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Studies of the Pancreas: Implications for Type 1 Diabetes Aetiology

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Abstract

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Type 1 diabetes (T1D) is a disease of severe insulin deficiency through loss of β cells in the endocrine pancreas. The T1D dogma maintains that a precipitating event unleashes autoimmunity in at-risk individuals, often measured through autoantibodies against β cell antigens. This is followed by the death of β cells at the hands of autoreactive cytotoxic T cells. However, several findings have not found their place within this dogma; first, the immune cell infiltrate in islets is usually located outside the islets, and second, there is a pronounced impact on the exocrine pancreas with lower pancreatic weight and fibrosis surrounding the ducts. In this thesis, pancreata from human subjects without diabetes (ND) as well as with T1D or type 2 diabetes (T2D) have been examined in an attempt to clarify the aetiology of T1D.

The consensus definition of insulitis (≥15 CD45+ cells per islet in ≥3 islets) was validated against ND pancreata. In **paper I** we show that this definition cannot sufficiently discriminate between the findings in T1D and T2D pancreata, due to an increase in exocrine infiltration in T2D, predominantly made up by macrophages. As exocrine infiltration is also a common finding in T1D, we propose a new definition. In **paper II** we found tissue resident memory T (T_{RM}) cells in association to islets in both ND and T1D pancreata, and they made up a significant proportion of the insulitic lesion in T1D. Islets contain on average 60% β cells. In **paper III** we found that despite the seeming loss of this predominant cell type in the T1D islets, islet size remained the same. Instead, islet density was markedly reduced. The islets contained mainly α cells, some of which expressed PDX1, a transcription factor marker of β cells. In **paper IV** we examined pancreata from ND organ donors aged 1-81 years. For the first time, the islet transcriptome was analysed without prior enzymatic digestion of the tissue. We corroborate earlier findings of reduced cell cycle activity and increased senescence with increasing age, as well as present a hypothesis of how islet age might affect T1D.

The findings in this thesis sprout an alternative hypothesis that disturbed establishment of β cells in early life, due to lower islet density and lower pancreatic weight, would lead to β cell stress as insulin demand increases with physical growth. However, as islets do not decrease in size, we suggest that the disappearance of β cells could be explained by transdifferentiation into glucagon-producing cells.

Keywords: Pancreas, Type 1 Diabetes, Insulitis, Beta cells, Immunology, Tissue resident memory T cells, Islets of Langerhans

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Till Åke
Världens bästa gubbjävel
och min morfar

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. (2017) Insulitis in human diabetes: a histological evaluation of donor pancreases. *Diabetologia* 60:346–353

II Kuric, E., **Seiron, P.**, Krogvold, L., Edwin, B., Buanes, T., Hanssen, K.F., Skog, O., Dahl-Jørgensen, K., Korsgren, O. (2017) Demonstration of Tissue Resident Memory CD8 T Cells in Insulitic Lesions in Adult Patients with Recent-Onset Type 1 Diabetes. *American Journal of Pathology*, 187(3):581–588

III **Seiron, P.***, Wiberg, A.*, Kuric, E., Krogvold, E., Jahnsen, F.L., Dahl-Jørgensen, K., Skog, O., Korsgren, O. (2019) Characterisation of the endocrine pancreas in type 1 diabetes: islet size is maintained but islet number is markedly reduced. *Journal of Pathology: Clinical Research*, 5(4):248–255 *Authors contributed equally

IV **Seiron, P.**, Stenwall, A., Hedin, A., Granlund, L., Esguerra, J.L.S., Volkov, P., Renström, E., Korsgren, O., Lundberg, M.*, Skog, O.* (2020) Transcriptional analysis of islets of Langerhans from organ donors of different ages. *Manuscript* *Authors contributed equally

Contents

Abbreviations

T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
ND	Non-diabetic
DiViD	Diabetes Virus Detection Study
ES	Enrichment score
FDR	False discovery rate
GSEA	Gene set enrichment analysis
HLA	Human leukocyte antigen
LCM	Laser capture microdissection
nPOD	Network for Pancreatic Organ Donors
TCR	T-cell receptor
T_{RM}	Tissue resident memory T cell
PP	Pancreatic polypeptide
SASP	Senescence associated secretory phenotype

Introduction

Diabetes mellitus is the common name for diseases in the glucose homeostasis. Translated from ancient Greek into "passing through" for the excessive amounts of urine and "sweet" for the taste of the urine. Mentions of this sweet- or honey-tasting urine dates back to ancient Egypt. It took several thousand years before it was linked to the pancreas, when Joseph von Mering and Oskar Minkowski in 1889 saw diabetes in dogs who had undergone pancreatectomy and that implanted pancreatic tissue prevented this[1]. In 1921 Frederick Banting and Charles Best managed to prepare a pancreatic extract that could reverse diabetic symptoms, by first causing the major exocrine region of the pancreas to atrophy through duct ligation. The resulting substance was named insulin and was successfully tested in humans in 1922.

Today diabetes is estimated to affect 463 million people across the globe[2]. A majority of which suffer from type 2 diabetes (T2D), a disease characterized by insulin resistance and a relative deficiency of insulin secretion[3]. This leads to hyperglycemia and over time to chronic complications: atherosclerotic cardiovascular disease and microvascular complications such as retinopathy, neuropathy and chronic kidney disease[4,5]. Apart from these chronic complications, individuals with type 1 diabetes (T1D) who almost or completely lack the inability to produce insulin, face the daily risk of dying from diabetic ketoacidosis or hypoglycemia if they do not get the insulin they need or if they get too much, respectively[6]. Albeit improved over the past century, insulin therapy remains as the only available treatment for type 1 diabetes.

