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Characterization of the Pancreas in Type 1 and Type 2 Diabetes

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Abstract

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Diabetes is recognized by hyperglycaemia and polyuria. Complications, reduced quality of life and staggering health-care costs are all derived from the disease. Two subclasses of diabetes are Type 1 diabetes (T1D) and Type 2 diabetes (T2D). The beta cell mass is reduced in T1D, which is generally considered to be caused by an immune-mediated beta-cell destruction, but definitive evidence for this hypothesis remains absent. Development of insulin resistance and dysfunctional beta cells are commonly recognized as important factors that contribute to fulminant T2D. The literature that describes human T1D and T2D pancreata is sparse due to the limited number of specimens available for study. If more features of the respective pancreata are described, we might be able to elucidate the mechanisms involved in the pathoetiology of the diseases.

Accordingly, in this thesis pancreatic biopsies obtained from subjects with T1D or T2D have been examined with the aim to provide a more comprehensive picture of the respective pancreata. **Paper I** reports that aggregates of leucocytes substantiated mostly by macrophages are present in several T2D pancreata. Furthermore, as 28% of the T2D pancreata met the consensus definition of insulinitis developed for T1D, a redefinition of insulinitis is proposed. In **Paper II**, the density of parasympathetic axons was found to be reduced in the exocrine compartment in recent-onset T1D subjects compared to non-diabetic and long-standing T1D subjects. However, no alteration was discovered in islet-associated parasympathetic axons. In **Paper III**, interferon-stimulated genes were found to be over-expressed in recent-onset T1D islets, but no inducer explaining this expression has been discovered. **Paper IV** shows that T2D islets exhibit a stress response on a transcriptional level, and expression of these genes were investigated in islets from subjects with elevated HbA1c levels but without a clinical T2D diagnosis.

In conclusion, this thesis explores several new areas of the pancreas in both T1D and T2D, and demonstrate several important findings that increase our knowledge on how diabetes develops.

Keywords: Type 1 diabetes, Type 2 diabetes, HbA1c, interferon-stimulated genes, parasympathetic, axon, cellular stress, islets, exocrine, immunology, leucocytes, inflammation, human, pancreas

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*Dedicated to my father who
introduced me to science fiction.*

List of Papers

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

- I **Lundberg, M.**, Seiron, P., Ingvast, S., Korsgren, O., Skog, O. Insulinitis in human diabetes: a histological evaluation of donor pancreases. *Diabetologia* 2017 Feb; 60 (2): 346–353
- II **Lundberg, M.**, Lindqvist, A., Wieurp, N., Krogvold, L., Dahl-Jørgensen, K., Skog, O. The density of parasympathetic axons is reduced in the exocrine pancreas of subjects recently diagnosed with type 1 diabetes. *PLOS ONE* 2017 Jun 19;12(6):e0179911
- III **Lundberg, M.**, Krogvold, L., Kuric, E., Dahl-Jørgensen, Skog, O. Expression of Interferon-Stimulated Genes in Insulitic Pancreatic Islets of Patients Recently Diagnosed with Type 1 Diabetes. *Diabetes* 2016 Oct; 65(10): 3104-3110
- IV **Lundberg, M.**, Stenwall, A., Tegehall, A., Korsgren, O., Skog, O. Expression profiles of stress-related genes in islets from donors with progressively impaired glucose metabolism. *Islets* 2018 Feb 15:e1433980

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Abbreviations

BMI = Body mass index
CXCL10 = C-X-C motif chemokine 10
DiViD = Diabetes virus detection study
dsRNA = double-stranded RNA
FDR = False discovery rate
FoxP3 = Forkhead box P3
GWAS = Genome-wide association study
HbA1c = Glycated haemoglobin
HLA = Human leucocyte antigen
IF= Immunofluorescence
IFN = Interferon
IHC = Immunohistochemistry
IPEX = immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
ISG = Interferon-stimulated gene
LADA = Latent autoimmune diabetes of the adult
LCM = Laser capture microdissection
NOD = Non-obese diabetic
nPOD = Network for Pancreatic Organ donors with Diabetes
PCR = Polymerase chain reaction
PP = pancreatic polypeptide
qPCR = Real-time polymerase chain reaction
T1D = Type 1 diabetes
T2D = Type 2 diabetes
Treg = T regulatory cell
UPR = Unfolded protein response
VIP = Vasoactive intestinal peptide

Introduction

Diabetes is a widespread and prevalent disease that affects approximately 415 million individuals globally [1]. Both reduced life expectancy and quality of life are consequences of diabetes [2, 3]. Long-term exposure to hyperglycaemia damages microvasculature, that may lead to retinopathy, neuropathy, nephropathy and slow wound healing [2]. In severe cases, amputation, blindness and kidney replacement are unfortunate results. Although aggressive treatment with insulin reduces the risk for complications, it may also have the adverse effect of inducing increased episodes of hypoglycaemia, hypoglycaemia unawareness, as well as coma/death [4-6]. Diabetes is estimated to affect approximately 642 million in 2040 [1]. Therefore, diabetes will continue to be a major global epidemic that demands much from the affected individuals as well as from society. Complications, reduced quality of life, as well as 174 billion dollars in yearly health care costs in the US alone [5], are all derived from diabetes, and taken together, the incentive to find a curative treatment is strong.

Insights into the aetiology and pathology of a disease constitute an important foundation when preventive and curative strategies are considered. More than 50 000 articles state that type 1 diabetes (T1D) has an autoimmune aetiology, and several risk factors for type 2 diabetes (T2D) are known. Furthermore, T2D is generally considered to be caused by a combination of resistance to insulin action and an inadequate compensatory insulin secretory response [8]. This implies that the aetiology and pathology of the respective diseases to a large extent have been determined. However, current knowledge is based on a limited amount of human data. This has led us to carefully approach the question of what may contribute to the development of diabetes.

In this thesis, features present in pancreas biopsies obtained from subjects with T1D or T2D have been examined and described with the aim to elucidate the aetiology and pathology of the conditions.

A historical perspective on diabetes

Records originating from ancient Egypt, India and China mention a condition where excessive thirst, honey-like urine and weight loss were prominent features [5]. The same condition was, during the 2nd century, named *diabetes* by Aretaeus, meaning “siphon” to address the excessive amount of urine associated with the disease [9], and in 1675, Thomas Willis complemented this description by adding the Latin term *mellitus* for “sweet honey” to the name of the condition [10]. In 1869, Paul Langerhans described clusters of cells spread out within the pancreatic parenchyma [11], and Eduard Laguesse later deduced that the islets had an endocrine function, and, in recognition of Langerhans’ work, named them “islets of Langerhans” [12]. In 1889, pancreatectomy of dogs proved to give symptoms similar to human diabetes, which was reversed by placing a small portion of pancreas back into the dogs [13]. By examining cadaver pancreata collected from human subjects with diabetes, Eugene Opie 1901 connected damaged islets to the condition [14]. Two decades later, in 1921, the “insulin” of islets was isolated and injected into dogs [15], and the following year, the first human patient was successfully treated with insulin [16].

Type 1 and type 2 diabetes

Classification, diagnosis and treatment

In the 19th century, Lancereaux recognized that there were at least two forms of the diabetic condition and named these two forms *diabetes maigre* and *diabetes gras*, which translates to diabetes of the “thin” and “fat”[17]. By 1979, juvenile diabetes, or insulin-dependent diabetes mellitus was reclassified as type 1 diabetes (T1D) whereas adult onset, or non insulin-dependent diabetes mellitus, was reclassified as type 2 diabetes (T2D) [18].

