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Studies of the human pancreas to understand the pathologic events leading to type 1 diabetes

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Abstract

Granlund, L. 2023. Studies of the human pancreas to understand the pathologic events leading to type 1 diabetes. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1961. 57 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1854-7.

Type 1 diabetes (T1D) is classically described as a disease emanating from beta-cell loss, and as such, the beta cells have been the main target of investigation. However, recent years have witnessed a shift in perspective, with T1D being increasingly recognized as a condition that affects the entire pancreas. This shift in focus emphasizes the importance of investigating not only the islets but also the exocrine pancreas and endocrine cells beyond the islet perimeters. In this thesis, pancreases from individuals with and without T1D have been investigated with regard to the exocrine tissue, islets, and other endocrine cells in an endeavour to shed light on the aetiology of the disease.

In **Paper I**, the exocrine part of the pancreas was investigated in donors with and without longstanding T1D. While no histological evidence of acinar atrophy in T1D was found, transcriptional alterations were identified. The absence of atrophy supports the idea of a reduced number of acinar cells as an explanation of the reduced pancreas volume, and the transcriptome analysis demonstrated the impact on the exocrine pancreas in T1D. In **Paper II**, islets from control and T1D subjects were examined. Islets from T1D subjects showed upregulation of transcriptional pathways related to vasculature and angiogenesis, along with increased vascular density. As endothelial cells are important for proper beta-cell function, the changes in vasculature might be a reaction to the loss of beta-cells. **Paper III** aimed to characterize extra-islet endocrine cells in healthy donors aged 1-25. Similar frequencies of these cells were observed in all age groups, indicating that new cells are continually formed as the pancreas grows, in part likely through replication as mitotic cells were found. Notably, many of the insulin- and glucagon-positive extra-islet cells lacked the expression of their corresponding transcription factors, PDX1 and ARX, suggesting they may be newly formed or plastic. The focus of **Paper IV** was to examine endocrine extra-islet cells in T1D donors compared with matched controls. The density of extra-islet insulin cells was decreased, whereas the density of extra-islet glucagon cells was increased. The latter might be due to a compensatory response to impaired alpha-cell function or beta-to-alpha-cell conversion.

These findings contribute to our understanding of the entire pancreas's function in both health and disease, enhancing our knowledge of T1D development and progression.

Keywords: Pancreas, Type 1 Diabetes, Islets of Langerhans, Exocrine tissue, Acinar cells, Islet Microvasculature, Endocrine cells, Extra-islet cells, Insulin, Glucagon

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Till min första kärlek - Ulrika

List of Papers

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

I Granlund L, Hedin A, Wahlhütter M, Seiron P, Korsgren O, Skog O, Lundberg M. Histological and transcriptional characterization of the pancreatic acinar tissue in type 1 diabetes. *BMJ Open Diabetes Research and Care*. 2021

II Granlund L, Hedin A, Korsgren O, Skog O, Lundberg M. Altered microvasculature in pancreatic islets from subjects with type 1 diabetes. *PLoS One. 2022.*

III Granlund L, Korsgren O, Skog O, Lundberg M. Extra-islet cells expressing insulin or glucagon in the pancreas of young organ donors. *Manuscript.*

IV Granlund L, Korsgren O, Lundberg M, Skog O. Loss of insulin-expressing extra-islet cells in type 1 diabetes is accompanied with increased number of glucagon-expressing extra-islet cells. *Manuscript.*

Additional Publications

V Gandasi R N, Arapi V, Mickael E M, Belekar A P, Granlund L, Kothegala L, Fredriksson R, Bagchi S. Glutamine Uptake via SNAT6 and Caveolin Regulates Glutamine-Glutamate Cycle. *International Journal of Molecular Sciences*. *2021.*

VI Seiron P, Stenwall A, Hedin A, Granlund L, Esguerra S L J, Volkov P, Renström E, Korsgren O, Lundberg M, Skog O. Transcriptional analysis of islets of Langerhans from organ donors of different ages. *PLoS One. 2021.*

Paper	Aim	Methods	Results	Conclusion
I	To examine the exocrine pancreas on a histological and transcriptional level in longstanding T1D donors.	The histology of acinar tissue was analysed using IHC and IF. The transcriptome of LCM:ed acinar tissue was examined by AmpliSeq RNA sequencing.	The acinar nuclei density was preserved in T1D, with no support of acinar atrophy. Transcriptome analysis identified the gene sets *Myc Targets V2* and *Estrogen Response Early* to be upregulated in T1D.	The absence of atrophy supports the idea of a reduced number of acinar cells as an explanation for the reduced pancreas volume. The transcriptome analysis provides evidence of exocrine affection in T1D
II	To characterize the islets from donors with and without T1D using LCM, to as close as possible reflect the *in vivo* situation.	Islets were excised using LCM and sent for transcriptome analysis. Histological analysis was performed by IF and IHC.	The transcriptome analysis showed several upregulated genes related to vasculature and angiogenesis in T1D. Histologically, vascular density was increased in T1D.	The transcriptional alterations together with increased vascular density, suggest an altered microvasculature in islets in T1D. By using LCM, this study closely reflects the *in vivo* situation.
III	To characterize extra-islet cells expressing insulin or glucagon in the pancreas of young non-diabetic subjects.	Multispectral imaging was used to examine PDX1, ARX, Ki67, INS and GCG expression in extra-islet cells in tissue from donors aged 1-25 years.	Extra-islet cells were frequent in all donors. The density was relatively constant regardless of age. Many of the INS- or GCG-positive cells were negative for both PDX1 and ARX. Ki67 were rare, but present in all age groups.	The preserved density indicates that new cells are continuously formed. Part of this increase may be due to replication, as Ki67+ cells were found. The absence of PDX1 and ARX suggests cell immaturity and/or plasticity.
IV	To investigate and compare the extra-islet insulin or glucagon-positive cells in organ donors with and without T1D.	Multispectral imaging was used to examine extra-islet cells by staining for INS, GCG, ARX, PDX1 and Ki67.	Decreased density of INS-positive and increased density of GCG-positive extra-islet cells were observed in T1D. Proliferating cells were present both in donors with and without, T1D.	The increase of GCG-positive cells in T1D could be a compensatory effect in response to impaired alpha-cell function, or a sign of beta- to alpha-cell transdifferentiation.

Contents

Abbreviations

ARX	Aristaless Related Homeobox
CAMERA	Correlation Adjusted MEan RAnk test
cDNA	Copy/Complementary Deoxyribonucleic Acid
DAB	Diaminobenzidine
DAPI	4′,6-Diamidino-2-Phenylindole
DEGs	Differentially Expressed Genes
EMT	Epithelial-Mesenchymal Transition
ER	Endoplasmic Reticulum
EV	Enterovirus
FDR	False Discovery Rate
FFPE	Formalin-Fixed Paraffin-Embedded
GSEA	Gene Set Enrichment Analysis
HLA	Human Leukocyte Antigen
HRP	Horse Radish Peroxidase
IF	Immunofluorescence
IHC	Immunohistochemistry
Ki67	Marker of proliferation Ki-67
LCM	Laser Capture Microdissection
LPS	Lipopolysaccharide
MAFA	MAF bZIP transcription factor A
MAFB	MAF bZIP transcription factor B
MSigDB	Molecular Signatures Database
NKX6.1	NK6 homeobox 1
NOD-mouse	Non-Obese Diabetic mouse
ORA	Overrepresentation analysis
PCA	Principal Component Analysis
PDX1	Pancreatic & Duodenal Homeobox 1
PP	Pancreatic Polypeptide
RNA	Ribonucleic Acid
scRNA-Seq	Single-cell RNA Sequencing
TCR	T-cell Receptor
TLR	Toll Like Receptor
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TSA	Tyramide Signal Amplification
UPR	Unfolded Protein Response

Introduction

Diabetes – from discovery until today

Although it has been known since antiquity, Diabetes Mellitus remains an incurable, chronic disease. The pancreas was first described by the Greek surgeon and anatomist Herophilus around 300 BC. The name "pancreas", made up of the Greek words *pan*, meaning "all", and *kreas* meaning "flesh", was coined about 400 years later by another anatomist named Ruphos (1). However, it took a long time to discover the function of the organ, and even longer to establish its connection with diabetes. There are many examples of early descriptions of excessive urination and thirst, accompanied by honey-tasting urine. The first accurate account of the disease, and the first time the term diabetes is used, is by Aretaeus of Cappadocia during the 2nd century AD (2). The term mellitus was later added by the English anatomist and physician Thomas Willis (1621-1675) and is referring to the sweetness of the urine of diabetic patients. Willis incorrectly thought diabetes to be an ailment of the blood, and it would take until the late 1880s that the disease was connected to the pancreas, by experiments done by Minkowski and von Mering. Not long before this, in 1869, Paul Langerhans was the first to describe the islets of Langerhans, although he thought them to be cell clusters of nerves or pancreatic lymph nodes. Some years later, in 1893, Laguesse identified their true function, as responsible for the combustion of sugar, and named them after Langerhans (1). Approximately 30 years later, in 1922, the administration of insulin became the first treatment of diabetes. Before this, the only treatment available was very strict diets bordering on starvation (3). Work by Banting, Best, MacLeod and Collip lead to the discovery of insulin and its glucose-lowering effects. The discovery was awarded the Nobel Prize in Medicine in 1923 and insulin became commercially available the same year. Until this day, 100 years later, this is still the only available treatment for type 1 diabetes (T1D), despite intense research.