In this thesis human pancreata have been interrogated for features of the immune cells and the pancreatic islets that could lead to new insights of type 1 diabetic aetiology.

The Pancreas

Situated dorsally in the abdomen as a secondary retroperitoneal organ, the pancreas is to 98% an exocrine gland weighing about 50-100 grams[7]. It consists of a branching ductal system surrounded by acinar cells that produce the exocrine enzymes; trypsin, chymotrypsin, lipase and amylase. They flow out through the main pancreatic duct, joining the common bile duct, before being secreted into the duodenum through the ampulla of Vater where the enzymes aid in digestion of nutrients[8]. The remaining 2% is made up of the endocrine

compartment, the islets of Langerhans, that are tiny micro-organs scattered throughout the pancreas and made up of five endocrine cell types; α cells, β cells, δ cells, ε cells and γ cells that produces glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide, respectively.

Anatomically the pancreas is divided into the three regions, the head (caput), body (corpus) and tail (cauda). It originates from two separate locations during embryogenesis. The ventral anlage from the common bile duct and the dorsal anlage directly from the foregut. When these fuse in week 7 of embryological development, the ventral anlage becomes part of the head, containing the common bile duct and ampulla of Vater, as well as the uncinated process while the dorsal anlage makes up the remainder of the head as well as body and tail of the pancreas[8].

The Islets of Langerhans

The endocrine compartment has a mass of about 1-2 grams in adults from a few million islets[9]. Numbers and mass of islets and their constituting endocrine cells show marked heterogeneity between individuals, but also in different areas of the pancreas of the same individual. Islets can range in size from clusters of just a few endocrine cells to several thousand. On average, β cells are the most prominent cell type, constituting about 60% of the islet cells, followed by α cells at 30% and δ cells at about 10%[10,11]. PP-cells make up a significant fraction of the islets in the PP-rich regions of the uncinate process[12]. In murine islets, these cells organize with α cells and delta cells as a mantle around the periphery of the islet, with β cells in the islet core[13]. In human islets, all cell types can be seen throughout the islet. It has been suggested that this represents subunits each resembling the mantle-core architecture of murine islets[14]. Or a laminar architecture with a layer of α cells covering blood vessels, with layers of β cells in between[15]. While α cells are preferentially located adjacent to blood vessels, it is not exclusive[16]. A recent study examining islets three dimensionally could not support either of these suggested architectures, only confirming that the endocrine cell types have a weak preference of clustering together with the same cell type[11].

In order to release their hormones systemically, the islets are richly perfused, receiving a blood flow 5-10 times higher than the surrounding exocrine parenchyme[17]. This blood flow is controlled by pericytes by contracting the vessels[18] and in mice, islet blood flow is markedly increased during hyperglycemia[19]. Further facilitating efficient protein transport is the highly fenestrated capillaries[20]. Most islets receive only one afferent arteriole, regardless of islet size[21]. This arteriole and the capillaries of the islet is covered by a double basement membrane with both a vascular sheet and an endocrine sheet[22]. Similarly, the islets are surrounded by both an endocrine and an exocrine basal membrane, in most islets the space between these sheets is filled with a thin layer of collagen, often referred to as the islet capsule[23].

Glucose homeostasis

The human body normally maintains blood glucose levels in the narrow range 4-6 mmol/L, regardless of intake or increased need. The endocrine pancreas is a key player in maintaining this balance.

Insulin, is produced as a prohormone, preproinsulin, that is converted rapidly into proinsulin. When proinsulin is cleaved by prohormone convertase 1/3 it results in insulin and c-peptide, the latter which is a stable marker in blood of insulin production and release[24]

Insulin is released when β cells are exposed to higher levels of blood glucose. Glucose influx leads to rapidly increasing ATP levels, in turn turning of an ATP-sensitive K+ channel. This leads to depolarization of the cell membrane and Ca2+ influx, which leads to exocytosis of insulin granules[25]. The glucose-induced insulin secretion can also be affected by the metabolic amplifying pathway, which is also glucose-dependent but independent on membrane depolarization. Instead it is affected by secretagogues such as the incretin GLP-1 which can potentiate insulin secretion, by increasing cAMP, after being released from the gut after feeding[26].

Insulin, once released into the bloodstream, acts upon the insulin receptor. This has several downstream effects with translocation of GLUT4 to the membrane and subsequent glucose uptake being the most well known[27]. It has a general anabolic effect, stimulating fatty acid synthesis and glycogen synthesis, inhibiting glycogenesis and production of ketone bodies. On the other hand, glucagon, which is a counter-regulatory hormone, stimulates glycogenolysis, gluconeogenesis and ketogenesis, to maintain blood glucose levels in the fasted state.

Islet development and aging

During pancreatic organogenesis, endocrine progenitors originate from the same early system of epithelial tubules, also known as the plexus, as the exocrine compartment's acinar and ductal cells[28]. Endocrine progenitors bud and form islets at the outside of these tubules[29]. Postnatally through childhood and adolescence, expansion of the endocrine compartment is suspected to occur through continued islet neogenesis and an increase in islet size, with the latter being the main contributor[30]. The postnatal islet neogenesis is thought to originate from pancreatic ductal glands from a population of Sox9 positive cells[31].

Beta cells in adults are generally regarded as post-mitotic, based on several findings. Levels of Ki67, a proliferation marker present throughout active phases of the cell cycle, diminishes with age[30,32]. Instead $p16^{ink4a}$, a tumour suppressor coded by *CDKN2A* is a marker for senescent cells. It is present in adult β cells and increases with age[33,34]. Several findings support the view that β cell turnover is mostly limited to the initial two to three decades of life. Perl et al studied both incorporation of radioactive nucleotides into the β cells of

autopsy subjects previously participating in clinical cancer trials as well as comparing levels of carbon-14 isotopes present in β cells with the levels in the atmosphere at different time points across the 20th century[35]. Incorporation was limited to the first 20 and 30 years of life, respectively. The same conclusion was reached by measuring the accumulation of lipofuscin bodies in β cells[36]. Shortening of β cell telomeres is rapid during childhood and adolescence attributed to cell division but decreases past 20 years of age, though there is a slow continuous decline in adulthood[37].

