzing data from isolated islets, there are some factors that must be taken into consideration. The isolation procedure is quite volatile, and as such it may induce stress-related responses in the islet cells [115]. As the islets are removed from their pancreatic context, the islets are studied in a milieu that is not representative of their natural

environment and the consequent results may not be entirely representative of pancreatic islets. When isolating islets, it is impossible to sort them based on their metabolic properties or inflammatory states, meaning there may be islets with or without inflammation and islets in various states of insulin deficiency included in research populations.

Laser micro dissection

Laser-assisted micro dissection (LMD) is a method developed in order to allow the researcher to selectively remove and analyze specific tissue of interest from its surrounding histological context. The main principle behind the technique is based on that of light microscopy where tissue morphology is analyzed and evaluated. LMD allows the researcher to extract the tissue of interest based on visual assessment, and to preserve it for down-stream processing [116].

There are several commercial systems available that adhere to the main principle of selective removal of tissue, and two of these methods have been used in the research included in this thesis – gravity-assisted micro dissection (paper I) and polymer-cap based laser capture micro dissection (paper II and III). Gravity-assisted micro dissection is based on the principle of gravitational pull, where the tissue of interest is placed upon a thin polymer membrane, which in turn is placed over a collection vessel. The tissue of interest is cut out by a laser beam, and subsequently falls down into the collection tube where it is preserved during the session, awaiting downstream processing. In the case of polymer-cap based laser capture, a small plastic cap covered by a thin polymer coating is placed on top of the tissue and the polymer is melted from above in order to adhere to the tissue selected for removal, and is subsequently cut by a laser beam. As the cap is removed from the tissue, so is the tissue adherent to the plastic cap [117].

The utilization of LMD allows for specific analysis of certain tissue areas, cell populations, cell clusters or even single cells depending on the tissue. As such, it is a valuable tool in analyzing pancreatic tissue, where the Islets of Langerhans can be extracted. If used in conjunction with immunohistochemistry (IHC), islets with or without specific morphological features can be extracted or avoided. In paper I, the tissue prepared for laser micro dissection was stained utilizing an RNAse-free HE protocol, whereas no staining was performed directly on the tissue prepared for dissection in paper II and III as the use of auto-fluorescence made it unnecessary for identifying the islets. Instead, consecutive sections were stained on conventional glass slides for navigation purposes, and in order to avoid islets containing CD45+ cells in paper II.

However, there are limitations to the methods that must be considered when interpreting the results. As the process of laser micro dissection is performed, there is a risk of contamination from the tissue surrounding the area of interest, as microscopic parts of the tissue sample may fall into the collecting tube below, or stick to the plastic cap. This is avoided by microscopically inspecting each collection tube or cap prior to downstream analysis in order not to carry over any contamination or debris. There is also a risk of selection bias where the largest and the smallest islets are not extracted due to technical difficulties. This may result in an islet population representing a relatively homogenous population with regards to size.

The process of laser micro dissection is time consuming and user dependent, and as the pancreas is rich in endogenous RNAse activity, the RNA is rapidly degraded once thawed and placed in the apparatus in room temperature, resulting in the risk of poor RNA quality [118]. This is partially avoided by the thorough dehydration of the tissue. In this thesis, it should also be pointed out that the method is applied on entire islets and not a specific islet cell type, which must be accounted for when interpreting the results. Nevertheless, laser-assisted micro dissection remains a well-established and validated method for selecting and extracting specific tissue for gene expression analysis, and by continuously evolving the protocols, the method will most likely allow for higher resolution analysis with improved selectivity.