In present time, hyperglycaemia constitutes a common denominator for all types of diabetes [19], and further classification is based on clinical assessment and laboratory testing. T1D is, at diagnosis, characterized by marked hyperglycaemia recognized by acute insulin insufficiency, polydipsia, polyuria, and polyphagia [8]. T2D, on the other hand, is recognized by symptoms that initially are less severe and that have developed for an extended period of time before diagnosis [8]. As the previous classifications of the respective conditions imply, T1D (juvenile diabetes) commonly affected young, lean patients, whereas T1D (adult onset diabetes) affected old, often obese patients. Indeed, T2D is associated with obesity, physical inactivity and age [5]. When clinical assessment of the patient is not sufficient to make the

diagnosis, laboratory testing of auto-antibodies often present in T1D [20] and severe loss of c-peptide levels support the diagnosis of T1D [21].

Classification of diabetes may appear uncomplicated. However, about 50% of T1D patients are diagnosed in adulthood, and clinical presentation occurs at all ages, as late as in the ninth decade in life [22]. Furthermore, T2D affects patients in their adolescence, and the frequency of patients with auto-antibodies within this group has been reported to be as high as 30% [23]. Conclusively, the current diagnosis criteria for diabetes contain contradictions and are insufficient to clearly classify all patients without overlaps. However, according to the American diabetes association, assigning a type of diabetes to an individual depends on the circumstances at the time of diagnosis, and *“for the clinician and patient, it is less important to label the particular type of diabetes than it is to understand the pathogenesis of the hyperglycemia and to treat it effectively”* [8].

Latent autoimmune diabetes of the adult (LADA), also known as type 1.5 diabetes, is often misdiagnosed as T2D due to a similar clinical phenotype at the time of diagnosis [24]. LADA patients do not require insulin treatment for a period after diagnosis. However, LADA patients progress to insulin therapy more rapidly, have higher HbA1c levels, lower BMI, and the beta cell function declines faster than in adult T2D patients [24]. Additionally, LADA patients are islet-auto-antibody positive. In comparison to T1D patients, LADA patients have a less severe loss of beta cell function and fewer multiple islet auto-antibodies [24]. The name of the condition, “latent autoimmune diabetes of the adult”, is derived from the presence of islet auto-antibodies. However, known islet auto-antibodies target intracellular, and not surface antigens of the beta cell [25]. This precludes a direct pathogenic mechanism of the auto-antibodies [25]. Furthermore, auto-antibodies are present in non-autoimmune conditions [26]. Additional diabetes classifications are monogenetic diabetes, idiopathic diabetes, gestational diabetes, and, more recently, additional sub-stratifications of diabetes have been presented based on clinical disease progression and complications [8, 27]. These are, however, not subject of further discussion in this thesis.

Insulin-insufficient patients (e.g. T1D and LADA) require exogenous insulin replacement. Patients with resistance to insulin action and an inadequate compensatory insulin secretory response (e.g. T2D and gestational diabetes) are often initially treated with exercise, weight loss, and glucose-lowering agents [8].

Epidemiology

Diabetes is estimated to affect approximately 439 million in 2030 [28] and 642 million in 2040 [1]. The majority (90-95%) have T2D while the minority (5-10%) are diagnosed with T1D [29].

The incidence of T1D is highest in Finland with an annual incidence of 60 in 100,000, and lowest in Caracass (Venezuela) and Zunyi (China), with an incidence of 0.1 in 100,000 per year [35]. The incidence has been increasing for several decades worldwide [36] but has recently plateaued in Sweden [37]. Two neighbouring populations from the Karelian Republic of Russia and Finland have equally genetic susceptibility for T1D. However, the incidence of T1D is close to sixth-fold higher in the Finish population, suggesting that environmental factors contribute to the difference in incidence rate between these two regions [38].

Age [30], diet, lifestyle [31], increased BMI, and genetic factors influence the risk for T2D [32]. The major burden of T2D is now taking place in developing rather than developed countries [28]. Of T2D cases, 80% live in less developed countries and areas [28]. China has recently emerged as the diabetes epicentre in the world. In 1980, <1 % of the Chinese population was affected by diabetes [33], compared to 9.7 % in 2008 [34]. Furthermore, the prevalence of T2D, traditionally considered exclusive for adults, has increased in adolescents as well as in children. The prevalence of T2D in youth varies substantially by ethnicity and is higher among ethnic groups such as Australian Indigenous, Native American, Hispanic, African American, Asian and Pacific Islander populations [28].

The Pancreas

The pancreas contains the insulin-producing beta cells that are affected in both T1D and T2D. The pancreas is a 15-20 cm long, approximately 70 g heavy, retroperitoneal organ, positioned just below the stomach [39]. It is divided into three main parts; head, body and tail, that are composed of lobes and smaller 1-10 mm lobules [40]. The largest portion of the pancreas is composed of exocrine cells that, through the ducts, release digestive enzymes into the duodenum. The minority of the pancreatic cells (1-2 %) are clustered into islet-like structures scattered in the pancreatic parenchyma (see islets of Langerhans mentioned in historical perspective). These highly vascularized islets are on average 140 μm in diameter and are partially surrounded by a fibrous capsule [41, 42]. The islets consist of cells with different endocrine functions; the beta cells produce insulin, alpha cells produce glucagon, delta cells produce somatostatin, PP cells produce pancreatic pol-

ypeptide, and epsilon cells produce ghrelin. The beta-cell mass is the largest (~55%), followed by alpha cells (~33 %), while the other endocrine cells comprise a lesser part of the islet [43]. This relative distribution is, however, varying in different parts of the pancreas [44]. Compared to mice, human islets have reduced vessel volume, limited interaction between vasculature and beta cells, and a more homogenous distribution of endocrine cells within the islet, which may have functional implications [45, 46].

Pancreatic innervation

The autonomic nervous system appears to affect islet secretion as administration of drugs that inhibit the neurotransmission across autonomic ganglia impairs the insulin response during the cephalic phase [47]. Furthermore, vagotomy, auto-transplantation and transplantation of pancreas, all reduce pancreatic polypeptide (PP) secretion in response to hypoglycaemia [48-50].

Already in the 19th century, Paul Langerhans described nerve innervation of the pancreas in cat and rabbit [51]. Since then, an extensive literature derived from animal models has accumulated that describes the anatomy, targets, and functions of the pancreatic innervation [51]. Literature derived from human pancreata was sparse until 2011, when Rodriguez-Diaz et al. described human islets to be mainly innervated by sympathetic axons that target vascular smooth muscle cells [52]. Rodriguez-Diaz et al. further demonstrated that mouse islets are more innervated compared to human islets, and that exocrine tissue contains both sympathetic and parasympathetic axons (the latter being in accordance with previous and recent reports [52-57]). VIP-containing parasympathetic axons can be found within and close to pancreatic islets [53-57] but the number of axons has only been described to a limited extent [52].

In animal models, parasympathetic axons stimulate glucagon secretion in response to hypoglycaemia and insulin secretion in response to hyperglycaemia. Sympathetic axons inhibit insulin secretion and stimulate pronounced glucagon secretion during hypoglycaemia [58]. However, this should not be translated to human physiology as the axon density and innervation target of axons are dissimilar [52]. Exocrine secretion is also regulated by the autonomic nervous system in animal models [59]. There may be a similar function in humans, but the mechanisms and extent remain to be determined.