Despite these findings, it is unclear what level of adaptability that is held by the β cell population. In a 2014 study, insulin-resistant non-diabetic subjects had a significantly higher density of islets, larger islets, as well as an increased level of small islets (<8 endocrine cells) and ductal cells positive for insulin[38]. Similar results of increased β cell mass, an increased number of small islets and insulin-positive duct cells has been seen in human pregnancy[39]. While Ki67 expression in β cells of adults is usually low in studies, a possible confounder could be how the organ is procured. In mice and pigs a delay before fixation, such as is the case with human autopsy samples, led to a marked decline in Ki67 positive cells[40]. In organ donors, Ki67 has been seen to increase after a prolonged stay in intensive care prior to brain death and organ donation, suggesting at least a capability for proliferation[41,42]. And even if only 0,1% of β cells express Ki67 at a given timepoint, it would still mean that the turnover rate of the β cell population would be roughly three years[43].

Type 1 Diabetes

Type 1 diabetes onset can be dramatic, in many children presenting with diabetic ketoacidosis. A state of severe insulin deficiency leading to hepatic gluconeogenesis leading to hyperglycaemia and production of ketone bodies leading to acidosis[44]. A less dramatic onset can be polydipsia and polyuria due to the osmotic diuresis caused by hyperglycaemia. While onset seems sudden to patients and clinicians it is usually preceded by several years of dysglycaemia, often in the form of a reduction in the first phase insulin response, with an accelerated decline approximately 2 years before onset[45–47]. A common model to describe T1D pathogenesis is that of Eisenbarth[48]. It postulates that a "precipitating event", brought on by genetic predisposition and possibly environmental factors, initiates islet autoimmunity. During a period of several years, β cell mass is continuously lost until endogenous insulin production drops beneath a critical threshold and glucose homeostasis can no longer be upheld. Islet autoimmunity is often measured by the presence of autoantibodies. This is reflected in a 2015 statement by several associations involved in diabetes research and treatment where T1D progression was divided into three stages[49]. Stage 1 represents more than 2 autoantibodies toward islet autoanti-

gens but no effect on glucose homeostasis. Stage 2 is the addition of dysglycaemia as seen for instance in an oral glucose tolerance test. Stage 3 represent clinical onset of disease with the addition of clinical symptoms. In children at risk of T1D the peak incidence for seroconversion of autoantibodies is prior to 2 years of age[50,51]. The risk of developing autoantibodies is then estimated to decline exponentially[52].

While T1D occurs at all ages, incidence is highest in childhood and adolescence, a period in life of physical growth and hence increasing insulin demand[53]. Indeed, the risk of type 1 diabetes in childhood is associated to increased height and weight as well as with rapid early growth[54,55]. This could suggest that not only β cell loss, but also an inability to expand β cell mass to compensate for this increasing demand could be a factor in T1D aetiology. After disease onset and initiation of insulin therapy many patients experience a partial clinical remission, colloquially known as "the honeymoon period", wherein the need for exogenous insulin declines and metabolic control improves, reflecting a partial β cell recovery and improved insulin sensitivity[56]. In the best of cases, patients can maintain euglycemia without exogenous insulin. Indeed, islets from recent-onset type 1 diabetics improve their insulin secretion when cultured in a non-diabetogenic milieu[57]. Reflecting a component of β cell stress at T1D onset that can be improved by normalising metabolic factors. Beyond this period, C-peptide levels and correspondingly β cell mass continue to decline, albeit at a slower rate in patients with older age at onset[58]. Despite this continuous decline after diagnosis, many patients retain detectable C-peptide levels even after more than 50 years[59]. Having a higher level of C-peptide was again, associated with older age at onset, and among these patients several responded with an increased insulin production to a mixed meal tolerance test. Postmortem examination of these patients show that all had residual β cells, often scattered as single cells or clusters, but a majority also had a few β cells left within a few islets[60].

Epidemiology, genetics and environment

While T1D incidence rate is higher in childhood and adolescence, it is more common to get the diagnosis in adulthood, on the basis of it being longer than childhood. In an American study of 32 thousand cases, 13 thousand were 0-19 years at diagnosis, compared to 19 thousand at 20-64 years of age. But the incidence rate was 34,3 cases vs 18,6 cases per 100,000 person-years for ages 0-19 years and ages 20-64, respectively[61]. In Sweden type 1 diabetes incidence has doubled across the last 4 decades[62]. In line with numbers from other Western countries[63]. T1D incidence rate varies greatly by country, by as much as 400-times between Finland, the country with the highest incidence, and China and Venezuela, with the lowest known incidence[63].

Most of the genetic risk for T1D is associated to the HLA 2 locus on chromosome 6, conferring about 50% of the genetic risk. The HLA-DR3-DQ2

(DQ2) and HLA-DR4-DQ8 (DQ8) haplotypes are associated to the highest risk. Remaining genes are both related to the immune system and to the β cell, such as *IL27* or *INS*, respectively[64]. However, several findings prove that the genetic risk is not always the same. Comparing Finland and Russian Karelia the incidence is 4 times higher in Finland despite sharing common ancestry and HLA profiles[65]. The rise in incidence across the last decades has seen a rising frequency of lower risk HLA haplotypes compared to the highest risk DQ2/DQ8 heterozygote[66,67]. Children born in Sweden to immigrant parents showed a 60-70% higher incidence than those children arriving in Sweden after birth[68]. And the concordance between monozygotic twins is only 23-61% despite sharing the same set of genes[69]. This proves that there must be environmental factors of importance for T1D incidence. Many studies have focused on dietary differences in the first year of life, such as breastfeeding, the introduction of gluten or cows' milk and vitamin D levels. While some studies show an increase in T1D incidence, others show the opposite[70]. Caesarean section and antibiotic treatment during the first year of life has been seen to slightly increase the risk for T1D[71,72]. Higher growth rate in childhood as well as obesity also increases the risk[54,55,73].