Approaches to gene expression analysis

Quantitative polymerase chain reaction (qPCR) is a standard method for analyzing gene expression levels. qPCR can be utilized in various study designs and is suitable for analyzing specific gene expression levels through the use of primer pairs specifically amplifying a few genes of interest, as well as for analyzing groups of specific genes by using qPCR-arrays containing groups of primer pairs. Analysis by qPCR is highly specific and sensitive even to small amounts of template RNA. There are trade-offs to increasing the scope of the analysis by expanding the number of genes in the analysis in an array-based fashion as well as to the use of whole-transcriptome analysis. qPCR arrays and even more extensive transcriptome analyses generate huge amounts of data, and as the number of statistical tests increases, there is an increasing risk of finding significant results purely by chance. This can be compensated for in various ways by using statistical methods such as Bonn-Ferroni corrections or by defining false discovery rate. However, applying statistical methods for reducing the amount of false positive results may increase the number of false negative results, which may consequently result in true differences not being discovered.

In paper I, a customized panel of selected primers was used, according to a design philosophy adhering to the principle of a directed analysis and gene expression analysis was performed using standard qPCR protocols. In paper II, four pre-designed PCR arrays, each containing 84 genes, were used in order to cover a wide range of genes associated with different aspects of cellular biology in the context of cellular stress. Relative gene expression levels were calculated using the 2^(−ΔCt) method, by which the expression of each gene of interest is normalized to the expression of 2-3 references genes.

The transcriptome-analysis in Paper III was carried out by utilizing AmpliSeq, which is an ion-torrent based method for transcriptome analysis. As with qPCR, the method is based on the synthesis of complementary DNA from template sample RNA, followed by a pre-amplification step and the subsequent quantification of the amplicons by ion-torrent sequencing. Ion-Torrent sequencing allows for multiple parallel reactions in thousands of microwells, making the system capable of large-scale sequencing in relatively short time [119, 120], making it suitable for transcriptome analysis.

In paper III, gene set enrichment analysis was used to identify previously defined cellular signaling pathways as being either up-regulated or down-regulated with increasing age. Gene set enrichment analysis (GSEA) is a method for identifying a set or multiple sets of genes that are defined prior to analysis based on biologic knowledge, and to find out whether the expression levels of these genes are highly expressed, expressed at low levels or randomly distributed [121]. This is done in order to indicate whether entire signaling pathways or systems appear to be significantly altered in a data set, which in turn may imply biological consequences.

Immunohistochemistry

Immunohistochemistry (IHC) is a method for visualizing the presence of antigens in a histological context through antibody-antigen interaction. In this thesis, an indirect approach was used where a primary antibody specific to the antigen of interest is applied to the tissue sections mounted on glass slides, and a secondary enzyme-conjugated antibody specific to the primary antibody is added afterwards. When a substrate is added to the tissue containing the enzyme-conjugated secondary antibodies, a chemical reaction results in a color precipitation that can be visualized through light microscopy. In order to minimize interference by background precipitation, blocking agents are added to minimize the effects of endogenous peroxidase. There is also a risk of cross-reactivity where the antibodies bind to incorrect antigens, which is minimized by carefully validating the staining protocols by includ-

ing positive and negative control samples where the correct antigen is present and non-present respectively.

IHC can be used as described above to detect the presence, localization and relative distribution of an antigen in 2-dimensional space. However, IHC cannot adequately quantize the expression levels of antigens and the analysis is usually limited to thin sections of tissue. It is also user-dependent when not performed in an automated fashion, and as such it is sensitive to user bias.

Statistics

In this thesis, data sets from a heterogenous cohort of individuals have been compared. The data was not expected to follow a Gauss distribution and therefore we chose to utilize non-parametric tests such as Mann-Whitney when comparing two groups, and Kruskal-Wallis for multi-group comparisons, in paper II.

When performing multiple comparisons as when comparing large gene expression data sets, the risk of type I errors is high if no adjustment is made for multiple comparisons. However, if co-variation of gene expression levels is expected, adjusting for multiple comparisons may result in type II errors. One approach to this problem is to allow for a certain amount of type I errors, or *false discoveries*, by setting a false discovery rate (FDR) for a specific p value. We utilized the Benjamini-Hochberg method for calculating the FDR in paper II, and III.