Diabetes Prevalence and Incidence

Diabetes is one of the fastest-growing global health emergencies, with the number of people living with diabetes estimated to rise from 529 million in 2021 to an unfathomable 1.3 billion by 2050 (4,5). The vast majority of people

living with diabetes have type 2 diabetes (T2D) (approximately 90 %), leaving close to 50 million people suffering from T1D (6,7). Even though the number of incidents of T1D differs a lot between countries, with Finland presenting more than 60 cases/100000 people /year, compared to some East Asian populations with only 0.1 cases/100000 people /year, the rate of increase is rapid all over the world (6). Sweden has one of the highest incidents in the world, and it has doubled over the last 40 years (8,9). Diabetes is associated with short-term complications such as hypoglycaemia and ketoacidosis, which in severe cases can cause coma or even death. Furthermore, long-term hyperglycemia reduces life expectancy due to an increased risk of cardiovascular disease and a number of microvascular complications (10). Taken together, this imposes a tremendous both social and economic burden on society, providing a high incentive for better treatments or possible cures (5,11).

The Pancreas

The pancreas, located below the stomach and weighing between 50-100 grams, is typically divided into three main anatomical areas; the head (which is adhering to the duodenum), the body and the tail (12). The tissue is further divided into densely packed lobules, ranging in size between 1-10 mm. It is a curious organ consisting of two distinctly different tissue types; exocrine and endocrine tissue. The acinar cells of the exocrine tissue produce digestive enzymes that are released via the ducts of the pancreas through the ampulla of Vater to the duodenum. These acinar cells make up the vast majority of the organ and the remaining endocrine cells, the islets of Langerhans, only constitute a few per cent (1-4 %) of the total mass of the gland (13). There are about one-two million islets, scattered throughout the pancreas, although there is a huge variation between individuals (12,14). The islets consist of several different endocrine cell types; e.g. beta cells, alpha cells, delta cells, Pancreatic Polypeptide (PP) cells and epsilon cells that produce insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin respectively (12). The composition of islet cells, as well as their number and size, is highly inter-individual and differs between different regions of the pancreas (14). Beta cells correspond to roughly 60 % of the total islet mass, alpha cells represent up to 30 % while the remaining cell types constitute a lesser amount of the total islet endocrine mass. PP-cells are more abundant in the uncinate process, the lower part of the head of the pancreas which has another embryonic origin (12).

The Exocrine Pancreas

While it has generally been believed that T1D is a disease exclusively affecting the beta cells, atrophy of acinar cells in the exocrine pancreas (15–17), and

a reduced pancreas size in patients with T1D compared with non-diabetic subjects (18–22), are frequently observed. Decreased pancreas weight has been demonstrated both at onset and in patients with long-standing T1D, regardless of being children, adolescents, or adults (18). A reduced pancreatic size has also been reported in first-degree relatives of patients with T1D as well as in autoantibody-positive donors without diabetes, indicating that the reduction in pancreas size precedes clinical onset of T1D, or perhaps that being born with a smaller pancreas carries a risk of later developing T1D (18,19,23,24). The lower pancreas weight in relatives of patients with T1D has been interpreted to indicate a shared genetic or environmental factor that might predispose certain individuals to T1D (23). But there are some conflicting results on whether or not there is a correlation between disease duration and pancreatic weight or volume. No correlation is reported in some studies (17,18), while others see a continued decrease in size after diagnosis (21,22). Exocrine dysfunction (assessed by direct stimulation test or indirect enzyme measurement) is also more frequent in patients with T1D, even though not all patients suffer from this (25–28). About 40 % of the patients developing T1D show a defect in exocrine function (27) and a correlation between the severity of exocrine dysfunction with disease duration has also been found (28). A theory aiming at explaining the reduced pancreas size and function is that insulin has a trophic effect on the exocrine tissue, regulating growth and amylase synthesis (15,29–31). However, this would not explain the reduced pancreas size in pre-diabetics and first-degree relatives. The effect of T1D on the exocrine pancreas is also manifested in the formation of autoantibodies targeting exocrine enzymes (32–34), as well as signs of pancreatitis and inflammation (35,36). Hence most T1D patients seem to have an ongoing inflammatory process within the exocrine pancreas as well as the endocrine pancreas. However, despite our current understanding of the effect of T1D on the exocrine pancreas, literature describing the histology and the molecular biological profile in this area remains somewhat contradicting and much is yet to be learned.

The Endocrine Pancreas

The Islet Architecture

The islets of Langerhans have been described many times, in both health and disease. However, the field of islet research has traditionally relied on rodent-based investigations, but these findings have been challenging to apply to humans. Rodent- and human islets have been shown to differ both in architecture and composition (37–39). In mouse and rat islets, the beta cells make up the core of the islet, surrounded by a mantle of alpha, delta and PP cells. In humans, the arrangement of cells is more complex, and as a result, highly debated with contradicting views (38). Several studies propose that human islets

have a comparatively less structured architecture, where the different endocrine cell types are intermingled with each other (37,40). This results in a high degree of heterogeneous contact between cell types, with most endocrine cells also being in contact with the blood vessels of the islets. Conversely, evidence for a more distinct arrangement of the endocrine cells, similar to that in rodents, exists as well. In these studies, especially smaller islets exhibit beta cells residing in the core of the islets and alpha cells mainly in the mantle (41–43). These contradicting results are in part likely a result of different experimental methodologies. How the human islet architecture affects paracrine and endocrine islet cell interactions is not fully understood, but undoubtedly an important key in understanding the full function of these micro-organs.

The Islet Vasculature

The islets are richly perfused to be able to handle the high metabolic activity of the islet cells and maintain glucose homeostasis (44,45). In rodents, the islets receive up to 20 % of the total pancreatic blood despite only constituting 1-2 % of the total pancreatic volume (46). Islet capillaries are highly fenestrated, demonstrating up to tenfold more fenestrations than exocrine capillaries. They do as well display a larger diameter, and higher vascular density compared with the exocrine pancreas, further illustrating the close interaction with the circulation and islet cells (46,47). Three models for how islet blood perfusion is organized have been suggested, all primarily based on animal studies. The models are: “periphery-to-center”, “center-to-periphery” and “pole-to-pole”. Depending on the islet cell architecture, the different models would allow for different cells to first come in contact with the blood flow. Factors secreted by these cells may then affect or modulate the response of downstream cells. In the “pole-to-pole” model, however, blood would reach all cell types simultaneously, allowing for very intricate crosstalk between cells (45,48). More work on human islets is required to elucidate the exact arrangement of the islet vasculature.

Beta Cells

The central role of the loss of insulin-producing beta cells in T1D pathology is undisputed. The beta cells produce and secrete insulin mainly in response to increased glucose concentrations, but amino acids, fatty acids, hormones and neurotransmitters are also capable of enhancing insulin release (49). This process is highly demanding; insulin alone accounts for up to 10 % of the total protein content of the beta cell (50). The need for tightly controlled glucose homeostasis makes the beta cells susceptible to stress (51). During the gradual loss of the cells in T1D development, the demand on the remaining beta cells increases. Ultimately, the blood glucose rises to fatal levels. Despite our extensive knowledge of beta cell physiology, the mechanisms of beta cell death are still partially benighted, as is the contribution of the beta cells themselves to their own demise. In 1986, the article “Death of a Beta Cell: Homicide or

Suicide" was published by Bottazzo (52). Herein, it is discussed whether the pathogenesis of the disease is mainly due to immune responsiveness, i.e. homicide, or if the inherent vulnerability of the beta cells leads to apoptosis, i.e. suicide. This concept has been reassessed later as well (51,53). The extremely high secretory demand on the beta cells makes them prone to ER stress, as the ER protein folding capacity reaches its limits when a high amount of insulin is to be secreted. This can lead to the release of chemokines by the beta cells, as a response to damage and stress, as well as apoptosis, if the unfolded protein response (UPR) does not succeed in restoring normal function (54). The released chemokines will attract immune cells, which through the rich vascularization of the islets, required for insulin sensing and release, have easy access to the islets. The immune cells may then damage the beta cells e.g. by release of cytokines. The beta cells have also been proven to be very sensitive to cytokine-mediated damage, which can alter many functions within the beta cells (55). The cellular damage inflicted by the cytokines increases the ER stress and hence the vicious cycle begins again (51). The beta cells hence seem to play a central role in their own demise, which should not be neglected (56).