Autoantibodies in type 1 diabetes

Today four autoantibodies are used for T1D diagnosis and for determining risk of T1D in studies. These autoantibodies are directed to insulin (IAA), tyrosine phosphatase (IA-2), glutamate decarboxylase (GAD65) and zinc transporter 8 (ZnT8)[3]. Recently, a new autoantibody was identified against tetraspanin-7. Similar to the other autoantigen epitopes the tetraspanin-7 epitopes are located in the cytoplasmic domain. And similar to GAD65 and IA-2 it is also present in brain among other tissues[74].

The autoantibodies are an important diagnostic and screening tool. Children with multiple autoantibodies run a severe risk of developing type 1 diabetes. Within 10 years of seroconversion to multiple autoantibodies, 70% of children develop the disease. However, presence of only one autoantibody against an islet autoantigen is much less predictive of disease, as only 15% develop T1D within 10 years[50]. Among those with only one autoantibody, 35-53% revert back to being antibody negative, depending on age at initial seroconversion, below or above 8 years of age respectively[75].

In adults, presence of autoantibodies is more complicated. Among pancreata from adult organ donors with one or several antibodies, only very rarely can islet inflammation be detected. In one study only 2/62 donors had islet inflammation[76], in another 0/32 donors exhibited the finding[77]. Also, β cell mass seems to remain unaffected in these autoantibody positive adults[76–78].

Type 1 diabetics also often have antibodies directed against autoantigens in exocrine tissue such as carbonic anhydrase II, lactoferrin or cytokeratin[79,80], suggesting that the exocrine compartment is also affected by disease. In fact,

when staining for C4d, a marker of classical complement pathway activation initiated by binding of antibodies, it seems to affect ducts and blood vessels to a higher degree than the islets[81].

T-cells and insulitis

The term *insulitis* was coined in 1940 by von Meyenburg to describe immune cell infiltration of islets[82]. In a seminal paper by Gepts in 1965 this was seen in 15/22 patients with recent onset disease and was often more pronounced at the islet periphery, what is today referred to as *peri-insulitis*[83]. In 2010 Peter In't Veld did an overview of the literature to find in total 150 cases of insulitis published between 1902-2010 and could conclude that the lesion was most common in young subjects (<14 years of age) with recent onset disease (<1 year)[82]. The definition of insulitis has however varied between studies, for example 5 T-cells per islet[84]or even as low as 2 T-cells[85]. In 2013 a consensus definition was reached: ≥15 CD45+ cells in at least 3 islets, to account for presence of other immune cells e.g. B cells, macrophages. Included in the definition is the presence of pseudo-atrophic islets – islets not containing insulin[86]. There is a large variation in how many insulin-containing islets that are affected by insulitis, ranging from a few percent to a majority of islets[84,87]. While insulitis in humans can be discreet and often only present outside the islets, in the NOD mouse, the most widespread animal model for T1D, the immune cell infiltration is massive. Immune cells can make up 40% of the total cell count within the islet[88], even up to the point of aggregating into tertiary lymphoid follicles, a structure normally present in lymph nodes[89].

The most prominent immune cell in the insulitis is the CD8+ cytotoxic T cell[84]. Capable of killing adjacent cells by secretion of granzymes and perforin. However, it has been suggested that children below the age of 7 have a predominant B cell infiltration[90].

Several studies have shown autoantigen-specific T cells to a wide range of β cell epitopes, such as preproinsulin or ZnT8a, in peripheral blood of type 1 diabetes patients[91–93]. Comparable frequencies of autoreactive cells can be seen in non-diabetics, however, in some cases, showing less signs of being antigen experienced[91,92]. The presence of these T cells in healthy individuals could be explained by lacking or divergent expression of β cell epitopes in the thymus, leading to poor negative selection of these cells[94]. In context, a T cell receptor capable of killing β cells *in vitro* was estimated to be capable of binding to a million different decamer peptides[95]. Though non-diabetic individuals rarely have autoantigen-specific T cells in the pancreas, they have been found in a patient with chronic pancreatitis[93]. In T1D, the insulitic lesions of nearby islets are populated by T cells of different specificities[96]. To this day there is no proof of T-cell mediated killing of β cells in the insulitic lesion[97], in fact, most T cells are not even in contact with β cells[16] and are of an unknown specificity[96].

Islet cells in type 1 diabetes

While initially thought of as innocent victims of the immune assault, islets and β cells are now suspected of playing their part. Loss of tolerance to β cells by T cells could be explained not only by a defective negative selection in the thymus. The stress on β cells to keep up with the demand to produce insulin could lead to translational errors forming neoepitopes that are not part of the negative selection in the thymus[98]. Beta cells also seem to downregulate IDO1, an immune regulatory molecule, prior to insulitis and β cell loss[99]. B cells can for instance produce IL-1β in response to hyperglycaemia[100]. This, in turn, can lead to production of cytokines such as CXCL9, CXCL10, and CXCL11, especially in the presence of IFN-γ[101].