In paper III, the aim was to investigate the effects of ageing on the Islet transcriptome. To this end, age was used as an independent variable in a simple linear regression model. FDR was calculated for each gene, were considered to vary by age when $FDR < 0.05$.

Principal component analysis (PCA) is a statistical method that reduces the dimensionality of the data set while retaining most of the variation. This is achieved by identifying the directions, or principal components, in which the variation is most pronounced. Through this method, each sample can be represented by a much smaller set of numbers, making it much more feasible to visually identify similarities and differences between groups and individual samples [122, 123]. PCA was utilized in paper III together with hierarcical clustering analysis in order to detect outliers and to elucidate whether any donor clustering on the basis of age could be found in the material.

Results and discussion

Paper I – Gene expression analysis in a subject at onset of type 1 diabetes

No apparent signs of inflammation, ER-stress or apoptosis in islets showing morphological signs of hydropic degeneration in a donor at onset of type 1 diabetes

Hydropic degeneration has been considered a hallmark of islet morphology in T1D in the early 20th century [36, 37, 124] and this cellular edema has been suggested to stem from an alteration in the electrolyte homeostasis of the beta cells [125]. Morphological analysis of the islets from a donor that died at onset of T1D showed that a majority of the islets (73%) were affected by extensive hydropic degeneration, while only a few cells in 8% of the islets from a nondiabetic donor showed signs of hydropic degeneration. The percentage of insulin-positive islet cells on paraffin-fixed tissue slides was only slightly decreased in the T1D donor compared to the non-diabetic donor. On a transcriptional level, there were no apparent signs of ongoing inflammation, apoptosis or ER-stress in tissue from the donor with T1D compared to islets from a non-diabetic donor.

Establishing and validating protocols for laser micro dissection of pancreatic tissue

In paper I we successfully developed and established protocols for utilizing gravity-assisted laser micro dissection to extract and analyze specific tissue from frozen pancreatic biopsies. This development process included the handling and sectioning of the tissue in -20°C, the subsequent handling of the POL membrane slides for mounting the tissue, and the laser assisted micro dissection procedure. The qPCR results from isolated islets were similar to those from islets retrieved through laser micro dissection in both the non-diabetic donor and in the donor with recent-onset T1D. Thus, we arrived at the conclusion that laser micro dissection is a feasible method for analyzing pancreatic tissue and Islets of Langerhans.

Paper II

Donors diagnosed with T2D show differential expression of stress-related genes

The impaired glucose tolerance in T2D has been attributed to several factors, where many revolve around impaired function of the beta cells. Cellular stress due to prolonged exposure to elevated blood glucose levels could be a cause of altered cellular function in the beta cells. The ER has been found to be morphologically and functionally different in islets from T2D patients [87, 126], and the mitochondria in beta cells from T2D patients seem to have an altered function and morphology [85, 87] suggesting that these factors may be important in understanding beta-cell dysfunction in T2D.

In paper II, laser-capture micro dissection was utilized in order to collect islets from deceased organ donors with progressively impaired glucose tolerance. The donors were categorized in four different groups depending on HbA1c levels; control donors with normal HbA1c levels (<5.5%), semi-diabetic donors with elevated HbA1c levels (6.0-6.5%), donors with high HbA1c levels (>6.5%) but without a T2D diagnosis, and donors diagnosed with T2D. Islets containing CD45-positive cells were excluded in the laser-capture process, so as not to include any immune cells in the analysis. The expression levels of 330 stress-related genes were analyzed using four different pre-designed qPCR arrays including genes related to ER-stress, unfolded protein response (UPR) and mitochondrial biology. Relatively high variation in gene expression levels were seen between donors in the respective groups, suggesting there may be other pre-mortal factors besides HbA1c levels influencing the expression of stress-related genes in deceased organ donors.

In donors with T2D, 44 out of 330 genes were differentially expressed compared to control islets. Over-expressed genes were mainly related to the UPR and under-expressed genes were mainly associated with mitochondrial function. These results are somewhat in contrast to another study based on laser-capture micro dissection of islets from T2D donors and non-diabetic donors where no apparent difference could be detected [127], though it should be pointed out that the analysis was based on a different sequencing method covering a much larger number of genes.