Pathophysiology and aetiology of type 1 diabetes

In 1965, Gepts published his landmark paper where autopsy samples from a large collection of individuals with T1D were reported to contain peri- and intra-islet inflammatory infiltrates [60]. This phenomenon was coined “insulinitis”, and Gepts, with support from other literature of the time, pointed out the possibility of an immunological derangement in connection to the aetiology of the disease. Later, Eisenbarth put forward a model for the natural history of T1D that remains the most referenced [61, 62]. According to Eisenbarth’s model, individuals are born with varying degrees of genetic predisposition to develop T1D. Environmental triggers initiate autoimmunity, and relapsing immune-mediated destruction of the beta cells decreases the beta cell mass over time until the remaining cells cannot compensate for their low mass, and clinical symptoms appear.

The defining criteria for an autoimmune disease

In an attempt to evade a subjective debate regarding a potential autoimmune disease’s origin, Witebsky’s postulate was designed in 1957 [63] and updated 1993 [26]. According to Witebsky’s postulate three criteria need to be fulfilled to define a condition as autoimmune in origin. These are: 1) Direct evidence of pathogenic antibodies or pathogenic T cells; 2) indirect evidence based on reproduction of the autoimmune disease in experimental animals; 3) circumstantial evidence from clinical clues.

Witebsky’s first criterion is direct evidence of pathogenic T cells or pathogenic antibodies. 15-year-old children from western countries with a susceptible HLA allele or with first degree relatives with T1D, have a diabetes risk that is 12.7 % with one islet auto-antibody, and 79.1 % with three auto-antibodies [64]. Islet auto-antibodies are therefore biomarkers of T1D. However, there is currently no evidence supporting a pathogenic role for auto-antibodies in T1D [25, 65, 66]. In fact, the discovered auto-antibodies target intracellular beta cell antigens, rather than surface antigens, which preclude a direct pathogenic role [25]. In this context, it is important to mention that auto-antibodies have been reported to be present during non-pathogenic conditions [26], and it is therefore possible that islet auto-antibodies are non-pathogenic.

The presence of T cells reactive against islet antigens has been reported in the pancreas of several, yet a limited fraction of subjects with T1D [67]. However, the majority of T cells found within the pancreas of subjects with T1D are of unknown specificity. In fact, a substantial proportion of CD8+ cells discovered in the insulitic infiltrate display a tissue resident memory T cell phenotype, arguing against a conventional cytotoxic response [68]. Fur-

thermore, autoreactive T cells are equally frequent in peripheral blood of recent onset T1D and non-diabetic subjects [69]. Therefore, the presence of a limited number of autoreactive T cells in a limited proportion of T1D pancreata, should not necessarily be interpreted as causal to beta cell destruction. In conclusion, a pathogenic role of islet auto-antibodies or T cells is not evident, and thus Witebsky's first criterion is not fulfilled.

Witebsky's second criterion is indirect evidence based on reproduction of the autoimmune disease in experimental animals. Due to the lack of availability of human pancreatic tissue from subjects with T1D, a majority of studies on T1D have been performed in animal models. The NOD mouse [70] and the BB rat [71], share several genetic and immunological characteristics with human T1D subjects, and have therefore been considered the best available options to model T1D in humans [72]. The islet inflammatory lesions (insulinitis) found in the animal models have proved to contribute to a beta cell specific autoimmune destruction [73]. As diabetes is replicated through an autoimmune mechanism in an experimental model, this suggests that Witebsky's second criterion is fulfilled [26]. However, insulinitis was in a recent meta-analysis limited to 73% of T1D patients aged <14 years and in patients >14 years old, insulinitis was only found in 40% [74]. Additionally, the human insulinitis is discrete, is heterogeneously distributed, and affects few islets in comparison to the NOD model. Several immunoregulatory treatments have been successful in studies performed on the NOD mouse and BB rat, but when translated to a clinical setting, these treatments are much less promising [75]. Therefore, the NOD model does, at the very least, not represent all human T1D subjects. Conclusively, Witebsky's second criterion is not fulfilled.

Witebsky's third criterion is circumstantial evidence from clinical clues. According to this criterion, circumstantial evidence such as a statistical association between disease and certain HLA haplotypes, lymphocytic infiltration of target organ, and association to other autoimmune diseases within the same individual or within the family is not sufficient evidence to define a disease as autoimmune in origin but is an incentive for future research [26]. A monogenetic disease named immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) is caused by a mutation in FoxP3. FoxP3 is essential for the immune-suppressive function of T regulatory cells (Tregs) [76]. When there are no functional Tregs, massive immune infiltration of the pancreas as well as diabetes develop at a young age. This demonstrates that a genetic mutation, with consequent dysfunctions of the inhibitory regulation of the immune system, in turn results in the development of diabetes with an autoimmune aetiology. Of note, however, is that histological examinations of subjects with IPEX reveal that the immunopathological situation appears to vary substantially from the one reported in T1D [77]. In

addition to monogenetic mutations, alleles of the HLA-region have been associated to T1D although it remains undetermined which of the hundreds of genes in the region that cause the association [78]. Additional genes associated to T1D have been discovered with genome wide association studies (GWAS). Many of the associated genes have an immunological function. However, the associations are weak and fail to explain the pathogenesis of the disease [78]. As mentioned above, these findings are interesting and may be used as an incentive for future research. However, they are not sufficient to define T1D as an autoimmune disease.

Collectively, according to Witebsky's, postulate there is insufficient evidence to define T1D as a disease with an autoimmune origin.

Several features present in T1D argue against an autoimmune origin of the condition

There are several features connected to T1D that are rarely mentioned in relation to the aetiology of the disease. Besides the islet auto-antibodies that are commonly found in subjects with T1D, auto-antibodies targeting cells of the exocrine compartment are also evident among a noteworthy proportion of T1D patients. Auto-antibodies targeting either carbonic anhydrase II, or lactoferrin, that are expressed in exocrine cells, have been reported to be present in 77 % of T1D patients [79]. Additionally, auto-antibodies against bile salt dependent lipase is present in about 70 % of T1D patients and in about 30 % of pre-T1D patients [80]. In 39 % of T1D patients, auto-antibodies against cytokeratin can be found [81]. The role of these exocrine auto-antibodies is unelucidated. Just as is the case of the role of islet auto-antibodies, they are most likely non-pathogenic biomarkers of tissue damage and generated as a secondary by-products of exocrine cell destruction.

The volume of the pancreas is reduced approximately 30% on average in patients examined shortly after the time of diagnosis [40, 82]. As the endocrine tissue mass constitutes a small proportion of the pancreas, this decrease is more than can be explained by the loss of beta cells [83]. Furthermore, there is an insufficiency of the exocrine pancreatic secretion in a majority of T1D patients, suggesting that the pancreas in T1D also has a loss of function [84]. The weight reduction of the pancreas has been suggested to be explained by a loss of trophic effects exerted by insulin on the exocrine tissue when the beta cells are diminished in T1D [85]. However, there is a loss of exocrine mass already at onset and even in auto-antibody positive donors [39, 40], where insulin secretion is still normal or at least affected to a limited extent. This suggests that the lack of local insulin trophic effect does not

explain the whole phenomenon as to why the exocrine compartment in T1D is negatively affected [85].

Signs of pancreatitis is often discovered in subjects with T1D, already reported by Gepts in his classical work [60], and collectively, the whole pancreas appears to be negatively affected in T1D but with the main clinical symptoms emanating from the loss of beta cells. Of special note, C-peptide levels are still detectable several years after onset of disease [75]. This suggests that although the beta cell is the cell type mainly affected in T1D, it is not completely eliminated as would be expected from an autoimmune response.