Exocrine pancreas in type 1 diabetes

T1D was long considered a β cell-specific disease. However, in many patients there is a component of exocrine dysfunction. 27 to 56% of T1D patients have low levels of faecal elastase, a common marker of pancreatic exocrine function[102]. In line with this, pancreatic weight is reduced in T1D patients. This difference does not increase with time after onset, suggesting that this happens prior to or in connection with clinical onset[7]. Curiously, first-degree relatives of T1D patients seem to have a slightly lower pancreatic volume measured by MRI[103]. Which could represent an underlying predisposition for disease.

The exocrine pancreas in T1D contains more immune cells than the non-diabetic pancreas[104]. Indeed, the overall immune cell density is higher in exocrine tissue than in islets with regards to cytotoxic T cells, T helper cells, macrophages and neutrophils[16]. Immune cell infiltrates can be seen at the pancreatic ducts[83]. In T1D, these ducts are commonly surrounded by periductal fibrosis[105]. Apart from the effect on ducts, the exocrine pancreas in subjects with T1D contains more non-functional area in the form of fibrosis and fat[106].

Aims

Paper I

Leucocyte infiltration of islets has a central role in the pertaining view of type 1 diabetes aetiology. In 2013 a consensus definition was decided to make results more coherent between studies and groups. This consensus definition was set as ≥15 CD45+ cells per islet in at least three islets[86]. This consensus definition separates type 1 diabetic pancreases from those of non-diabetic controls. However, type 2 diabetes has also been suggested to be adversely affected by islet inflammation.

- Characterise inflammation in type 2 diabetic pancreata.
- Evaluate if the consensus criteria for insulitis can distinguish between findings in type 1 and type 2 diabetes.

Paper II

The presence of T cells at the islet in T1D has been known for long. A few of these T cells have been shown to have an autoreactive T cell receptor (TCR)[96]. However, many clinical trials aimed at perturbating T cells responses have been unable to save β cells in the long term[107]. The knowledge of T cell subsets has increased over the past decade. One such subset is tissue resident memory T cells (T_{RM}). While central memory T cells recirculate through lymphoidal tissue and effector memory recirculate through non-lymphoidal tissue, resident memory T cells remain at the site of the initial response[108]. Mostly these cells are present in epithelial barrier tissues such as the intestines or airways.

- Investigate the presence of tissue resident memory T cells in islets from pancreata of non-diabetic controls and individuals recently diagnosed with type 1 diabetes.

Paper III

Loss of β cell mass is believed to occur for several years before diagnosis until normoglycemia can no longer be upheld and clinical symptoms appear[109]. We postulate that if the β cells, making up a major fraction of islets, die due to apoptosis then islets would decrease in size.

- Assess islet size and density in pancreata of subjects with recent-onset T1D, long-standing T1D and non-diabetic controls.

Paper IV

It is well known that glucose tolerance generally decreases with age and that the incidence of type 2 diabetes rises[110]. Previously studies have been undertaken to study this phenomenon in mice and in isolated human islets that have been digested into single cells. However, islet isolation, culture and dissociation could affect the transcriptomes. Laser capture microdissection (LCM) can be used to selectively analyse islet tissue without these steps.

- To find genes in islets that co-vary with age and could explain the age dependant decline in islet function.

Research Design and Methods

In this section are general considerations on the methods used throughout this thesis. For specific methods please read the respective material and methods sections in papers I-IV

Biopsy material from type 1 diabetic pancreata

Since the literature review by In't Veld[82], listing 150 cases of insulitis throughout the last century, several ambitious endeavours have been undertaken, such as nPOD and DiViD to increase our knowledge of what happens inside the pancreas prior to and in connection with T1D onset.

They represent two different ways of acquiring this material. The Network for Pancreatic Organ Donors (nPOD)[111] is set up to collect pancreases from organ donors that have type 1 diabetes or screen positive for autoantibodies. Organ donors still have beating hearts and as such are to be preferred over material gathered at autopsy, such as the Foulis material[112]. However, organ donors are not healthy – prerequisites include being intubated before brain death sets in. As such, many donors have had critical, life-threatening conditions and been subjected to treatment at intensive care units.

The Diabetes Virus Detection study (DiViD) was instead set up to acquire biopsies from living type 1 diabetic patients 3-9 weeks after diagnosis through laparoscopic minimal tail resection of the pancreas[113]. While avoiding the disadvantages of organ donor material, the procedure proved to yield unexpected adverse events, such as post-operative bleeding and pancreatic leakage. Due to this the study was aborted after 6 patients had been included. Material from these 6 donors have been used in Paper II and III.

All four papers have utilised material procured through the Nordic Network for Islet Transplantation. This biobank collects tissue from organ donor pancreata mainly from non-diabetics that are received with the intention of islet isolation and subsequent transplantation. From each pancreas received, biopsies are saved in the biobank. In the case of type 1 or type 2 diabetics additional biopsies are taken or islets isolated for research purposes.

Visualisation of protein expression – Immunohistochemistry, Immunofluorescence and the Opal Polaris platform.

Throughout paper I, II and III different methods of visualising protein expression in pancreatic sections have been used. All of them rely on the binding of primary antibodies to the protein being interrogated. In many cases, secondary antibodies targeting the primary antibody is used to amplify the signal. Both of these need to be evaluated for optimal concentration, incubation time and temperature to achieve true staining with minimal background. This is often done using positive control tissue, where the protein is known to be expressed. Negative controls can be tissues where the opposite is true, however a true negative control is only achievable through removing protein expression in the tissue being investigated, for instance through the use of knockout mice.

Blocking is performed before antibody incubation to minimize unspecific binding and binding to Fc-receptors, this can be done with specific blocking agents, sera or even milk. When using secondary antibodies, blocking is usually performed with sera from the same species.