We conclude, based on our findings in T2D donors, that mitochondrial function and biology may indeed be compromized in islets from donors with T2D, and that this may in turn affect beta-cell function and the ability of the beta cells to adequately respond to glucose stimulation. We also conclude that the UPR seems to be up regulated in islets from T2D donors, which may

indicate a partially compromised secretory function of the cells. It should be pointed out that the study was conducted on whole Islets of Langerhans and not on beta cells specifically. This needs to be accounted for when interpreting the results.

Donors with slightly elevated HbA1c levels show more signs of stress than donors with high HbA1c levels

In the semi-diabetic (HbA1c 6.0-6.4%) group, 26 out of 330 genes were differentially expressed, pointing towards some degree of cellular reaction to stress. However, in the undiagnosed T2D (HbA1c >6.4%) group, only 7 out of 330 genes were differentially expressed. This was somewhat unexpected, as we hypothesized that prolonged exposure to increasingly high levels of blood glucose would induce a correspondingly higher levels of cellular stress. One explanation could be that the donors in the high HbA1c group has been adapted to a higher blood glucose level, but this idea is somewhat contradicted by the higher expression levels of stress related genes in the established T2D group. There may also be differences in how the patients were treated pre-mortally, that may in turn influence the expression of stress-related genes in the islets, but that remains a speculative explanation as we do not have access to that information. In order to fully elucidate the dynamics of cellular stress due to high HbA1c levels, further research is needed on islets also from individuals with HbA1c levels in the 5.5-6.0% range.

The array-based method for gene expression analysis is a feasible approach

In paper II, the cap-based laser capture micro dissection method was used for tissue extraction according to previous successful projects [75], and the analysis was up-scaled from one array of 84 genes to four arrays with a total of 330 genes, while still yielding robust results. This shows that laser-capture micro dissection is a feasible and robust way to extract sufficient amounts of RNA from frozen pancreatic tissue for a wider analysis comprising hundreds of genes. We also conclude that while PCR is the gold standard method for analyzing gene expression levels, even the inclusion of 330 genes does not provide a complete coverage of cellular functions. However, it should be pointed out that an array-based or limited approach can be advantageous in answering specific questions. As other methods for analyzing the entire transcriptome are now sensitive enough to handle the limited RNA quantities available from pancreatic tissue obtained through laser microdissection, these methods should be considered for utilization in future projects.

Paper III – Transcriptional analysis of Islets of Langerhans from organ donors of different age

Transcriptome analysis of islets from 26 organ donors aged 1 to 81 revealed 20 genes that co-varied significantly with age

Great scientific effort is directed in determining the effects of ageing on human beta cell physiology. Glucose tolerance is decreased in the aging human as a result of impaired insulin secretion and increased peripheral insulin resistance [96-98], implying decreased beta-cell functionality with increasing age. This has been attributed to a compromised physiology of the cells and a decline in the numbers of physiologically active beta cells [99]. Several factors have been proposed as being responsible for the progressively impaired physiology, such as an age-dependent decrease in the coordination of Ca^{2+} signaling between beta cells [108] and an age-related decline in mitochondrial function. The idea of a gradual loss of beta cells have been attributed to an age-related decline in replicative ability [19, 100-102, 128] and increased rate of apoptosis and progressive de-differentiation resulting in beta cells losing their phenotype [94].

In paper III, laser-capture micro dissection was performed on frozen pancreatic tissue from 26 non-diabetic organ donors aged between 1 and 81 years, in order to selectively extract the islets. The transcriptomes were analyzed utilizing AmpliSeq and the gene expression levels were compared using linear regression analysis and through hierarchical clustering. We found 20 genes in total that varies significantly with advancing age: 18 genes were up regulated and 2 genes were down regulated. The genes that varied significantly with age did not have an easily identifiable common denominator with regards to cellular pathways or functions.