Interferon-stimulated genes in T1D

Both viral and bacterial infections have been implicated [86, 87] but never proven to have an etiological role in T1D. Expression of interferon-stimulated genes (ISGs) can be regarded as “viral foot prints” that link innate and adaptive immunity as they are induced by viral infections [88]. However, ISGs are also induced by other factors [89, 90], and may therefore be considered a more general sign of inflammation. In response to viral and bacterial infection as well as exposure to their components (such as dsRNA and lipopolysaccharide), interferons are produced [91]. Interferons put the nearby cells in an anti-viral state by upregulating the expression of several genes [91]. All nucleated cells can produce Type I IFNs as a response to stimulation of pattern recognition receptors (PRRs). Viral nucleic acids are recognized by cytosolic PRRs such as RIG-I and MDA5 and by several toll-like receptors (TLR) [92]. Type I IFNs can also be induced by bacterial components that are recognized by TLR-4 [93]. Intracellular signaling cause the transcription factors IRF3 and IRF7 to bind to a transcription factor binding site called interferon-stimulated response element (ISRE), which activates gene transcription of several genes, including Type I IFNs [94]. Type I IFNs have an autocrine and paracrine effect by binding to the cell surface receptors IFNAR1 and IFNAR2, which, through activation of the JAK-STAT signalling pathway [95, 96], ultimately results in transcription of ISGs.

One example of an ISG is members of the 2'-5' oligoadenylate synthase (OAS) family that activate the endoribonuclease RNase L to degrade viral transcripts [97]. Tetherin is another example of an ISG, that prevents virus budding by trapping virions on the cell surface [98].

Immunohistochemical (IHC) studies have suggested the expression of Type I IFNs [99, 100] and some ISGs [101, 102] in T1D islets, which has been presented as indirect evidence for a viral infection of the islets. However, other

studies have been unable to confirm the presence of Type I IFN in T1D islets [103]. Furthermore, as mentioned above, ISGs are induced also by other factors than viruses [89, 90].

In summary, the aetiology for T1D remains undetermined and should not necessarily be assumed to be autoimmune in origin. Although there is a great limitation of the available human pancreatic samples obtained from subjects with T1D, one of the most pressing needs in the field of T1D aetiology is to evaluate this tissue with an unbiased approach to define the features that characterize the pancreas in T1D.

Aetiology and pathogenesis of type 2 diabetes

Age [30], diet, lifestyle [31] and increased weight affect the risk to develop T2D. However, there is also a high heritability for T2D [32]. For example, the concordance rate for either T2D, or impaired glucose tolerance, is 96%, 15 years after twins have been ascertained discordant for diabetes [104]. Impaired glucose metabolism develops gradually and overt hyperglycaemia is often present for a period before diagnosis [105]. Hypersecretion of insulin in early stages of the disease suggests an initial crucial role of insulin resistance [105]. Furthermore, excessive liver fat has been suggested to play a key role in the early pathogenesis of T2D, as energy-restrictive diet lowers triacylglycerol stores in the liver which leads to normalized glucose metabolism [106]. In later stages of the disease progression, a reduction in insulin secretion is observed, leading to fulminant T2D and in some cases, eventual loss of endogenous insulin production [105].

Beta-cell loss

Insulin-positive cells have been reported to be reduced in several histological studies, and part of the c-peptide insufficiency in T2D has been attributed to this reduced beta-cell mass [107, 108]. However, Marselli et al. recently examined T2D islets both by insulin immunohistochemistry (IHC) and electron microscopy. Insulin-positive cells were substantially reduced in T2D but there was only a minor reduction of beta cells when electron microscopy was utilized [109] and others have found a similar lack of difference when electron microscopy is used to assess beta cell number [110]. These reports should be carefully interpreted as electron microscopy only allows a limited number of islets to be examined. However, in addition to electron-microscopic examinations of islets, endocrine mass measured by endocrine tracers in vivo, is not decreased in subjects with T1D in relation to reduced c-peptide levels [111]. Furthermore, dedifferentiation of beta cells in response to long exposure of triacylglycerols has been suggested [106]. Taken

together, this may suggest that the reduced number of insulin-positive cells in T2D is derived from exhausted or partly dedifferentiated beta cells rather than through apoptosis.

Beta-cell dysfunction

The metabolic demand and the dysfunctional secretion are expected to correlate to an altered phenotype of the islet. Indeed, the mitochondria and ER have altered morphologies in T2D, indicating that the pathways related to these organelles are affected [112, 113]. Furthermore, islets obtained from T2D donors show some signs of ER stress [113, 114], oxidative stress [114] and mitochondrial dysfunction [112, 115] on a transcriptional level. However, these transcriptional patterns have been surprisingly weak, indicating that the islets are somewhat functionally intact. Whether the altered transcriptional phenotype is a cause, or a consequence of the hyperglycaemia in T2D remains undetermined. Furthermore, it is currently unknown how the islets vary in their morphological and transcriptional pattern as pre-diabetes progress to fulminant T2D. As loss of insulin secretory function is imperative for the development of T2D, this area requires additional study.

Aims

The literature that describe human T1D and T2D pancreata is sparse due to the limited number of specimens available for study. If more features of the respective pancreata is described, we might be able to elucidate the mechanisms involved in the pathoetiology of diabetes. With the ultimate aim to elucidate the aetiology and pathology of diabetes, this thesis examines pancreatic biopsies obtained from subjects with T1D or T2D to provide a more comprehensive picture of the respective pancreata.

Paper I

A preliminary examination of CD45+ cells in a T2D pancreas indicated the presence of an elevated number of leucocytes. Consequently, the first hypothesis of Paper I was that a substantial proportion of T2D pancreata demonstrate an elevated number of leucocytes and signs of inflammation. This lead to the next hypothesis: namely, that T2D pancreata can meet the definition of insulinitis developed for T1D due to higher background levels of leucocytes. Moreover, if one characteristic described as a hallmark of the T1D pancreas is present in T2D pancreata, an additional hypothesis was that other characteristics associated with the T1D pancreas—such as insulin-deficient islets and HLA Class I hyperstained islets—might be as well. All of these hypotheses are addressed in Paper I.

Paper II

Sympathetic islet innervation has recently been reported to be reduced in T1D [116]. Parasympathetic innervation of the pancreas has not previously been systematically examined in T1D. The aim in Paper II was to investigate the distribution of parasympathetic axons in T1D pancreata.

The first hypothesis was that the number of islet-associated parasympathetic axons would be altered in T1D. The second hypothesis was that the density of parasympathetic axons would be altered in the exocrine compartment of the pancreas in T1D.

Paper III

Activation of the innate immune response has been suggested to be an important step in an inflammatory process of the islets in T1D. The first hypothesis in Paper III was that expression of ISGs is differentiated in insulitic islets from subjects with T1D compared to non-diabetic, non-insulitic islets. If ISGs are differentiated in insulitic islets, an additional objective was to determine whether the presence of enteroviruses can be linked to the phenomenon. The second hypothesis was that the ISGs has a higher expression in the islet core of an insulitic islet than in the peri-islet area containing leucocytes.

Paper IV

Islets in T2D become dysfunctional and have been suggested to lose their phenotype as the disease progresses [106]. However, islets in pre-T2D and established T2D have not been well examined.

The aim of Paper IV was to evaluate the cellular-stress response in islets from donors with elevated HbA1c and established T2D by examining the expression of genes related to the mitochondria, unfolded protein response (UPR) and oxidative-stress response. The hypothesis was that differentiation of stress-related genes is apparent in established T2D islets and is present in donors with elevated HbA1c.