Immunohistochemistry is the oldest of the techniques used herein. It is based on the secondary antibodies carrying an enzyme that can cleave a substrate, leaving a permanent stain on the section. The most commonly used combination is horseradish peroxidase (HRP) and diaminobenzidine (DAB). This is lastly combined with a counterstain e.g. haematoxylin, to visualise non-stained tissue for analysis in a bright-field microscope.

Immunofluorescence instead utilises fluorophores, molecules that can be excited by light at one wavelength and emit light at another wavelength. This allows for simultaneous detection of more proteins on a single section. The technique is limited by overlap in excitation and emission spectrum of different fluorophores and more advanced fluorescence or confocal microscopes needs to be used to distinguish between signals. Fluorophores also have the advantage of being able to be conjugated easily to either primary or secondary antibodies. In immunofluorescence, nuclear stains such as DAPI or Sytox Orange usually serve as the counter-stain.

The Opal Polaris platform is a combination of both these methods. In essence it is an immunohistochemical protocol with the exception that the substrate is a fluorophore binding covalently to the tissue. To visualize more than one epitope, the previous antibodies are removed through microwave treatment, while the fluorophore remains. This can be repeated up to 5 times for simultaneous analysis of 6 proteins in a single section. Visualising more antigens in the same section allows for deeper phenotyping of cells, analysing multiple surface markers or transcription factors in one cell type. Other novel methods for multiplex tissue analysis is imaging mass cytometry and the Akoya CODEX platform. Imaging mass cytometry utilises heavy-metal conjugated antibodies and laser ablation of the tissue coupled with detection in a

mass spectrometer. For this method, the limitations are time required for analysis and resolution, as tissue must be incinerated pixel-by-pixel. However, thanks to the incredibly narrow spectrums of heavy metals, there is no bleed between channels and theoretically all markers can be individually identified in the same cell. The CODEX platform utilises oligonucleotides barcodes conjugated to antibodies. Detection is performed of three antibodies/barcodes at a time through hybridisation with fluorescently labelled detector sequences. However, at the end these of analyses, only the digital image remains.
These novel technologies promise a future where even more secrets can be elucidated from tissue sections. However, as automation of analysis becomes a necessity, the importance of optimising e.g. antibody clones and incubation conditions increase exponentially. Inherent disadvantages of antibody-based detection such as non-specific staining remains.

Laser Capture Microdissection

Laser capture microdissection (LCM) is a technology whereby specific regions of tissue sections are extracted for subsequent analysis, e.g of the transcriptome. This allows for analysis of selected cells without the risk of changing them through the process of tissue dissociation, which is necessary for flow cytometric cell sorting of select cells.

In paper II and III, we have used the Arcturus platform. Frozen sections are placed on PEN membranes and kept at -80 C. Sections are dehydrated in increasing alcohol concentration before they are put in the machine. Islets are then identified through autofluorescence using excitation with light at a green wavelength and emission caught by a long-pass filter above 515nm. Islets were then dissected from surrounding exocrine tissue using a UV laser. Finally, an IR-laser is used to melt the plastic of a collection cap down onto the section. The cap is removed from the section, bringing with it the dissected tissue.

As the work is done on non-fixed frozen sections, RNA is at risk of degradation during the LCM procedure. As such, swift execution is of the essence.

Transcriptomics – PCR array and AmpliSeq analysis

There are different ways of analysing RNA levels, ranging from qPCR of single genes to whole genome transcriptomics. In **paper II** a qPCR array was used. In **paper IV** AmpliSeq was used.

The array performed in **paper II** was done using a designed panel by Qiagen, focused on pathways of an inflammatory immune response. RNA was prepared followed by cDNA synthesis and pre-amplification of the 84 genes. Finally, expression levels of the pre-amplified genes were analysed by qPCR. Readout is fluorescence from finished transcripts after each cycle. Normaliza-

tion is done with house-keeping genes, expressed at similar levels in all samples, to remove variation caused by varying levels of genetic starting material in different samples.

In **paper IV**, a whole transcriptome analysis was used, differing on a few main points from the protocol in **paper II**. Briefly, cDNA was pre-amplified using AmpliSeq kit containing primers encompassing the entire transcriptome. The prepared amplicons are referred to as a library. These are then conjugated to wells on a chip. Each nucleotide sequence is sequenced using next-generation sequencing, utilising fluorescent nucleotides and detecting each incorporation. Readout is sequences from all amplicons on the chip. These can be aligned to the genome and finally converted into raw counts. There are several important steps to analyse this massive amount of data. Filtering is the step where genes expressed at low levels, or in only a few samples, are removed as it will not be possible to extract important information from them. Normalisation has to take into account, not only the library size (the total amount of reads in sample) but also the library composition. For instance, if a set of genes are very highly expressed in some samples, they make up a greater proportion of the library and thus skew the relative expression of other genes downward. To combat this, normalisation is performed against genes whose average expression is similar in most samples. This then yields a conversion factor for each sample that all genes are multiplied with.

After significance testing of, in the case of **paper IV** 14.794 genes, many genes reach a *p*-value of 0.05 or below. However, after correcting for multiple comparisons not many are left. This leads to a high degree of type II errors, as many truly significant differences are discarded. The Benjamini-Hochberg method of determining false-discovery rate (FDR) uses the assumption that all non-differentially expressed genes will have *p*-values evenly distributed from 0 to 1. While true positives will always be skewed towards 0. Choosing genes with an FDR-adjusted *p*-value of <0.05 then means 5% are false positives.