GSEA revealed four KEGG pathways that were positively correlated with age. Non-parametric testing of the gene expression levels of the 10 youngest donors compared to the 10 oldest donors identified 52 genes that were differentially expressed. The expression levels of genes included in the KEGG pathways of insulin secretion, cellular senescence and cell cycle were seemingly randomly distributed over all donors. PCA analysis and hierarchical clustering showed no apparent clustering according to donor age. As in paper I and II, the study was conducted on whole islets and not on beta cells specifically. This means that the results have to be interpreted in the context of entire islets.

The expression of the negative cell-cycle regulator $p16^{INK4a}$ is increased with advancing age in human islets

Several researchers have reported an age-related increase in cell-cycle regulator proteins and $p16^{INK4a}$ has attracted particular attention as it is up-regulated with age both in mouse islets [103, 104] and in isolated human islets [105]. Genes involved in cell-cycle progression, mitosis and UPR are expressed in juvenile human islets according to the same study, and interestingly, the changes induced by age were mostly shared by both alpha and beta cells, suggesting a somewhat similar response to aging [105]. However, the role of an age-dependent increase in the expression of negative cell cycle regulators has been subject to debate [129], and as beta cell replication is comparatively low in young individuals the relevance of these findings in the context of diabetes is not clear.

The islet transcriptomes of juvenile and elderly donors are relatively similar

We hypothesized that ageing would induce substantial differences in the islet transcriptomes, but found only a minuscule part of the genes to vary significantly with age. This raises the question of islet cell longevity: are the islet cells of an 81-year old practically identical to those of a 1-year old, or are the cells in fact renewed during the life-span of a human being? Preservation of beta cell function in a base-line beta cell population would seem somewhat unlikely given the high metabolic and secretory activity of the beta cells. And, as the evidence for beta cell replication in the adult human is scarce, a possible explanation could be a continuous beta cell renewal from a hitherto unknown source. This would imply that the age of the islets of an 80-year old could be in roughly the same range as those of a five-year old, which in turn would explain the surprising similarities between islets from donors of different age. There is some support for this concept, as the expression levels of $p16^{INK4a}$ in rodent islets have been shown to vary between islets from the pancreas of the same individual [130], a finding that could indicate that the islet population is composed of islets of varying age.

AmpliSeq is a feasible way of performing transcriptional analysis on pancreatic tissue obtained through laser-capture micro dissection

In this paper, we utilized the same laser micro dissection methods and protocols as in paper II, with the purpose of increasing the depth of the analysis. A minimum amount of 30 islet sections per donor was stipulated. The laser-capture procedure yielded enough RNA of sufficient quality and purity to

successfully perform AmpliSeq sequencing of the transcriptomes, resulting in expression level data from more than 20k genes.

Paper IV – Characterization of host defense molecules in the human pancreas

Seven out of nine host defense molecules show positive staining patterns in human pancreatic tissue from non-diabetic donors and in a donor that died at the onset of T1D.

Immunohistochemical analysis showed the expression of seven host defense molecules in the human pancreas, and two of the included peptides showed no positivity at all. Five molecules showed positive staining patterns in the exocrine niche of some or all non-diabetic donors, and five peptides showed positively stained cells in the Islets of Langerhans in some or all non-diabetic donors. For three of the molecules, cells were stained positively in both the exocrine and the endocrine niche of the pancreas. We do report inter-individual differences in the expression of these molecules, with varying degrees of positivity between donors as well as some donors showing negative staining patterns of molecules being positive in most donors. Most of these molecules have not been shown previously to be expressed in the human pancreas.

In a donor with acute-onset T1D, seven host defense molecules showed positive staining patterns. Two different biopsies representing two different parts of the pancreas were analyzed from the donor with acute-onset T1D; one part (CD45hi) with intense immune cell (CD45+) infiltration, and one part (CD45lo) with very few infiltrating immune cells. Four host defense molecules were non-detected by staining in the CD45lo biopsy, but showed positive staining patterns in the CD45hi biopsy. Defensin β1, which was not expressed in pancreata from any of the non-diabetic donors, was positive in scattered cells throughout the parenchyma of the CD45hi biopsy.