Alpha Cells

Beta cells have traditionally been the main target of investigation in T1D research. However, dysfunction of other pancreatic hormone-secreting cells is becoming more frequently reported and is demanding more research focus (57–60). Many patients with T1D show impaired glucagon secretion leading to an inadequate response to hypoglycemia as well as exaggerated hyperglycemia, indicating an alteration in the function of the pancreatic alpha cells (61). The disturbed response to hypoglycemia has been known for a long time. In 1973, a lack of glucagon response to hypoglycemia in patients with T1D was demonstrated (62). In a more recent publication, it was even suggested that glucagon excess, rather than insulin deficiency, is the main perpetrator of T1D-associated hyperglycemia (63). The T1D alpha cell has indeed been shown to be functionally impaired with a reduced glucagon secretion capacity (in response to hypoglycemia) and a reduced expression of the alpha cell transcription factors MAFB and ARX (64). The focus of the field is thus broadening due to the recognition that other endocrine cells than solely the beta cells are dysregulated in T1D.

Delta Cells

Even less attention has been directed towards the third most common cell type in the islets, namely the somatostatin-secreting delta cell. The delta cells have a curious morphology, resembling neuronal cells, with long filopodia-like extensions enabling close contact with other islet cell types even at long distances from the cell body (65). This elongated cell morphology, together with the important regulatory function of somatostatin on both beta and alpha cells, gives the delta cells a crucial role in maintaining glucose homeostasis (66).

Somatostatin exerts an inhibitory effect on both insulin and glucagon secretion; glucose activates both beta- and delta cells, initiating an insulin secretory response, and at the same time stimulating the release of somatostatin to counterbalance the insulin release (65). Likewise, when glucagon secretion is stimulated (e.g. by arginine), the delta cells are activated and secret somatostatin to limit alpha-cell response (67). Moreover, impaired delta cell function has been suggested in diabetes (66,68), and an early study demonstrated improved glycemic control in diabetic patients when treated with somatostatin infusions, despite a reduced insulin dose (69). Altogether pointing towards a pivotal role of the delta cells in maintaining glucose homeostasis.

The (Re)Generation of Islets and Beta Cells

The endocrine cell population could originate both from replication of existing cells as well as neogenesis from different progenitor cells (70,71). For many years, an accepted concept was that one was born with all the beta cells one would ever have. This notion has however changed (72). The beta cell mass has been shown to expand massively during infancy and early childhood as compared with other islet cell types, after which the growth is slowed down, with no spike during adolescence although this represents a phase of rapid growth in children (71,73). However, even in adults, the beta cells have exhibited abilities to expand both in mass and numbers (73,74), although the mechanisms for this are poorly understood. Indeed, theories raised 100 years ago are still debated today. This includes the following mechanisms; (I) transdifferentiation of acinar cells into endocrine cells and vice-versa, (II) neogenesis, defined as the formation of new endocrine cells from the ductal epithelium, and (III) replication of existing endocrine cells (1). Acino-insular transdifferentiation has been observed *in vitro* many times, nevertheless, this phenomenon has not been proved beyond these artificial conditions and the question remains whether human acinar cells exhibit this plasticity (75–77).

The involvement of both neogenesis and replication of preexisting beta-cells in the renewal of the beta-cell population are more adopted theories (78). However, which mechanism is the driving force is contested. Neogenesis is difficult to prove as clear identifying markers are lacking. The presence of small clusters and/or isolated beta cells scattered within the exocrine pancreas, as well as hormone-positive cells budding off from the duct epithelium are often described as signs of neogenesis (79,80). Progenitor cells within the ductal epithelium have been seen to evolve into islet cells *in vitro* (73,81), and during pregnancy, an increase in beta-cells has been reported, although not as massive as most reports in rodents. Contrary to the duplication of beta cells in existing islets reported in rodents during pregnancy, human pregnancy is not accompanied by an increased replication frequency, supporting the notion that

neogenesis can happen in adult human pancreases (79,82,83). The most commonly used marker for replication is Ki67. During the neonatal period and infancy, replication is considered to be a major source of new beta cells and islets (84). However, most reports on human beta-cell replication in the adult pancreas show very little replicative activity, and replication has therefore often been dismissed as an origin of new beta cells in adults (85–87). However, many of these reports may have underestimated the rate of replication due to postmortem changes in Ki67 expression, and the effect of even the lowest replication rate on the massive number of beta cells that do exist in adults is not taken into consideration (88–91). Even a low replication rate could have a massive impact on the overall number of beta cells.

Taken together, both neogenesis and replication are potential mechanisms for the generation of new beta cells and islets (1).

Development and Progression of Type 1 Diabetes

T1D can have its debut at any age, however, onset in children and adolescents is often more aggressive and abrupt, while disease progression is slower in adults (92). It is one of the most common chronic diseases of childhood and usually presents itself with polydipsia, polyuria, polyphagia and weight loss – symptoms arising from hyperglycaemia. The clinical diagnosis is often based on fasting blood glucose levels ≥ 7.0 mmol/l (126mg/dl) (93). In 1986, Eisenbarth put forward a model to describe the development and progression of T1D. This has since been modified but remains the most referred. According to this model, each individual is born with a certain genetic predisposition and susceptibility to later develop T1D. Environmental triggers during life, such as an inflammatory event, then initiates beta cell destruction. The highest genetic risk for developing T1D has been attributed to the HLA haplotypes HLA-DR3-DQ2 and HLA-DR4-DQ8 (94), but, as mentioned above, these alone do not initiate disease. There is substantial evidence for an important environmental factor as well. The dramatic increase in incidence and prevalence of T1D seen during the past decades (92,95), the difference in incidence in populations sharing the same genetic predisposition but living in different geographical areas (96), as well as the concordance between monozygotic twins being only about 40 % (97,98), all testify to an important environmental aspect. However, it should be noted that the cumulative incidence between monozygotic twins increases in long-term studies (to about 65 %) (99). There are many proposed environmental triggers, and viral infections are among the most commonly referenced. Among these, enteroviruses (EVs) are often considered the primary suspects. It seems as if beta cells are worse at protecting themselves against an EV infection, compared to e.g. alpha cells. A persistent

EV infection could potentially in time lead to upregulation of HLA-1 molecules, among other things, that could facilitate antigen presentation to autoreactive T-cells, eventually leading to beta-cell destruction (100). Symptoms only arise when the beta-cell mass can no longer produce enough insulin to maintain glucose homeostasis, which might take years (93).