In order to make sense of the hundreds or thousands of supposedly true positives one can use pathway enrichment analysis. Basically, interpreting a long list of genes into a shorter list of pathways. While there are many different softwares and pathway databases to use, the ones used in **paper IV**, GSEA[114] and g:profiler[115], represents two different methods. Comparing either all genes or a list of genes passing a significance threshold, respectively. In g:profiler, a list of genes deemed to be the most differentially expressed, e.g by a threshold of FDR-adjusted *p*-value and fold change, are used as input. The list is then compared to the pathways of choice in order to find pathways in which the genes of interest are over-represented (i.e. enriched). In GSEA, all genes are ranked based upon a chosen metric, for instance -log10(*p*-value) multiplied by the sign of log fold change. Starting at the highest ranked gene, the pathways enrichment score (ES) increases if the gene is a part of the path-

way and decreases otherwise. The final sum of the ES is then normalized relative to pathway size, yielding a normalized enrichment score (NES). P-values and false-discovery rates are calculated through re-running the analyses with permutated gene sets. In the "leading edge" one can then identify the genes that contributes most to the ES. As there is often considerable redundancy between pathways, software such as Enrichment Map[116] can be used to visualize this overlap and aid in interpretation.

Results and Discussion

Paper I – Insulits in Human Diabetes: a Histological Evaluation of Donor Pancreases

Current definition of islet inflammation is not specific for type 1 diabetes

Using the consensus definition ≥15 CD45+ cells in more than 3 islets, 14 out of 50 (28%) type 2 diabetic donors fulfilled the criterion. The mean frequency for insulitis in these donors was 2.8%, a number comparable to a study in type 1 diabetics[84]. In the consensus definition CD45 was used as a marker in order to reliably diagnose insulitis even when a significant proportion of immune cells are not T cells, such as shown in children when B cells make up the majority of the insulitic infiltrate[90]. The limit of 15 CD45+ cells per islet was used to increase specificity against the level of inflammation that could be seen in the non-diabetic pancreas. As for instance, inflammation in non-diabetic pancreata generally increase with time in intensive care[42]. This limit held true in this material as well as only 1 out of 2130 islets had ≥ 15 CD45+ cells per islet. However, the insulitis definition was not validated against type 2 diabetics. In this paper we show that the current definition for islet infiltration cannot differentiate between T1D and T2D. This only worsened when the old, less stringent criterion ≥5 CD45+ cells in ≥3 islets was used. 42/50 donors with T2D and 10/44 non-diabetic donors fulfilled the criterion, illustrating the need for a consensus definition.

In the T2D pancreata, most immune cells were not preferentially located near the islets. Instead, several were classified as insulitic due to immune cells scattered in the surrounding exocrine parenchyme or in some cases, in the surrounding fibrosis. The most frequent cell type in the immune infiltrate were CD68+ cells at 102 per mm^2, interpreted as macrophages, followed by MPO+ neutrophils and CD3+ T cells at 49 and 20 cells per mm^2, respectively. CD20+ B cells were only rarely found. Even when not exacerbated by surrounding inflammation, islet-associated macrophages have been seen to be increased in type 2 diabetes[117,118]. T cells were however less prevalent in our material. The highest number of CD3+ cells in or adjacent to an islet was 18, however apart from that outlier, multiple islets had 6-10 CD3+ islet-associated cells. To dif-

fer between the infiltrate seen in T1D and T2D we therefore suggest a threshold of ≥15 CD3+ cells per islet. As a single section can contain thousands of islets we suggest that "≥3 islets" is an arbitrary number. Instead, we suggest that in sections containing more than 100 islets, a threshold of ≥3% of islets should be used. Both acute onset donors in this material fulfilled our new modified criteria, as well as recent onset T1D donors in another study[87]. Neither long-standing T1D donors or T2D donors fulfilled the modified criteria.

Consequence of islet inflammation in T2D

Islet inflammation has been suggested as an important detrimental factor in T2D[119]. Among all islets in the insulitic T2D donors, only a few were insulin-deficient or contained only a few β cells. However, in 5/14 HLA class I hyperstaining was seen in at least one islet. This pattern of hyperstaining has been a defining feature of T1D, however it is still unclear if it is due to increased transcription, a reduced turnover or cross-reactive proteins being produced by the β cells[120,121]. As in T1D[120], HLA class I hyperstaining in T2D was present in islets with and without insulitis.

Glucose-stimulated insulin secretion was perturbed in isolated islets of T2D donors compared to non-diabetic controls. But there was no difference between donors with and without insulitis. Neither was there any difference in BMI or in HbA1c. However, the analysed islets may or may not be affected by insulitis and BMI and HbA1c are affected by other factors, such as diabetes treatment.

Importance of a consensus definition

The clear presence of insulin-deficient islets easily allows for distinguishing a T1D from a T2D pancreas. However, as we have shown, the current criteria for immune cell infiltration does not. Insulitis is considered an important morphological hallmark of T1D. Exocrine inflammation is increased in T1D as well as in T2D[104]. Under the current definition, a similar level of diffuse immune cell infiltration could be classified as insulitis, simply by the co-occurrence of insulin-deficient islets. The importance of a sensitive and specific consensus definition will increase as more and more image analysis is automated.

Paper II – Demonstration of Tissue Resident Memory CD8 T cells in Insulitic Lesions in Adult Patient with Recent-Onset Type 1 Diabetes

CD8 T cells in the insulitic lesion coexpress the tissue residency markers CD69 and CD103

Consistent with previous studies CD8+ T cells were more numerous in islets containing insulin and preferentially located in the endocrine-exocrine interface[84,87]. CD8 T cells coexpressing the markers for tissue resident memory (T_{RM}) cells, CD69 and CD103, were also preferentially located at this site. On average CD8+CD69+CD103+ cells made up 43% of all CD8+ cells per islet. While the number varied greatly between islets and donors, cells with the T_{RM} phenotype were present in 35 of 37 (94,6%) islets containing at least one CD8+ T cell. Though the number of CD8+ cells differed between insulin-containing and insulin-deficient islets, the proportion of CD8+CD69+CD103+ cells did not. Another T cell population capable of tissue residency, γδ T cells, were found only very rarely in the pancreas.