Considerations on research design and methods

Some of the materials and methods used in Paper I-IV are discussed and described below. Detailed information can be found in the respective papers.

Human pancreata

Pancreata from heart-beating organ donors

Human pancreata available for research are sparse and the collective number that has been examined is very limited due to: 1) the inaccessibility of the pancreas, 2) the high risks associated with collection of biopsies and 3) the intense autolysis occurring at death due to the release of exocrine enzymes [72]. Most pancreata have been obtained from cadaveric donors with profound autolysis where reduced quality of morphology and molecules are unfortunate consequences.

The effects of pancreatic autolysis can be limited by examining pancreata obtained from heart-beating organ donors. Therefore, efforts to collect human pancreatic samples in biobanks have been made. Two such biobanks are the Nordic network for clinical islet transplantation, and the Network for Pancreatic Organ Donors with Diabetes (nPOD).

Limitations with pancreata from heart-beating organ donors

There is a high incentive to examine pancreata obtained from T1D or T2D donors, as motivated in the introduction. Pancreata from heart-beating organ donors may be among the best samples of diabetic pancreata available for examination. However, several predicaments remain with these samples.

One predicament is that it is unknown how well the picture seen in the examined pancreas represents the situation prior to organ donation and disease. Parameters within the pancreas (such as the migration of leucocytes), or within the peripheral blood (such as HbA1c), could be altered as a response

to events prior to brain-death (e.g., alcohol consumption, steroids and other medications) or due to the time between brain death and removal of the pancreas. However, no correlation between duration of hospital stay and frequency of CD45+ or insulin-positive cells has been found in non-diabetic donor pancreata [119].

An additional predicament is that, during the design of a study, it is not always known how the patient was treated prior to death, and therefore not easy to match for medications and treatments. However, it is possible to match other variables between groups that could have an impact on the outcome of the results, such as age, sex, BMI and HbA1c.

The nPOD biobank

The nPOD biobank, initiated in 2007, contains high-quality samples from well-defined donors and, as of May of 2018, it included 182 controls, 154 donors with T1D, 35 auto-antibody-positive donors without T1D, and 41 donors with T2D (<https://www.jdrfnpod.org/for-investigators/donor-groups/>) [117]. Although the number of T1D pancreata in the nPOD cohort is impressive, only three among the T1D donors were diagnosed for <1 year (0, 0.25 and 0.6 years, respectively) [118].

The pancreata of donors with more recent onset of T1D are of particular interest to study, as progressive beta-cell loss is ongoing. In long-standing T1D pancreata, it is rarer to discover insulin positive cells, and thus, more difficult to study beta-cell loss. However, already in recent-onset T1D pancreata, evidences to the initiating events that have led to beta-cell loss may be gone. Therefore, it is also of high interest to examine pancreata from pre-T1D donors. Auto-antibodies are biomarkers for T1D, but it is unknown if or when a donor with auto-antibodies (even multiple) would develop symptoms of T1D.

Nordic network for clinical islet transplantation biobank

The Nordic network for clinical islet transplantation biobank contains pancreata from approximately 2000 non-diabetic donors, 50 T2D donors, 31 auto-antibody positive donors, 12 donors with long-duration T1D and three donors that died at onset of T1D. Among the control donors are several with elevated HbA1c levels who would have been diagnosed with pre-diabetes [105] or T2D [122] given the HbA1c.

Pancreatic tail resections from T1D patients

The DiViD biobank

Because autopsy-based specimens are at risk of masking important information or introduce artefacts [120], the Diabetes Virus Detection study (DiViD) was initiated. After all appropriate ethical permissions had been obtained and informed consent from the participants had been acquired, this study, led by professor Knut Dahl-Jørgensen, proceeded to collect pancreatic tail resections by laparoscopy from six patients with short-duration T1D (3-9 weeks since onset) [121]. A larger sample size (i.e., additional patients) was initially planned for the study; however, due to an unexpectedly high complication rate, it was deemed unethical to continue the study [121]. The incentive to initiate the study was high, and the DiViD biopsies have the potential to provide essential information regarding numerous questions relevant to the pathogenesis of T1D [120]. However, whether this high-risk study was warranted will be known only after the data of the study is obtained and we have determined how it affected the development of preventive and curative strategies for T1D.

Pancreata examined

Despite the caveats described above, well-preserved pancreata obtained from organ donors/patients with multiple auto-antibodies, or recent onset of T1D, are of high interest to examine, as they are particularly likely to provide clues regarding the pathogenesis of T1D. Furthermore, T2D pancreata are of high interest to examine to elucidate the pathogenesis of the disease. It is further important to examine islets during different stages of the T2D disease progress—even before the donors have been diagnosed with the condition. This can be done by examining pancreata obtained from pre-T2D donors, i.e., donors with elevated HbA1c [105].

In this thesis, biopsies from the DiViD study as well as biopsies collected through the Nordic network for clinical islet transplantation (where a consent from the donor or next of kin is mandatory) have been examined. Donor pancreata from recent-onset T1D, long-standing T1D, established T2D or elevated HbA1c have been investigated.

Immunohistochemistry and immunofluorescence

With IHC and immunofluorescence (IF), an antibody is used to label a specific antigen. This is often performed on a thin tissue section to discover the location of the antigen within the cell, or its distribution in a tissue. The anti-

body may be either polyclonal or monoclonal. Monoclonal antibodies are identical antibodies which target the same epitope. A direct or indirect method can be used to detect the antibody in the section. With the direct method, an enzyme such as peroxidase or a fluorophore such as FITC may be directly coupled with the antibody. When a substrate is added to the tissue with enzyme-conjugated antibodies, a coloured product is produced that can be viewed under a light microscope. If the antibody is fluorophore-conjugated, the fluorophore will emit light when exposed to light in a defined spectrum of wavelength. The indirect method includes primary antibodies that target the antigen and secondary antibodies that target the primary antibody. The secondary antibody is conjugated to an enzyme or fluorophore. Because it is common that several copies of the secondary antibody can bind to a single primary antibody, the indirect method causes an amplification of the signal.

Background staining may be present because the primary and secondary antibodies bind non-specifically to the tissue. “Reactive sites”, which cause this phenomenon, are quenched by blocking with a buffer containing, for example, bovine serum albumin or serum from the same species as the secondary antibody. To limit the influence of the possible cross-reactivity of antibodies (i.e., such that they bind to antigens other than the one they were developed for), the protocol should be validated by including a positive control where the antigen is present and negative controls where no antigens, and thus no signals, are present.

IHC can be used to detect and locate the presence of an antigen, but it cannot be used to quantify the amount of antigen present, as the intensity of the staining is dependent on the type of tissue, the type of fixation and the staining technique [123].

In this thesis, IHC and IF are utilized extensively. Leucocytes, islet cells, and VIP-containing axons have been detected through the presence of cell-specific antigens such as CD45, CD3, insulin, glucagon, synaptophysin and VIP.

Laser capture microdissection

As IHC and IF do not permit quantification of molecules, other methods are used for this purpose. One way to quantify molecules is to homogenize tissue and then analyse the molecule of interest [124]. If this approach is used on a heterogeneous organ such as the pancreas, the molecule of interest is analysed in all islet cells, duct cells, connective tissue and acinar tissue present in the biopsy. An altered presence of the molecule of interest in smaller cell clusters, such as the islets, may become indiscernible due to a large dilu-

tion of islet cells. Therefore, optimally, only one tissue type should be analysed at a time. However, if there are several variants of the tissue, the problem of diluting the molecule of interest may once again appear if it is hypothesized that only one of the variants of the tissue has an altered level of the molecule. Laser-capture microdissection (LCM) addresses these issues, as the method permits sub-classifications of tissue type and selective extraction based on histological appearance [124]. The molecule of interest can subsequently be quantified in the extracted tissue.