A widely accepted dogma to explain the pathology of T1D is that this is a disease resulting from autoimmune destruction of the beta cells, primarily driven by T-cells. Hence, T1D is sometimes referred to as a disease of the T-cells. This notion is often fortified by the presence of *insulitis* – the phenomena of infiltrating immune cells in the pancreas and specifically in the islet periphery, commonly referred to as *peri-insulitis*. In 2013, a consensus definition of insulitis was decided on, demanding the presence of ≥ 15 CD45+ cells in at least 3 islets to be called insulitis (101). Nevertheless, this definition was soon deemed inadequate in distinguishing between the general inflammation in T2D and the more classical insulitic infiltrate consisting mainly of CD3+ cells and concentrated to islets described in T1D. Subsequently, another definition of insulitis requiring the presence of ≥15 CD3+ cells in ≥3 islets was proposed but later on debated (102–104). The concept of a strong connection between T-cells and T1D has however lately been questioned. The definition of insulitis can be put in contrast to the level of infiltrating immune cells seen in the NOD mouse, the most commonly used animal model in T1D research, where the infiltrating cells can make up almost half of the total cells within an islet. This kind of massive infiltration is hardly ever seen in human islets, and the discrepancies between the species may have contributed to a misguided focus towards the T-cells (105). Indeed, in the healthy population, both islet autoreactive T-cells and islet autoantibodies are frequently found, but most people never develop T1D (106,107). Many immune cells have however been identified in the pancreas of patients with T1D, with CD8+ T-cells being the most prevalent. Nevertheless, in a study on recent-onset T1D patients, many of the T-cells present in the insulitic lesion had a protective tissue-resident memory (T_{RM}) phenotype, rather than a cytotoxic, and an ongoing T-cell mediated killing of beta cells has been difficult to prove in humans (34,108). Indeed, sometimes no CD8+ cells within the islets can be found at all (34,35), and they instead locate at the border between the islet and exocrine tissue. Furthermore, the T-cells' affinity for specifically beta cells is weak at best (109,110). Instead, a single T-cell receptor (TCR) has been shown to recognize more than a million different peptides, further weakening the claimed specificity for beta cells (111). A final question about the role of T-cells comes from the area of clinical trials. The vast number of different immune-based therapies, where patients have been treated with potent immunosuppression, have not been effective in haltering disease development (112–114). Recently, however, Teplizumab, an anti-CD3 monoclonal antibody, showed promising results in extending the time to T1D diagnosis. Though the fact remains that even with this

fairly aggressive treatment, T1D cannot be fully prevented using immunosuppression (115). Together, this questions the alleged required role for cytotoxic T-cells.

Aims

Paper I

The hypothesis tested in this study was that the acinar tissue is altered, on a histological and transcriptional level, in subjects with T1D compared with subjects without diabetes. Additionally, we aimed to investigate whether such differences are particularly pronounced in exocrine regions in close proximity to islet tissue.

Paper II

The aim of Paper II was to examine the islet cells in subjects with and without longstanding T1D, in a way that closely reflects the *in vivo* situation.

Paper III

We aimed to describe extra-islet insulin- and glucagon-positive cells with regard to their density, distribution, expression of the transcription factors PDX1 and ARX, as well as their proliferation activity by Ki67 staining.

Paper IV

The aim of the study was to characterize insulin- and glucagon-expressing extra-islet cells in healthy controls and longstanding T1D donors. The expression of the transcription factors PDX1 and ARX was assessed, as well as the density and proliferation frequency of these cells.

Methodological Considerations

Below, important methods used in this thesis are described. The strengths and weaknesses of the chosen methods are discussed, but for a more detailed description of the research design and methods, please see this section in each individual paper.

Biopsy Material

All papers in this thesis utilized access to human pancreatic tissue through the Nordic network for clinical islet transplantation. Thanks to the consent given by the deceased organ donors, or their next of kin, to donate not only for clinical transplantation but also for scientific research, this thesis was made possible.

The availability of human pancreatic tissue of good enough quality to allow molecular studies is scarce due to it being notoriously difficult to access in combination with the high risk of pancreatitis associated with the collection of biopsies. The high content of digestive enzymes in the pancreas also results in the tissue from autopsy material being prone to extensive autolysis (105). Therefore, obtaining good quality human pancreas specimens is primarily restricted to organ donors, significantly limiting accessibility. In this thesis, tissue from heart-beating organ donors treated as intended for transplantation, have been used. The pancreas is prepared for clinical islet isolation, and if consent is given, both biopsy material, as well as isolated islets, are made available for research.

While the use of human pancreatic tissue is both a unique and important contribution to diabetes research, some predicaments remain. Since we are restrained to study deceased organ donors, we are only able to obtain a snapshot of a disease that progresses over many years, which can make it hard to place the observation into a greater picture. Another issue is the medical history of the organ donors – all donors have spent time at the hospital, sometimes even the intensive care unit, prior to death which may affect both local and systemic homeostasis of the body. It is also impossible to cover all pharmaceutical treatments, as well as general way of life which can influence what is later ob-

served in the organ. Although one important notion to make is that no correlation has been found between the length of hospital stay and immune cell numbers in pancreatic biopsies (116), however, this is of course only one of many factors to consider. Given that we are fairly limited in our knowledge about the donors, proper matching of controls and T1D donors becomes important to limit the influence of possible confounding factors. Age, sex and BMI are always known, and are hence the variables by which matching is done.

Laser Capture Microdissection

Laser Capture Microdissection (LCM) is a method that allows for specific extraction of regions of interest within a tissue section that can be used for gene expression analyses. Frozen tissue sections are dehydrated through a series of ethanol baths, before being placed in the machine - in **Paper I** and **Paper II**, an Arcturus XT LCM system was used. The islets of Langerhans can be identified based on their autofluorescence and are subsequently cut out from surrounding exocrine tissue using a UV laser. The tissue is then captured on a small plastic cap which is covered with a polymer that is melted on top of the cut tissue using an IR laser to enable removal. In both **Paper I** and **Paper II**, LCM was used together with IF, to be able to better select which islets to extract. Consecutive sections were used for either LCM or IF. The IF sections were stained for known islet markers, and could hence be used as a map guiding the LCM. Based on cell types, the islet's autofluorescence and the morphological appearance of the tissue sections, precise excision of the target tissue was made possible.

Transcriptome analysis of LCM-extracted tissue is a good method for examining gene expression. Study of LCM-extracted tissue has many advantages over e.g. studies of islets isolated by enzymatic digestion, where the rough and stressful environment of the isolation process, as well as the following culturing of the cells, is likely to influence and change the gene expression patterns of the cells (117,118). When islets are obtained using LCM, they have also been extracted directly from their natural habitat, i.e. still surrounded by exocrine tissue, which may better preserve the presence of other cell types vital for islet function, e.g. endothelial cells (119,120). However, there are some limitations of the protocol. The method is very user dependent and requires practice to work up speed without losing accuracy, and since the pancreas is rich in RNases, RNA is rapidly degraded. Therefore, the extraction process must be conducted rapidly, and the subsequent analysis should be robust enough to handle such a high degree of degradation. At the same time, there is always a risk of contamination from surrounding tissue when cutting your

cells or region of interest, requiring careful examination of the extracted tissue. Nevertheless, the method keeps evolving, and it is already now possible to extract single cells with high precision, further expanding the usage of this technology.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry (IHC) and immunofluorescence (IF) are common methods to visualize protein expression through antigen-antibody interaction. This is performed on thin tissue sections to investigate the localization and relative distribution of the target protein. A primary antibody is applied to the tissue. If the direct method is used, this is either conjugated to an enzyme, for IHC, or a fluorophore, for IF. If the indirect method is used instead, the primary antibody is uncoupled and instead a secondary antibody coupled to an enzyme or fluorophore is added. Using either protocol, blocking to prevent background and unspecific staining is required. This can happen when antibodies bind non-specifically to the tissue or to Fc-receptors (a receptor that binds the constant region of antibodies). Blocking is done by adding a blocking buffer containing e.g. sera, and this is usually added before the antibodies. Sera from the same species as either the conjugated primary antibody or the secondary antibody should be used. Appropriate controls, to be able to judge what is true staining, is also of importance. Most often a positive control, where the protein is known to be expressed, is used as a reference. The purpose of the negative control is to validate that the reaction visualized is in fact due to the interaction of the epitope of the target molecule and the antibody, and not unspecific binding. As a negative control, a section from a tissue known not to express the target antigen can be used. Isotype controls can also be used. These are primary antibodies that lack specificity to the target but match the class and type of primary antibody used in the experiment. This can help differentiate between unspecific background signals and true antibody signal. If using the indirect approach, omitting the primary antibody to validate there is no unspecific binding of the secondary antibody, is also a good confirmation. One advantage of working with the pancreas is its composition of two distinct tissue types – the endocrine and exocrine tissues. When examining an endocrine target, one will assume that there will be no expression in the exocrine tissue, and vice versa. Consequently, within the same tissue slide, both an 'internal positive control' and an 'internal negative control' are present (121). However, exceptions like the transcription factor PDX1 exist, which is expressed by both endocrine and exocrine cells.

IHC is, as mentioned, based on the antibody being conjugated to an enzyme, usually horseradish peroxidase (HRP). A substrate, e.g. diaminobenzidine

(DAB), is then added to the tissue and cleaved by the enzyme leaving a permanent stain on the tissue. The staining can then be visualized in a bright-field microscope.

In IF, fluorophores are used instead. These can be visualised in a fluorescence microscope since they emit light at a specific wavelength. Using IF, several target antigens can be studied in the same section, the limitation being the overlap in the excitation and emission spectrum of different fluorophores, which can make them difficult to distinguish from each other.