When comparing our results with gene expression data from islets obtained through laser-capture micro dissection from organ donors of different age in paper III and from isolated islets and exocrine cell clusters [132] there are generally low expression levels in both exocrine and endocrine tissue of most of the host defense molecules included in the paper except for three genes that were strongly expressed in both exocrine tissue and islets.

The pancreatic expression of host defense molecules in non-diabetic donors show no apparent correlation to duodenal expression, donor age or immune-cell infiltration

Immunohistochemical analysis of the presence of CD45+ and CD3+ cells in the pancreatic tissue show varying degrees of inflammation in the non-diabetic donors, with generally low levels of immune cell infiltration. Two donors show moderate CD45-positive infiltration and one donor show a relatively high level of CD45-positive infiltration. No apparent correlation was seen between the level of CD45-positive infiltration and host defense molecule positivity score. No correlation was seen between positivity score and donor age, and no correlation was seen between duodenal positivity score and the host defense molecule positivity score.

Inter-individual differences in expression patterns indicate varying base-line expression levels of host-defense peptides

We conclude that several host defense molecules are expressed on a protein level in the pancreata of deceased organ donors, and that the pancreatic expression of host defense molecules varies between individuals. This variation does not seem to be linked to age, on-going inflammation or the duodenal expression levels. Thus, we speculate that there may indeed be an inter-individual difference in the constitutional expression of host defense peptides, which in turn may influence susceptibility to pancreatic inflammation and consequently, to T1D. We also conclude that the expression of these molecules seem to vary between different parts of the pancreas in a donor with acute-onset T1D, and we conclude that inter-lobular or other definitions of regional differences in host defense molecule expression levels may indeed be present in both the healthy and afflicted pancreas and that this may have impact on pancreatic disease susceptibility. Due to the close anatomic proximity and physiological connection between the GI tract and the pancreas and as the gut microbiota is known to play an important role in pancreatits, it may also be an important factor in the development of T1D [59, 60] suggesting an important role for anti-microbial peptides in the pancreatic defense against bacteria and viruses [133, 134] and as mediators of an inflammatory response [135, 136].

Conclusions

Paper I

No apparent signs of inflammation, ER-stress or apoptosis were seen in islets showing morphological signs of hydropic degeneration in an organ donor that died at onset of T1D compared to islets from a non-diabetic donor.

Gene expression levels were similar in islets obtained through laser micro dissection compared to isolated islets.

Laser micro dissection is a feasible way of selectively obtaining tissue of interest from frozen pancreatic biopsies from deceased organ donors

Paper II

Genes related to cellular stress are differentially expressed in islets from all three sample groups, with the T2D donor group showing the greatest amount of differential expression compared with the control group.

No obvious clustering based on pre-mortal HbA1c levels can be seen on a gene expression level between the different donor groups when plotting the donors in a PCA diagram.

Islets from semi-diabetic donors with elevated HbA1c show more signs of cellular stress on a transcriptional level than islets from donors with undiagnosed T2D. We cannot explain these findings, and further research is needed on islets from donors with progressively impaired glucose tolerance including donors with moderately increased levels of HbA1c, in order to carefully and more precisely elucidate the dynamics of cellular stress imparted on islet cells by elevated blood glucose levels.

Laser-capture micro dissection is suitable for obtaining sufficient material for down-stream processing and gene expression analysis utilizing an array-based approach.

Paper III

The transcriptomes of islets from organ donors aged 1 to 81 are relatively similar, with only 20 genes co-varying significantly with age. We see no clustering of transcriptomes according to age, and the genes that vary with age do not seem to represent easily distinguished cellular functions.

The expression levels of the negative cell-cycle regulator $p16^{INK4a}$ increases with advancing age, as has been previously reported in mouse and human samples.