LCM has been used to examine human pancreatic samples in previous studies with some success and some difficulties [123, 125-128]. However, the histological information connected to the extracted tissue has been limited. Some histological information has been obtained from unstained or hematoxylin-stained LCM sections. However, to localize cells—e.g., insulin-positive cells or CD45+ cells—IHC is necessary. Precise histological information is mandatory for a study where very specific tissues are to be extracted. If the histological information is reduced in quality, the risk of analysing undesired tissues increases. IHC leads to increased degradation of RNA for one or several of the following reasons: fixation, antigen retrieval, blocking and long incubation periods [123]. Therefore, the sections used for IHC are not suitable for LCM and subsequent RNA analysis. To address this problem, consecutive sections may be used for IHC or LCM that together provide high-quality histological information and sections from which tissue-specific RNA can be extracted.

In Paper III, IHC was used to identify islets with ≥ 15 CD3+ cells, and LCM was used to extract the islets on the consecutive section [127]. However, the morphology can be altered in serial sections. Thus, it is advantageous to stain a section before and after the LCM-section. Accordingly, a protocol was developed that allowed separate extraction of islets and the peri-islet region containing leucocytes (see figure 1 in Paper III). In this protocol, every fourth section was stained, and the intermediate sections were used for LCM. If sections one and four, for example, had a similar morphology, the intermediate LCM-sections were assumed to also be similar. This protocol became a compromise, making islets viewable on two or more consecutive stained sections, and simultaneously minimized the quantity of unique pancreatic tissue that was required for the study.

A limitation of this optimized protocol is that it excludes smaller islets for extraction, as these can rarely be viewed in every fourth section due to the approximately 20 μm distance (10 μm /section) between them. Furthermore, the considerable preparation it requires (consecutive staining, IHC, analysis and preparation of extraction strategy for each section) is time-consuming. Moreover, the contrast between endocrine and exocrine tissue was, in some

LCM-sections, insufficient for confident extraction of islets. In Paper IV, the same protocol was used to exclude islets containing CD45+ cells. UV-treatment of PEN-membranes before sectioning did often make it easier to differentiate endocrine and exocrine tissues.

In this thesis, LCM have been used to extract selected tissues. The RNA expression has, thereafter, been analysed in the extracted tissues.

Interpretation of data

Several factors need to be taken into consideration during the analysis of data. One limitation encountered while preparing the current thesis was scarce access to human pancreata (i.e., the small sample size). In an attempt to compensate for the few pancreata included in examinations, previous studies [129-131] have considered every islet a sample and have categorized the islets into groups based on the condition of the donor from whom the islets were derived. This approach to data is unsuitable for a situation in which the hypothesis tested involves two groups that are compared (e.g., T1D and non-diabetes donors), as the statistical test does not test the hypothesis.

The risk for Type I errors—i.e., false inference that a difference exists—increases when a Gaussian distribution of data is falsely assumed or when multiple comparisons are not accounted for. In data derived from heterogeneous groups of human samples, as in the current thesis, the data may be considered not to have a Gaussian distribution. Therefore, non-parametric tests such as the Mann-Whitney U test (when two groups were compared) and the Kruskal-Wallis with Dunn's test (when several groups were compared) have been utilized.

In addition to Type I errors, there are Type II errors: i.e., that a null-hypothesis is falsely retained. If multiple comparisons are adjusted for (e.g., by Bonferroni correction), in a situation where the variables analysed are expected to co-vary, there is a high risk of Type II errors. Therefore, one approach is to analyse genes of the same pathway by allowing, but limiting, the amount of false discoveries by setting a false discovery rate (FDR). This adjustment provides information regarding how many false discoveries (Type I errors) are expected among comparisons with a p-value below, say, 0.05. Depending on how important it is to discover individual genes that deviate in their expression, the acceptable FDR may differ.

Results and discussion

Paper I - Insulitis in Human Diabetes: a Histological Evaluation of Donor Pancreases

Revising the definition of *insulitis*

Paper I reports that the consensus definition for insulitis that has been developed for T1D cannot be used to distinguish pancreata retrieved from individuals with T1D (31% fulfilled the definition) from those with T2D (28% fulfilled the definition). According to the common interpretation of the consensus criteria [132], an individual can be diagnosed with insulitis when ≥ 15 CD45⁺ cells are found within the parenchyma or in the islet-exocrine interface in ≥ 3 islets [133]. The consensus definition of insulitis was adopted at the nPOD meeting in February of 2013 and published in November of 2013 in an effort to enhance understanding of the pathogenesis of T1D and to facilitate comparisons between histopathological studies [133]. CD45 was chosen as a marker for immune cells because the insulitic infiltrates in T1D include T cells, B cells, macrophages and granulocytes [86, 133, 134]. The definition was designed to distinguish pancreata in individuals with T1D from pancreata with background inflammatory infiltrates, as observed in non-diabetic individuals [133]. However, the consensus definition did not consider background infiltrates in other donor groups such as T2D donors.

The highest number of CD3⁺ cells within or adjacent to an islet from an individual with T2D was 18, and we found multiple islets containing 6-10 CD3⁺ cells. We therefore suggest that an insulitic islet should be redefined so that a lesion can be established when ≥ 15 CD3⁺ cells are present in one section within the islet parenchyma or in the islet-exocrine interface.

The human pancreas contains several million islets of widely varying size [135-137]. Obviously, the size of an islet will influence the number of immune cells present per islet. Based on these considerations, specifying the number of islets in the definition of insulitis (i.e., ≥ 3 islets) should, if possible, be avoided. Instead, the proportion of islets with infiltrating CD3⁺ cells provides a better diagnostic criterion. In an effort to adhere to the consensus definition, we propose that, if < 100 islets are available for evaluation, ≥ 3 islets should contain ≥ 15 CD3⁺ cells. However, if ≥ 100 islets are available

for evaluation, $\geq 3\%$ should contain ≥ 15 CD3⁺ cells. Using this definition for T1D, both of the acute-onset T1D donors included in Paper I and six of six individuals with recent-onset T1D examined within the DiViD study meet the criteria [127]. Furthermore, the proposed definition should adequately distinguish between individuals with T1D and T2D, as none of the 50 T2D donors included in Paper I met these criteria.

Not taking leucocytes into consideration, fewer than five insulin-deficient islets were discovered in the T2D pancreata, whereas they were frequently found in T1D (especially in long-standing) pancreata.

Inflammation of the pancreas in T2D

Beta-cell stress has been suggested to cause the recruitment of immune cells in T2D pancreata [138]. However, the CD45⁺ cells found within the T2D pancreata that met the consensus definition for insulinitis, were seemingly randomly distributed throughout the gland rather than preferentially located to the islets.

An increased presence of leucocytes in the exocrine pancreas has previously been reported both in T1D and T2D [139, 140], but in Paper I, the CD45⁺ cells were frequently found to be concentrated in areas of fibrosis, in which islets were densely surrounded by clusters of leucocytes. Fibrosis constitutes the end-stage of inflammation, and it is likely that cellular inflammation maintained several years after diagnosis contributes to the loss of both exocrine and endocrine parenchyma in individuals with T2D [107].