Both IHC and IF are great qualitative methods. They provide information on the localization of the antigen of interest, the type of staining (i.e. membranous, intracellular or nucleus) and to some extent the intensities can be described as well. However, the semi-quantitative assessment of intensities should be done with caution as the evaluations are often subjective. Neither IHC nor IF are traditionally viewed as quantitative methods, as there has been such high inter-observer variability (122). In addition, the intensity of the staining depends on more things than strictly the expression levels of the protein (i.e. epitope accessibility) (123). Nevertheless, as both the methods and image analysis software programs are improving, there is hope of achieving more objective, reproducible quantitative data from multiplex IHC and IF in the future as well (124).

Multiplex Immunofluorescence

Paper III and **Paper IV** utilizes a multiplex staining method. This allows for staining of up to 7 markers, including DAPI, within the same tissue section. The procedure is similar to a standard IHC but based on the mechanisms of tyramide signal amplification (TSA). Any standard primary antibody can be used, after which an HRP-conjugated secondary antibody is applied. A fluorophore-labeled tyramide is then added and the HRP will catalyze the conversion of the labelled tyramide into a highly reactive form that will covalently bind to tyrosine residues at or near the epitope of interest (125). The antibodies are then removed through heat treatment, after which the procedure starts again with a new target. This method also allows for multiple primary antibodies to be raised in the same species, which is yet another advantage.

In **Paper III** and **Paper IV**, the Opal 6-Plex Detection Kit (Akoya Biosciences, Marlborough, Massachusetts) was used together with the Autostainer Bond RX system (Leica Biosystems, Wetzlar, Germany). The Akoya system is the most widely adopted multiplex IHC in use today. The staining can be performed manually, but is very labour-intensive, taking several days to complete which increases the risk of human errors. Therefore, the automated Leica

system was used (124). The Opals are as well very robust, thanks to the covalent binding the reactive tyramide remains bound to the target epitope even through multiple heat treatments. Nevertheless, heat treatment has been seen to decrease the accuracy of antibody staining when the antibody is applied at a later stage in the multiplex staining sequence. Therefore, careful validation of the appropriate order of the antibodies is necessary, which can then produce multiplex panels representative of the single-plex stainings (126). However, the Opal system has proven to be very stable, when the expression of target antigens was assessed by comparing singleplex - and multiplex staining with the Opal method, all examined markers performed similarly, independent of used alone or in a combination panel (127). This, together with a meticulous validation of the order of the antibodies, makes us confident that the multiplex protocol used in this thesis did not distort the antigen expressions within the tissue.

Transcriptomics

In **Paper I** and **Paper II**, whole transcriptomic analysis was done. Briefly, total RNA was reverse-transcribed to cDNA and the cDNA was pre-amplified using an AmpliSeq kit, consisting of primers for the entire human transcriptome. Next, adaptors were ligated to the amplicons which were further amplified – the product is referred to as a library. The amplicons were then ligated to wells on a chip, and fluorescent nucleotides were used to sequence every nucleotide sequence. The reads are then aligned before finally being converted into raw counts. Before analysing the reads, the data have to go through filtering and normalization. Filtering removes genes that are expressed only in very few samples, and at low levels. This is done because it will be close to impossible to extract meaningful information from them. Normalization is done to overcome differences in library size and composition between samples. One sample might have more low-quality reads than another sample, hence making it appear as if some genes are transcribed to a greater extent in the sample with more good-quality reads – which might not be the case. To circumvent this, a conversion factor, based on genes with an average expression in all samples, is calculated and multiplied with each gene. The obtained reads can then be analysed.

Studies of the transcriptome of the islets are rapidly increasing, as the methods to investigate this become more and more sophisticated as well as cheaper. An inherent flaw in using LCM to collect the tissue analysed, as done in **Paper I** and **Paper II**, is that the total amount of material collected is quite small, as the material is cut out from thin tissue sections. The alternative is to use isolated islets which, if the downsides of this method are accepted (discussed more above), renders plenty of material. Another limitation in using LCM to

extract entire islets is that the analysis becomes cruder, and the observed expressions cannot with certainty be attributed to a particular cell type. One must remember that not only endocrine cells, but endothelial- and immune cells among others, reside within the islets as well. Nevertheless, both LCM and sequencing techniques are evolving quickly. Bulk RNA sequencing has been the most widely used method, but single-cell RNA sequencing (scRNA-Seq) is gaining more traction as well. It is truly astonishing that we can detect islet cell gene expression at single-cell resolution today. Nevertheless, this should not be done without caution. The reproducibility of studies is unfortunately low, in part because we are operating at the very limit of reliable detection for a large majority of genes (128). With that said, scRNA-Seq still has the potential of unlocking key aspects of biological processes, but the limitations of the method should be carefully considered when evaluating the data.

Regardless of whether a bulk or single-cell transcriptomics approach is chosen, large amounts of data are produced. To be able to make sense of this, different methods can be used. In **Paper I** and **Paper II**, differential gene expression (DEG) and pathway enrichment analyses were performed. In a DEG analysis, genes that are differentially expressed between two groups are extracted based on preset rules to determine differential expression, e.g. FDR-adjusted P-value less than 0.05 while testing for an absolute log fold change $\geq \log2(1.2)$. Regarding pathway enrichment analyses, a plethora of options are available. The underlying idea is to organize the genes into gene sets or "pathways", i.e. biologically interpretable processes. These pathways are organized in collections known as gene set databases. In both **Paper I** and **Paper II**, the MSigDB database was used, and CAMERA was used together with the MSigDB Hallmark set of pathways. In brief, all genes are included in the analysis and are subsequently ranked based on a predefined measure. The genes with the highest rank are denoted the most significance. The ranked list is then mapped against gene sets, to see where the genes occur. In **Paper II**, an overrepresentation analysis using g:profiler was done as well. In this method, a list of genes, considered to be the most differentially expressed, is used as input. For **Paper II**, the already established DEGs were used. This list is subsequently compared against a number of pathways, to determine whether the genes are overrepresented in any of them. The methods used in this thesis are widely used but it is difficult, if not impossible, to say that one method for pathway enrichment analysis is superior to another - they all come with different shortcomings (129,130). The purpose of them is to simplify and make the data comprehensible.

Results and Discussion

Paper I

Similar acinar cell density in the pancreas from donors with or without type 1 diabetes

In previous reports, different morphological patterns of atrophy of acinar tissue in patients with T1D have been described. Foulis and Stewart reported severe atrophy of acinar cells in the vicinity of insulin-negative islets, and a later study by Löhr and Klöppel described a more general atrophy (15,17). However, neither of these observations was repeated in this study. The nuclei density was manually counted on formalin-fixed paraffin-embedded (FFPE) sections stained for synaptophysin and hematoxylin and was defined as nuclei/mm2, and even though the inter-individual difference was large, no difference could be seen between donors with T1D compared with non-diabetic donors. Neither was any difference observed between regions close to islets as compared with regions further away from islets. The discrepancies between these results and previous studies could be the use of donor pancreases as compared with autopsy material, where autolysis could have affected the observed atrophy. Our results conform to a more recent paper where the cell size was reported to be unchanged in patients with T1D, and the overall reduction in pancreas size was instead attributed to a reduced number of acinar cells (131).

Amylase-negative acinar regions were found in FFPE but not in frozen tissue

Regions in the exocrine pancreas devoid of amylase expression had been described by Kusmartseva as a common finding in non-diabetic subjects, and almost as common in subjects with T1D (132). We explored this phenomenon by staining FFPE sections by immunohistochemistry as well as performing IF on frozen sections from the same donors. 3 out of 7 donors with T1D as well as 1 out of 8 non-diabetic donors showed patchy amylase-negative regions. Nevertheless, this could not be verified by IF, in which the amylase expression was evenly distributed throughout the section. These discrepancies could be explained either by the fact that these amylase-negative regions may only appear locally in some lobules but not others, or they are artefacts of formalin

fixation and/or paraffin embedding. Indeed, many variables in both tissue fixation and processing have been observed to affect the extent and intensity of immunostaining, as well as give rise to nonspecific background staining (133). The differences in handling and processing the tissue might explain our inability to replicate previous results.