We speculate that the similarity in expression profiles could be due to the beta cells being of similar age, regardless of the age of the donor.

Laser-capture micro dissection is suitable for extracting tissue from frozen pancreatic biopsies in order to utilize standardized AmpliSeq protocols for transcriptome analysis.

Paper IV

We report the expression of seven host defense molecules in pancreatic tissue from non-diabetic organ donors. We find no correlation between the expression of host defense molecules in duodenal mucosa, donor age or the level of immune cell infiltration, and the expression levels of host defense molecules in non-diabetic donors.

We also report inter-individual differences in expression levels in the non-diabetic group, suggesting varying base-line expression levels of host defense molecules between individuals.

We speculate that this may influence pancreatic disease susceptibility in ways not yet elucidated, which may in turn have important implications in diabetes pathogenesis.

Future perspectives

The islets and the beta cells in diabetes

A wealth of research points towards the beta cells being affected in various ways in the context of diabetes. Damaged islets and intra- and peri-insular immune-cell infiltrates have been reported in T1D [36, 45, 46], and reduced beta-cell functionality [85, 86], reduced beta-cell mass [91, 92] and signs of cellular stress [88-90] have been described in T2D. Nevertheless, we do not have any clear explanations for the progress towards diabetic disease and it is clear that we do not fully understand the role of the beta cells in diabetes.

In order to further elucidate the biology of the islets and beta cells during the life span of the human organism, further studies are needed in relation to endogenous factors such as high blood sugar levels, aging and obesity. We have shown that RNA from islets obtained through laser micro dissection are of adequate purity and quality for transcriptome analysis through readily available standard methods such as AmpliSeq. Further studies should focus on expanding the scope of analysis on donors with different HbA1c levels, including donors in the 5.5-6.0% HbA1c range, by utilizing full transcriptome analysis. To investigate cellular stress in this material is of vital importance, as are the expression levels of genes and pathways related to cell proliferation and survival.

The analysis of pancreatic tissue from donors of different ages could also be expanded upon, and further transcriptional analysis on larger donor groups is needed in order to further elucidate if there are any signs of beta-cell renewal not visible in our material, and if there are further clues to the actual lifespan of the beta cells. Effort should also be directed at further developing the laser micro dissection protocols in order to achieve methods for single-cell analysis that in turn would enable the subsequent analysis of different cell types in the islets. This could provide clues to whether the cell types of the islets respond to aging differently from one another.

Another area of great importance is the emerging non-invasive studying of beta cell function and biology through the use of radiologic methods for measuring beta-cell mass [137] and the analysis of circulating bio-markers for beta-cell death [138].

The pancreas

There are regional variations to the incidence in T1D that cannot be completely explained by endogenous factors such as genetics, pointing towards the possibility of one or more factors related to environment. In order to understand how environment could affect the islets, it is of key importance to understand the pancreas on a physiological level, but also to understand pancreatic immunology.

We have shown the pancreatic expression of several innate host defense molecules, but much remains to be elucidated regarding the pancreas as an immunologic niche. One way to do this is to histologically and transcriptionally examine pancreata from organ donors with and without diabetes, and with different pancreatic afflictions at time of death, such as pancreatitis or malignant disease. By deepening our understanding of the immunology of the exocrine and endocrine pancreas, we may be able to better understand the possible impact of inflammatory events on the organ. This could possibly be combined with the analysis of beta cell bio markers in peripheral blood as discussed above, but also with the analysis of the systemic inflammatory status of the donor.

The human body

In order to understand the pathological processes resulting in overt diabetes, we must also understand the context in which the beta cells, the Islets of Langerhans and the pancreas exists. Therefore, more research directed in understanding extra-pancreatic factors such as variations in the gut microbiome and the possible effects it could have on diabetes susceptibility is needed. Furthermore, the effects of systemic metabolic stress need to be further explored in islet and beta-cell biology, so as to deepen the understanding of our findings on an islet- and beta cell level.

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