CD68-positive cells (macrophages) constituted the largest fraction of the CD45-positive cells, and inflammation mediated by these may also directly affect islet function and survival. These considerations suggest a direct role of these cells in the pathogenesis of T2D. Supporting this notion, LCM-extracted islets from T2D pancreata express cytokine and chemokine patterns which suggest that a sustained mild inflammation affects the islets [141, 142] that may contribute to islet dysfunction. This notion was not, however, supported by the functional characterization of isolated islets in the present study. Though we found a pronounced decrease in glucose-stimulated insulin release in islets isolated from pancreata obtained from individuals with T2D when compared with islets from non-diabetic individuals, no difference in function could be observed between islets from T2D donors with and without insulinitis. Similarly, insulinitis does not correlate with the level of HbA1c.

HLA Class I hyperstained islets in T2D

Of the T2D pancreata with insulinitis, 36% contained islets that hyperstained for HLA Class I. The presence of HLA Class I hyperstained islets in T2D pancreata has also been described in previous studies [123]. Such islets are described as hallmarks in T1D and have been said to reflect a biological hyperexpression of the proteins [130]. They have also been proposed to create a “fertile field” for the development and enhancement of islet autoimmunity in T1D [143]. However, the finding reported in Paper I regarding HLA Class I hyperstained islets in T2D argues against this hypothesis.

Paper II - The Density of Parasympathetic Axons is Reduced in the Exocrine Pancreas of Subjects Recently Diagnosed with Type 1 Diabetes

No difference in islet innervation could be detected in T1D compared to controls

Previous studies have reported that VIP-immunoreactive axons are located within and in proximity to pancreatic islets [53-56]. However, we found that only 4.8% of non-diabetic islets had islet-associated, VIP-immunoreactive axons. Although only a thin, close to 2D picture, was provided in 6 um sections, this suggests that the islets are sparsely innervated by parasympathetic axons. In agreement with our findings, Rodriguez-Diaz et al. suggest that parasympathetic axons, identified by staining for vAChT and ChAT, are rarely seen in the islets [52].

The exact innervation targets and the functional capacity of the axons are not examined in Paper II. However, the density of parasympathetic axons within islets did not differ between pancreata from T1D and non-diabetic individuals, which suggests that parasympathetic innervation of the islets is not affected in T1D.

Innervation of the exocrine compartment in recent-onset T1D was reduced

Paper II reports a reduced density of parasympathetic axons in the exocrine pancreas of recent-onset T1D individuals, but the density discovered did not differ between long-standing T1D and non-diabetic pancreata.

Bacteria, viruses, mechanical trauma, inflammatory factors, or hyperglycaemia can induce axon degeneration [144, 145]. Reflecting this phenomenon, long-standing T1D patients with a prolonged exposure to hyperglycaemia

can be expected to be at higher risk of polyneuropathy in the pancreas, but no such difference was discovered in the research conducted for Paper II. It is unlikely that hyperglycaemia caused the reduced parasympathetic-axon density in the exocrine compartment of recent-onset T1D subjects due to the short duration of the disease. Thus, the reduced density is better explained by one or several of the other pathogenic options.

As the reduced density of parasympathetic axons was not present in long-standing T1D, it is possible that the ongoing processes in recent-onset T1D pancreata also affects parasympathetic axons, and that the axons re-establish their function/presence in the pancreas when these processes subside. An alternative explanation is that the volume of the pancreas is further reduced after onset whereas the number of axons remains unaltered. This would lead to the appearance of an elevated density of axons in long-standing compared to recent-onset T1D pancreata. The volume of the pancreas is reduced in T1D [40, 60, 83, 146], but larger studies and longitudinal-imaging studies are required to determine whether the pancreas is reduced further after initial symptoms [147]. It is therefore difficult to determine whether the absolute number of parasympathetic axons is unaltered or elevated in long-standing T1D pancreata.

The secretory function of the exocrine pancreas is regulated in part by parasympathetic axons in animal models [59, 148], and exocrine insufficiency is common in humans affected by T1D [59]. The reduced number of parasympathetic axons could therefore contribute to an impaired exocrine secretory function in T1D patients. However, due to the limited knowledge of the normal function of parasympathetic innervation of the exocrine tissue in humans [59], the exact impact of reduced parasympathetic axons in T1D requires further investigation.

Paper III - Expression of Interferon-Stimulated Genes in Insulitic Pancreatic Islets of Patients Recently Diagnosed with Type 1 Diabetes

Several ISGs were over-expressed in insulitic islets

26 of the 84 ISGs investigated by qPCR were either at-least five-fold upregulated or had a rank-based p-value < 0.05 in the T1D-extracted insulitic islets compared to non-diabetic islets. This indicates that a pathway leading to the induction of ISGs is activated in insulitic islets of subjects with T1D. The ISG over-expression pattern observed was similar to that demonstrated in isolated islets infected *in vitro* with enteroviruses [149], or exposed to Type I

IFN [89] or Type II IFN [90, 149]. However, Type I IFNs were rarely detected and were not over-expressed in the insulitic islets. Furthermore, no enteroviral genome was detected with PCR. This argues against viral infection as a source of the ISG induction. Therefore, our data points towards the possibility that a yet-undefined upstream inducer that may not be a virus is responsible for the over-expression of ISGs in the T1D insulitic islets.

ISG expression varied in the islet core region compared to the peri-insulitic region

To delineate if the over-expression discovered was derived from leucocytes part of the insulitic lesion, or from the non-infiltrated islet core, the respective areas were extracted separately by using LCM. This was achieved in a limited number of islets collected from DiViD patient 3. Thus, it is unknown if the findings are representative, and the results should consequently be carefully interpreted. The over-expression of ISGs including *EIF2AK2*, *HLA-2*, *IFI6*, *OAS1*, *TLR3*, *GBP1* and *STAT1* appeared to be mainly derived from the islet core region. However, *CXCL10* was nearly 10 times over-expressed in the peri-insulitic region. This is in contrast to IHC-based studies where this chemoattractant has been reported to be upregulated in the beta cells of subjects with T1D [102, 103]. However, it is more in line with the finding that the leucocytes part of the insulitic lesion are most often located in the peri-islet rather than in the intra-islet region [127, 150].

Paper IV - Expression Profiles of Stress-Related Genes in Islets from Donors with Progressively Impaired Glucose Metabolism.

High inter-donor variation of stress-related genes

LCM and qPCR arrays of 330 genes related to mitochondria, oxidative stress, or the unfolded protein response were used to extract and analyse islets from organ donors with HbA1c <5.5%, elevated HbA1c (6.0–6.5%), high HbA1c (>6.5%) and established T2D. The LCM protocol optimized in Paper III was used to exclude islets containing CD45-positive leucocytes; therefore, the risk of analysing the transcription profile of leucocytes rather than endocrine cells was minimized. High inter-donor variations in the 330 stress-related genes was discovered. This variation suggests that expression of stress-related genes is determined more by variables at the time of organ removal, other than HbA1c, that the groups were not matched for. Such variables might be factors discussed earlier in the section on considerations on

research design: e.g., the condition of the donor before trauma/cause of death, treatments prior to brain death, and treatments prior to organ removal.

Donors with established T2D had a differential expression of several stress-related genes

When compared to control islets, 44 of 330 genes were differentially expressed in islets from subjects with T2D. Under-expressed genes were mainly related to mitochondrial function, whereas over-expressed genes were mainly related to the UPR. In contrast, a study including LCM islets from 10 controls and 10 donors with T2D did not find a differential expression of genes related to mitochondria or ER stress [128]. This may in part be due to the GeneChip array approach that was used with a larger number of genes analysed (approximately 38,500).