The acinar transcriptome

Given the emerging evidence of a role for the exocrine pancreas in T1D development and aetiology, we hypothesized that this would be reflected in the acinar transcriptome. We also assumed there would be alterations in expression patterns depending on the distance to the islets, and hence extracted three different exocrine regions by using LCM; Exo 1 – defined as regions adjacent to islets, Exo2 – defined as regions between 50-100 µm from the closest islet, and Exo3 – defined as regions 100 µm or more away from islets. However, no apparent clustering, based on disease status or exocrine region, could be visualized by principal component analysis (PCA). Few differentially expressed genes were identified, although in the region closest to islets many of the genes found were assumed to be contaminations from islet tissue. An explanation for the few differences being detected between the three exocrine regions could be that we are only examining a 2D image when extracting the tissue with LCM. Hence, an exocrine area defined as 100 µm from an islet, might be much closer to an islet, if observed from a 3D perspective. An endeavour to avoid this issue was made by examining a series of sections to approach a 3D view. Nevertheless, we cannot exclude that some exocrine regions were closer to islets than what was recorded in the study. A gene set enrichment analysis did despite this identify several significantly enriched gene sets, with the top two being *Myc targets v2* and *Estrogen response early* in all three exocrine regions. This suggests a more general transcriptional alteration in exocrine tissue, independent of the distance to islets, and the alterations also seem to be a result of small variations in many genes, rather than a few larger changes. Both the transcription factor c-MYC (which is part of the *Myc targets v2* gene set) and estrogen receptor-related pathways have been linked to cell growth and cancer, and the activation of these pathways could hence be a proliferation initiation, to compensate for a reduced acinar volume that is commonly linked to T1D.

Paper II

A comparison between islets from donors with and without type 1 diabetes

In this study, LCM was used to extract and analyse islets from donors with and without T1D. In this way, harsh procedures like islet isolation, dispersion into single-cells and culture, which likely affect the transcriptome, were avoided. The composition of different cell types in the islets examined was determined on both a histological (IF) and transcriptomic (MuSiC (Multi-Subject Single Cell) analysis) level. As expected, the islets from donors with T1D were insulin negative and clustered separately from islets from non-diabetic donors in a PCA. The loss of beta cells was evident in all bioinformatical analyses. Downregulated DEGs well associated with beta cells, such as insulin, MAFA, and PDX1 were identified, and an overrepresentation analysis (ORA) of these presented gene sets such as *Regulation of gene expression in beta cells*. A GSEA using MSigDB was performed as well, displaying two negatively enriched gene sets in T1D; *Pancreas beta cells* that are most likely derived from the lack of beta cells, and *Epithelial-Mesenchymal Transition* (EMT), which may indicate that islets from donors with T1D have a less active EMT and a lower degree of plasticity. However, the change in EMT could also simply be a result of the loss of beta cells.

Changes related to vasculature and angiogenesis in subjects with type 1 diabetes

The upregulated DEGs had a less clear role in islets, but the ORA, done to explore whether known biological functions or processes were overrepresented in these lists of DEGs, revealed many pathways linked to vasculature and angiogenesis. To validate that this was not only an effect of the beta cell loss, an ORA on sorted islet cells from a previous study was performed as well (134). However, the gene sets related to vasculature and angiogenesis were not seen to vary between the different islet cell types, and none of these gene sets has been reported to distinguish different pancreatic cell types from each other in other single-cell studies either (134,135). As such it was concluded that these gene sets are indeed changed as a result of the disease.

Vascular density

In consecutive sections to those used for LCM, the vascular density in islets was examined by staining for the endothelial marker CD31. The vascular density was increased in subjects with T1D as indicated by an increase in the total CD31+ endothelial length per islet area (0.015 $\mu m/\mu m^2$ and 0.0085 $\mu m/\mu m^2$ in subjects with and without T1D respectively). As CD31 is also present on

macrophages, the possible presence of immune cells might be mistakenly interpreted as an increase in vascular density. However, all type 1 diabetic donors included in the study were previously screened by staining for CD45, and donors with pronounced immune infiltration were excluded. Moreover, no gene sets related to inflammation were found to be upregulated in the type 1 diabetic subjects. As such, the increase in density of CD31+ cells was concluded to be due to an increased presence of endothelial cells. This, together with the upregulated gene sets related to angiogenesis, suggests an active vascular remodeling in islets from subjects with T1D. Indeed, the endothelial cells are important for proper beta cell function and proliferation, and the upregulation and increased density observed here might be a compensatory response to the loss of beta cells.

Paper III

Extra-islet cells are frequent with a limited change correlated to age

Extra-islet insulin- and glucagon-positive cells found scattered in the exocrine parenchyma were examined in human pancreatic biopsies from organ donors aged 1 to 25. The median density of the insulin- and glucagon-positive cells were 17.3 and 22.9 cells/mm^2, respectively, which is approximately 5-fold higher than previously reported islet density (136). The frequency of the extra-islet cells was relatively constant during the first 25 years of life. As the pancreas size is increased several folds during the same time period (137), this indicates that new extra-islet cells are formed throughout childhood and into early adulthood.

The transcription factors PDX1 and ARX are not expressed in many of the extra-islet cells

PDX1 was primarily expressed in insulin-positive cells and ARX was mainly found in glucagon-positive cells. About half of all extra-islet cells lacked expression of any of the transcription factors. As both PDX1 and ARX are important for maintaining a stable beta- and alpha-cell phenotype, respectively (138), this result could indicate that these cells are immature, newly formed or plastic. In islets, insulin- or glucagon-positive PDX1-ARX- cells were also found, although they were much rarer (6% and 7%, respectively). Double-hormone-positive cells, as well as insulin-positive cells expressing ARX and glucagon-positive cells expressing PDX1, were rare but found in all age groups. Transdifferentiation of alpha cells into beta cells and vice versa have been previously reported in *in vitro* studies (138). The results observed herein could indicate that the extra-islet cells are newly formed from e.g. ducts, and

that further differentiation can take place as a mechanism contributing to islet formation.

Proliferating cells were found in all age groups

Proliferating cells, assessed by Ki67 staining, were found in all age groups (0-2 % Ki67-positive cells). No significant difference was observed based on age ($p=0.8018$), and the replication rate hence seems to be rather stable and contributing to a small but significant number of new endocrine cells. Indeed, even with a replication rate of just 2%, as observed in this study, the entire endocrine cell population would double within a matter of years (90,91).

Paper IV

Increase in glucagon-positive extra-islet cells in T1D

Extra-islet cells positive for insulin or glucagon were examined in donors with and without longstanding T1D. No difference in the overall density of the extra-islet cells was observed. The density of insulin-positive cells was significantly lower in the T1D donors compared with the non-diabetic (ND) donors (median T1D: 0.44 cells/mm^2, median ND: 20 cells/mm^2, $p=<0.0001$). Surprisingly, the density of glucagon-positive cells was significantly increased in the T1D donors (median T1D: 51 cells/mm^2, median ND: 14 cells/mm^2, $p=0.008$). This could be a result of a compensatory mechanism to counterbalance the impaired alpha cell function observed in T1D, an effect of the reduced pancreas size in T1D, or the decrease in insulin-positive cells accompanied with the increase in glucagon-positive cells is a sign of transdifferentiation. Differentiation of beta cells into alpha cells as a response to stress has been observed in both animal models and human T2D (139,140), but this is less explored in T1D. The result in this study, with increased density of glucagon-positive extra-islet cells and decreased density of insulin-positive extra-islet cells, which is in line with what has previously been reported in T1D islets (136), could be interpreted as a sign of transdifferentiation of beta cells into alpha cells.

The presence of insulin-positive and proliferating extra-islet cells

The decrease in insulin-positive extra-islet cells is most likely a result of the same beta-cell destructive process causing the demise of the islet beta cells. Insulin-positive cells were however found in all but one T1D donor, and in another donor, insulin-containing islets were present as well. Some of these islets had extensive bleedings, whereas some had a normal phenotype. Re-

maining insulin-positive cells, even in longstanding T1D, have been previously reported (141,142), supporting the concept of a sustained beta cell provision long after disease debut. This could happen either through neogenesis from e.g. ductal cells, or through proliferation of already existing endocrine cells. Ki67-positive cells were found in both the T1D and non-diabetic donors, in a range between 0-3 per cent. No difference was observed between the groups (p=0.43), which is in line with a previous report of beta-cell proliferation within islets (86), and both insulin- and glucagon-proliferating cells were found. These actively proliferating cells could be a potential source of the extra islet cells found herein.

Many extra-islet cells lack the expression of the transcription factors PDX1 and ARX

Many of the insulin- and glucagon-positive cells lacked the expression of their corresponding transcription factors, PDX1 and ARX respectively, in line with what was observed in **Paper III**. The median proportion of insulin-positive cells that also express PDX1 were 20 % and 30 % in the T1D and non-diabetic group respectively (p=0.2), and the median proportion of glucagon-positive cells that also express ARX were 25 % and 19 % in the T1D and non-diabetic group respectively (p=0.8). Double hormone-positive cells were found in both the non-diabetic and T1D donors (in 9/11 ND donors and 2/11 T1D donors). Other unusual phenotypes did also occur, although they were rarer. The loss of transcription factor expression could indicate that these cells are plastic and that there is ongoing transdifferentiation. Loss of ARX expression has been shown to induce alpha-to-beta cell conversion in mice (143,144), and human beta cells that have been differentiated to alpha cells *in vitro* maintain their PDX1 expression (145), which could explain the origin of these extra-islet cells found herein. However, as these cells were very rare, and their frequency did not differ between T1D and non-diabetic donors, this might argue against ongoing transdifferentiation. In addition, these rarer phenotypes could also be false positives, where the expression of nearby cells is interpreted as coming from the same cell.

Summary of Conclusions

Paper I

- No histological alterations in the exocrine pancreas were found between donors with and without longstanding T1D, and no evidence of acinar atrophy in acinar T1D tissue was found.
- Similar transcriptional alterations were identified in all three examined exocrine regions, advocating for a general transcriptional alteration in acinar T1D tissue, independent of distance from islets. The upregulated pathways found in donors with T1D might be a possible link to the increased incidence of pancreatic cancer in T1D.
- In summary, the lack of histological findings may argue against acinar atrophy but is in line with a reduced number of acinar cells as an explanation of the reduced pancreas volume, and the results from the transcriptome analysis provide evidence of exocrine involvement and the impact of the entire pancreas in T1D pathology.

Paper II

- Transcriptional alterations related to vasculature and angiogenesis were found to be upregulated in islets from subjects with T1D compared to non-diabetic controls.
- Vascular density was as well increased, suggesting an altered microvasculature in islets in T1D.
- As endothelial cells are important for proper beta cell function the changes in vasculature could be interpreted as a reaction to the loss of beta cells.

Paper III

- The density of extra-islet insulin- and glucagon-positive cells were higher compared to what has been reported for islets, and the maintained frequency suggests that new cells are formed with increasing age.

- Mitotic cells were found in all age groups and might hence be a possible source of new extra-islet cells.
- Many extra-islet cells lacked the expression of PDX1 and ARX, which may suggest that these cells are immature, newly formed and/or plastic.
- In summary, the extra-islet cells could play an important role in expansion or turnover of the endocrine cell population.

Paper IV

- Extra-islet insulin-positive cells were decreased, and extra-islet glucagon-positive cells were increased in donors with T1D. This could be a result of an ongoing beta- to alpha cell transdifferentiation, as a mechanism for beta-cell loss.
- Although at low numbers, proliferating extra-islet cells were found in both non-diabetic and T1D donors.

Perspectives

Moving Forward

Being able to solely use material from donated human pancreases for all papers included in this thesis is a humbling opportunity. It provides exclusive insights into the physiology of this curious organ. Nevertheless, we are only provided with a snapshot, a brief moment in time. With the use of state-of-the-art techniques, we have tried to elucidate what is happening, before and after this snapshot, to shed light on the pathophysiology of T1D. In **Paper I and II**, LCM was used to cut out regions of interest from pancreatic tissue sections. This method has since evolved, and the precision improved. Using this technique to cut out specific cells of interest from stained tissue sections would render us maximum information of these cells whilst keeping the morphology intact. The transcriptome analyses performed in both **Paper I and Paper II** were somewhat hampered by our RNA levels being on the very limit of detection for many genes of interest. Nevertheless, single-cell transcriptomics is fast evolving, and the sensitivity of these analyses as well. Combining these state-of-the-art methods of LCM and single-cell transcriptomics would deepen our understanding of the function of the cells in the pancreas both in health and disease. To be able to do this on both control pancreases, as well as pancreases from newly diagnosed and longstanding T1D subjects, would enable us to build a timeline of disease development. It would be interesting to see if the changes in vasculature observed in **Paper II** are evident already in newly diagnosed patients, or if it is something that evolves later in the disease. Beyond healthy and T1D subjects, examining first-degree relatives using LCM and transcriptomics would be an interesting opportunity. As this group has been observed to have a reduced pancreas volume, much like T1D patients, it would be informative to know if the pathways found to be upregulated in T1D subjects in **Paper I** are upregulated in first-degree relatives as well (23). This would indicate that these pathways are not a direct result of T1D, but rather an effect of the reduced pancreas volume. However, the knowledge needed to properly handle the immense volume of data these transcriptome studies often generate is lacking in many research groups. Better collaborations and knowledge exchange, including proper online repositories, will be pivotal if we are to make sense of the data and be able to replicate and compare studies.

In **Paper III and Paper IV**, multispectral imaging was used to examine pancreases from control and T1D donors. 5 different markers were stained for, including DAPI, making it possible to extract a lot of information from one single tissue section. Although it should be noted, that this is just scraping the surface of what is achievable with multispectral imaging. Methods allowing for dozens of different protein targets to be detected are evolving, each technology using a different strategy to overcome the limitations of conventional IF and IHC (146). It would be exciting to use one of these methods to explore the extra-islet single-cells examined in **Paper III and Paper IV**, with regard to the expression of other transcription factors beyond PDX1 and ARX. For example, beta cells that have converted to alpha cells *in vitro* retain the expression of both PDX1 and NKX6.1 (147). Staining for multiple transcription factors, as well as insulin and glucagon within the same tissue section, would enhance our understanding of these cells. Furthermore, techniques for optical 3D imaging of complete human organs are becoming more successful (148). Being able to investigate these extra-islet single cells within the entire pancreas, not being restricted to the extremely small part of the pancreas that a tissue section represents, would help us to quantify these cells and translate the results to the organ as a whole.

This thesis aims at further characterizing the entire pancreas, in health and disease, not limiting the studies to solely beta cells and/or immune cells. Using different methods, like LCM and multiplex stainings, the aim was to widen the current views on disease pathophysiology and explore it from different points of view. To get to the heart of the matter regarding T1D aetiology, we need to approach the questions at hand from different starting points, to cover all possibilities. In light of this, some alternative origins of T1D are explored in the next section.

Alternative Origins of Type 1 Diabetes

T1D is most commonly referred to as an autoimmune disease, where the beta cells are targeted and killed by cytotoxic T-cells. This is the most common explanation and description of the disease aetiology, and many papers start in this way. That a virus infection would be the environmental trigger activating the immune response is also a common description of the cause of the disease, and the theory dates back almost a century (149). However, despite enormous efforts to elucidate the pathophysiology of the disease, little progress has been made both in terms of a precipitating event caused by viruses and the so-called autoimmune attack. Other theories have been gaining interest during the past years, broadening the research focus and helping expand the view of the origin and progression of the disease, by questioning old dogmas. Some alternative origins and perspectives are discussed below.

1. The high incidence of T1D in children and adolescents, coincides with episodes of rapid body growth, and inevitably also an increased insulin demand (150). Indeed, in **Paper III**, an increase in extra-islet cells is observed during the first 25 years of life, likely an effect of a growing pancreas and body. But an inability to expand the beta cell mass to accommodate the increased insulin need would put a lot of stress on the available beta cells, which ultimately could lead to hyperactive beta cells and subsequent beta cell death. The reduced total volume of the pancreas observed in T1D could also indicate an inability of the pancreas to expand as the body grows. In **Paper I**, no acinar atrophy was observed in the T1D exocrine pancreases, which might indicate that the reduced pancreas volumes rather stems from fewer total number of cells, than an effect of atrophy. The observed transcriptional alterations could be linked to cell growth and proliferation, possibly activated as a compensatory response to the reduced acinar volume. This scenario presents an alternative explanation of T1D aetiology, although the cause of an inability to increase the beta cell (and exocrine) mass is yet unknown (151).