Supporting the notion that mitochondrial genes overall are under-expressed in T2D islets, reduced expression of genes involved in glucose-stimulated insulin secretion has been reported in islets isolated from donors diagnosed with T2D [115]. Furthermore, the mitochondria in beta cells appear to be morphologically and functionally differentiated in T2D [112, 151]. Taken together, and due to the findings in Paper IV, it becomes increasingly convincing that the mitochondria are affected on a transcriptional, functional and morphological level in T2D.

In addition to the mitochondria, the ER has also been reported to be altered functionally and morphologically in T2D [112, 113, 151]. The expression pattern of UPR-related genes in Paper IV supports the notion that the ER is affected in T2D islets.

26/330 genes were differentially expressed in donors with HbA1c 6.0–6.5%. Although the number is limited, this argues for that the islets has started to adopt the phenotype seen in islets from donors with established T2D. This hypothesis, however, is contradicted by the expression pattern encountered in the group of donors with HbA1c >6.5%. It had only seven differentiated genes, thereby indicating that this groups' expression of stress-related genes was not different compared to non-diabetic controls. This discrepancy could be caused by the fact that the donors with HbA1c >6.5% had been treated differently prior to organ removal (we unfortunately cannot know this, as we do not have access to the medical journals of the donors.) Alternately, it could indicate that the expression pattern seen in donors with HbA1c 6.0 – 6.5% is not related to the elevated HbA1c.

Summary of conclusions

Paper I:

- Diagnosis of insulinitis in T1D should be made when ≥ 15 CD3+ cells, not CD45+ cells, are found in $\geq 3\%$ of islets.
- Insulin-deficient islets distinguish T1D pancreata from T2D pancreata.
- Inflammation of the pancreas is common in T2D.
- Macrophages substantiate the largest fraction of CD45+ cells in T2D pancreata.
- HLA I hyperstained islets are present in T2D pancreata.

Paper II:

- The null-hypothesis that the number of islet-associated parasympathetic axons is unaltered in subjects with T1D could not be rejected.
- There is a reduced density of parasympathetic axons in the exocrine compartment of subjects with recent-onset T1D.

Paper III:

- ISGs are over-expressed in insulinitic islets derived from T1D patients.
- The upstream inducer of this phenomenon remains undefined.
- Most of the over-expressed ISGs had a higher expression in the islet core area than in the peri-islet area containing leucocytes.
- An elevated level of *CXCL10* in the peri-islet region could explain why leucocytes are preferentially located in this area.

Paper IV:

- No apparent clustering based on donor HbA1c could be seen in islets' expression of stress-related genes.

- Overall, stress-related genes are differentially expressed in islets from donors with established T2D.
- Data from islets derived from donors with HbA1c 6.0 – 6.5% suggests a transcriptional cellular-stress pattern, whereas data from islets derived from donors with HbA1c >6.5% suggests no cellular-stress pattern. Due to this discrepancy, additional studies are required that can confirm or reject the preliminary conclusion drawn in Paper IV: that a transcriptional stress response is present in islets from donors with HbA1c 6.0 – 6.5%.

Future perspectives

An extensive literature describes diabetes treatments, epidemiology and findings regarding the pancreas of diabetes subjects, but a definitive aetiology has not yet been established. Impaired glucose metabolism can be reversed in early stages of T2D by energy-restricted diets [106], but it remains to be determined for how long this can prevent the progress of T2D. In contrast, no treatments are available that prevent or reverse T1D. Therefore, more information is necessary for the development of well-motivated, preventive and curative strategies. I will below mention different areas that I deem especially important to examine in future studies.

Improved diabetes classifications based on the pancreas of the patient

T1D or T2D is diagnosed based on clinical parameters. With the aim to reduce complications, diabetes diagnosis permits the physician to choose the optimal treatment option currently available for the patient [122]. However, from a pathological approach, the diagnosis only offers information that suggests whether the patient, 1) has beta-cell loss or 2) has an inadequate glucose metabolism and dysfunctional insulin secretion. This information is derived from that T1D is caused by a loss of beta cells [127], and fulminant T2D only appears when beta-cell function is impaired [152, 153].

As pathological beta cells are a common denominator in the pathogenesis of T1D and T2D, this strongly indicates that the pancreas is the key to understanding the development of diabetes. However, despite decades of research, the events preceding, and the processes driving the continued loss and impairment of beta cells, are undetermined. It would be unsurprising if there are several causes of beta-cell loss or dysfunction, which opens the possibility of sub-classifying, or re-classifying, diabetes based on the pathology of the pancreas.

To improve the diagnosis of diabetes based on the pathology of the pancreas, optimally, everything in the pancreas would be recorded to the smallest de-

tail by non-invasive methods. Such methods are unfortunately not currently available. However, a promising technique for studying the progress of beta-cell decline involves the use of imaging techniques such as PET/SPECT, in which beta-cell biomarkers are under development [154]. Furthermore, beta-cell death can indirectly be measured in peripheral blood (by presence of cell-free DNA that is un-methylated in the region for insulin) [155]. A parameter that may correlate with beta-cell decline is the immunological phenotype in blood, that can be examined primarily by flow cytometry. Moreover, the general distribution of leucocytes in the pancreas can be examined by PET [156]. Clinical data regarding estimated beta-cell function, HbA1c, age of onset, insulin resistance and BMI [27] could complement the diagnosis of diabetes. In summary, while it is not possible to record everything in the pancreas, methods are, or will hopefully soon be available that will make it possible to acquire a more comprehensive picture of the progressive pathology of the diabetic pancreas. Such a clarified picture may provide the foundation to re-classify diabetes based on the pathology in the pancreas, which may, in the long term, be a necessary step in the development of preventive strategies and curative treatments for diabetes.

Future studies on human pancreata

Additional examinations of donor pancreata obtained from diabetes subjects should be performed to get a more detailed picture of the pathological events in diabetes. One limitation of these studies is that the pancreata in a majority of cases are obtained from deceased organ donors; therefore, only a snapshot of the processes occurring in the pancreas is available (maybe at a time when it is not possible to determine what causes the beta-cell loss). Nevertheless, examination of pancreata may contribute with details that cannot be studied by radiological methods. The idea of a consensus definition for insulinitis [133] is commendable. The same approach to standardize concepts should be considered for other concepts often described in the diabetes research field, as pancreata collected through different procedures throughout the world are examined by different groups with different subjective interpretations of what is present in the sample. Standardization of some common features—such as exocrine fibrosis, ductal fibrosis and loss of insulin-positive cells—would facilitate a collective comparison of diabetes pancreata. Beyond these common features, more specific questions should be addressed: e.g., what phenotype the leucocytes in the pancreas of various diabetes subjects exhibit, what processes occur in the endocrine cells and how other tissues in the pancreas are affected. These questions can to some extent be answered if the phenotype of cells that are part of and present in the pancreas are described in more detail. The methods utilized in this thesis, such as IHC, IF and LCM will contribute to characterizing the cells, but additional methods which al-

low 3D-visualization of tissues and quantification of selected proteins within single cells will contribute additionally.

In summary, diabetes should be diagnosed based especially on the processes which occur in the pancreas. Descriptive studies may be used to further characterize pancreata, but results should not be over-interpreted if they are found in a small group of diabetes subjects. Despite the sizable available literature on diabetes, it seems probable that only the tip of the iceberg is known about the aetiology of the diseases.

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