2. Another explanation for the loss of beta cells can be found in the theory of dedifferentiation of beta cells to alpha cells. This is most commonly discussed in the context of T2D, where it is an accepted explanation for beta cell dysfunction (139). Loss of beta-cell identity is often characterized by the expression of beta-cell transcription factors in glucagon-positive cells (145). However, this theory has not been extensively explored in the context of T1D. A high insulin demand can lead to stress-induced exhaustion of beta cells, potentially triggering them to adopt an alpha cell phenotype as a protective mechanism against apoptosis, which is generally found to be low in T1D (141). **Paper IV** explores this theory, as an increase in glucagon-positive extra-islet cells, accompanied by a decrease in insulin-positive extra-islet cells, was observed in T1D donors compared with controls. As many of these cells also lacked the expression of transcription factors, which are important in maintaining a stable phenotype, they were hypothesized to be plastic, able to transdifferentiate. A dedifferentiation of beta cells into alpha cells would explain the reduction in insulin-positive cells and the simultaneous increase in glucagon-positive cells. This theory would also explain the maintained islet size seen in T1D, however, conflicting studies where the islet size has instead been shown to be decreased exist as well (131,136,152,153).

3. The hypothesis of a role for bacteria as a trigger of T1D is emerging as well (105). The connection between the bile duct system and the pancreas, through the ampulla of Vater, allows translocation of intestinal content, including bile and various bacterial species, from the duodenum to the pancreas. In the ductal system of the pancreas, the bacteria would get in contact with pancreatic juice containing antibacterial effects, causing the release of bacterial components like lipopolysaccharides (LPS) and other toxins. This could lead to an inflammatory response subsequently affecting the beta cells (34,154). Interestingly, the injection of bacteria in the ductal system of rats shows remarkable similarities with morphological findings in patients dying at onset of T1D (154,155).

4. Beta cell stress is often discussed in T1D aetiology, and ER stress specifically has been described both as a cause of apoptosis and as the culprit in exposing beta cells to the immune system, initiating selective destruction of beta cells by CD8 + T-cells (54). A source of stress is the high demand for insulin in response to a meal, which poses a great challenge for the beta cells. Protein folding is monitored carefully in the ER and ER stress will occur if misfolded proteins start to accumulate, which in turn will activate the UPR. The UPR is supposed to restore homeostasis within the cell, but if the stress is chronic and persistent apoptosis might be triggered (156). ER stress within the beta cells can also initiate release of chemokines as a response to the damage caused by the aforementioned stress. Immune cells attracted to the site will, among other things, release cytokines. Inflammatory cytokines have been shown to affect gene expression in beta cells, both on a transcriptional, translational and post-translational level, leading to misfolded proteins and activation of the UPR. These aberrant proteins may generate beta-cell epitopes leading to autoreactive T-cells (157). Indeed, the beta cells are by nature prone to stress, and this together with their inherently inadequate self-defence, makes them highly implicated in their own destruction. Together these responses would explain the appearance of auto-antigens and apoptosis of the beta cells (56).

5. The effect of gut microbiota on both health and disease has gained more and more interest during the past years, and its effect on the development of T1D is gaining more traction (158). The gut microbial composition has been proven to differ between individuals with and without T1D (159), and the microbiome is both more diverse and stable in healthy children compared with children who later developed T1D (160). The gut microbiota has been seen to influence the immune response through endotoxins like lipopolysaccharides

(LPS). LPS is expressed on the surface of gram-negative bacteria and is highly potent in raising the levels of proinflammatory cytokines, and individuals with T1D have been shown to have higher circulating LPS than non-diabetic individuals. This could trigger activation of TLR2 and TLR4, leading to a proinflammatory state that potentially harms the beta-cells (161).

6. The islets of Langerhans are richly perfused to be able to maintain glucose homeostasis (44). During high insulin demand, the blood perfusion in the islets is increased, as a result also increasing the islet capillary pressure (162). However, the islets have very few lymphatic capillaries and are therefore inherently bad at regulating the interstitial fluid levels (163). When blood perfusion, and hence also capillary pressure, rises, this increases the risk for microvascular bleeding within the islets (90). Hemorrhagic islets are a rather common finding, in healthy humans, in approximately 50% of the subjects examined, it has been reported that hemorrhagic islets account for 3-4% of all islets (164). This would ultimately lead to a continued loss of islets, which has to be counterbalanced by the generation of new islets, which is seemingly not an issue in healthy individuals. However, this process might be dysfunctional in T1D, e.g. periductal fibrosis is often observed, which may disrupt the neogenesis of islets (142,165). Given that the development of T1D is very slow, often taking many years to develop, the beta-cell damaging process must be of low intensity. Impaired neogenesis due to trauma affecting the ducts, in combination with loss of islets in hemorrhagic incidents, would cause a slow but steady decline in islet mass, creating a vicious cycle by leading to an increasing workload on the remaining islets, further increasing the risk for microvascular bleedings (90). Indeed, in **Paper II**, an increase in vascular density and an upregulation of pathways related to vasculature and angiogenesis was observed. This could be a compensatory effect to be able to handle the increased workload on the remaining islets, to try and avoid a hemorrhagic event.

If nothing else, these alternative views on the aetiology of T1D should inspire us to dare and explore new ideas in our quest to uncover the true causes of the disease. In a commentary by Donath, the role of autoimmunity in beta cell death is indeed questioned. (166). This is based on a study proving blood glucose levels to rise months before the detection of autoantibodies, suggesting beta-cell insult to precede the development of autoimmunity (167). This opens up a model in which various factors, including viral infections, genetic predisposition, or any of the aforementioned mechanisms, may contribute to beta-cell damage. These insults may then ultimately cause beta-cell death, releasing

beta-cell antigens that are picked up by antigen-presenting cells subsequently activating the immune system, starting an autoimmune attack on the remaining beta-cells. The role of autoimmunity might still be crucial in the disease progression – but have we misinterpreted what is the true starting point and who is the main villain in the disease progression?

Populärvetenskaplig Sammanfattning

Typ 1 diabetes (T1D) är en livslång, idag obotlig sjukdom som beror på att de celler som producerar hormonet insulin försvinner. Dessa så kallade betaceller återfinns normalt sett i bukspottkörteln, i små cellkluster kallade Langerhanska öar. När insulinet försvinner kan inte kroppen ta tillvara det socker vi får i oss genom födan, vilket leder till att sockernivån i blodet stiger, något som kan leda till döden. T1D behandlas idag genom att man tillför syntetiskt insulin med hjälp av sprutor eller en insulinpump, vilket i stort sett är samma behandlingsmetod som funnits i 100 år, allt sedan insulinet upptäcktes 1921. Att en så pass vanlig och allvarlig sjukdom inte haft någon utveckling, utöver rent tekniska framsteg, vad gäller behandlingsmetod är anmärkningsvärt. Vad beror detta på?

Den underliggande orsaken till att betacellerna försvinner är ännu inte helt känd för oss. Vi vet att både gener och miljö spelar in, och att det egna immunförsvaret verkar spela en avgörande roll i sammanhanget. Det finns många teorier kring sjukdomens orsak och uppkomst, men inga har visat sig helt kunna förklara händelseförloppet. I den här avhandlingen har vävnad från organdonatorer, med och utan T1D använts, för att försöka fylla i några av luckorna i vår förståelse av sjukdomen. Målet har varit att undersöka vävnaden utan styrning från befintliga teorier kring sjukdomen, alltså att titta på resultaten och beskriva vad vi ser utan fördomar och föreställningar om vad vi *borde* se.

I avhandlingens första arbete har den exokrina vävnaden i bukspottkörteln undersöks. Där upptäcktes vissa skillnader mellan donatorer med och utan diabetes, vilket tyder på att även denna del av organet är påverkat vid T1D. I det andra arbetet har istället de Langerhanska öarna undersökts. Vi kunde då se en förändring av blodkärlen i öarna som en följd av diabetes. I de två sista arbetena har endokrina celler utanför öarna undersökts. I tredje pappret undersöker vi förekomsten av dessa celler i friska donatorer i åldrar mellan 1-25 år. Vi ser då att dessa celler fortsätter bildas under uppväxten, och till viss del även delar sig för att öka sitt antal. Detta kan vara en viktig process för att öka antalet betaceller vid behov. I sista pappret jämförde vi dessa endokrina celler utanför öarna i friska kontroller jämfört med donatorer med T1D. Vi kunde då se en minskning av antalet betaceller, men en ökning av antalet alfaceller,

kanske som en följd av att betacellerna ändrar identitet och blir alfaceller i stressfulla situationer, som den när T1D utvecklas.

Sammantaget bidrar dessa arbeten till att öka vår förståelse av hur de olika celltyperna i bukspottskörteln fungerar, och hur deras funktion förändras vid T1D. Genom att undersöka hela organet, och inte begränsa oss till att titta på de celler som vid första anblick verkar vara huvudrollsinhavarna (betaceller och immunceller), kan vi bredda vår förståelse och komma närmare T1D-etiologin.

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