In T_{RM} cells CD69 blocks S1P1, a receptor for sphingosine-1-p hosphate and mediator of tissue egress, leading to cell retention in the tissue. CD69 is however most well known as a marker of T cell activation[122]. Curiously, in the insulitic lesions of recent onset T1D patients, no CD8+ T cells were CD69+ without also expressing the other residency marker CD103. This suggests that the non-T_{RM} cells in the lesion are not conventionally activated cytotoxic T cells. In support of this, CD8+CD69+CD103+ cells were also found in islets of non-diabetic organ donors, making up a majority of islet-associated CD8 T cells in most islets, indicating that this population is not an artifact of T cell activation. This also corroborates earlier findings that CD8+CD69+CD103+ cells are present in association with islets in non-diabetics[123].

Insulitic islets lacks gene expression indicative of a conventional cytotoxic response

Insulitic islet sections were captured using laser microdissection and analysed with an array for expression of 84 genes associated with inflammation of autoimmunity. 19 of these were not even detected. Surprisingly, among the genes not detected were several associated with activated cytotoxic T cells (*IL2R, CD40LG, FASLG*) and with an acute cytotoxic response (*TNF, CCR7, LTA, IL1A*). This is in line with a previous study where the gene expression in insulitis differed greatly when compared to the acute cytotoxic response of graft rejection[87]. Several genes connected with inflammation and the innate immune system (*IFNG*, *IL15*, *IL18* and *IL22*) and several chemokines

(*CXCL1, CXCL9, CXCL10, CCL3, CCL4, CCL5, CCL7, CCL11* and *CCL19*) were upregulated in T1D donors compared to non-diabetic donors. It is impossible to infer with certainty what part of this expression pattern can be attributed to T_{RM} cells. For instance, CCL5 has been seen to be expressed in islets in when peri-insulitis is present[124]. However, several of these cytokines and chemokines have been seen to be released by T_{RM} (IFN-γ, IL-22, CCL3, CCL4) or by the local environment after T_{RM} activation (CCL5, CXCL9, CXCL10)[125,126].

Several theories have been put forward for pathogens such as viruses or bacteria as causative agents in type 1 diabetes[127,128]. The presence of T_{RM} cells could be a result of a previous pathogenic insult. In case of a repeat insult T_{RM} cells could recruit more T cells. Curiously, IL-15 and IL-18 that were upregulated in insulitic islets have been known to induce bystander inflammation, an innate-like TCR-independent activation of T cells[129,130].

Paper III – Characterisation of the Endocrine Pancreas in Type 1 Diabetes: Islet Size is Maintained but Islet Number is Markedly Reduced

Type 1 diabetic islets – Loss of β cells but preserved architecture

In non-diabetic donors ≥60% of the endocrine area stained for insulin in 97% (92-100%) of islets. In subjects with recent-onset disease, 13% (3.0-40%) stained for ≥60% insulin area. 13% (5.1-22%) stained for 20-60% insulin. 74% stained for ≤20% insulin containing area. The T1D donors with long-standing disease had no insulin-containing islets but in 5 out 7, single insulin-positive cells were found scattered throughout the exocrine parenchyma. Despite the loss of β cells in most islets of the subjects with recent-onset T1D and all islets of those with long-standing disease, most islets maintained their architecture without areas of fibrosis or necrosis corresponding to the proposedly lost β cells. Using circularity, a measure of the roundness of an object compared to a perfect circle which equals 1, there was a tendency to lower circularity in recent-onset disease, however this did not reach statistical signifance.

Islet size is maintained, but islet number is reduced

Diabetic islets contained no trace of the β cell loss. The median of the mean in each donor was 4813 (1673-11 807) μm^2 in control donors as compared to 6127 (3623-6721) μm^2 in recent-onset T1D and 3678 (2569-4608) μm^2 in long-standing T1D. Corresponding to a diameter of 78, 88 and 66 μm^2, respectively, for a circle with the same area. While islets from the recent-onset

T1D subjects were significantly larger than from subjects with long-standing T1D ($p<0.05$), there was no statistically significant between the control donors and any of the T1D groups. In order to confirm that there was no size difference between the islet populations, an exponential curve ($y = a * e^{-bx}$) was fitted to the islet size distribution, in the form of mean number of islets per mm^2 per size category in each donor. There was no statistically significant difference when comparing the slope coefficient *b* between groups. However, the constant *a* differed greatly between controls and T1D ($a= 9$ in control donors, 2.5 in recent-onset T1D subjects and 2.8 in long-standing T1D donors). This reflected the significantly lower median islet density in the recent onset T1D (1.7 islet per mm^2, range 1.3-1.8) and long-standing T1D donors (1.8 islet per mm^2, range 0.6-2.4) compared to the non-diabetic control donors (4.4 islet per mm^2, range 3.4-7.7) ($p<0.005$).

Reduced islet density was seen as early as in the 1965 Gepts paper[83]. However, the cause of this is unknown. There are two possible scenarios. Either whole islets have perished, or they were never formed. While it cannot be excluded, it would be farfetched to assume that the mild peri-insulitic infiltration seen in T1D would lead to destruction of entire islets. Instead, a near ubiquitous finding in T1D pancreata is periductal fibrosis[105]. As islets have been suggested to stem from the ductal epithelium, damage to this locale might preclude further islet neogenesis.

α cells in type 1 diabetic pancreata express β cell marker PDX1

In this patient material, islet size is maintained in T1D and most islets preferentially contain glucagon. This could be due to a compensatory expansion of the α cells however no such findings have been published in human diabetes. Contrarily, β cell dedifferentiation has been seen in several contexts of β cell stress. Dissociation and reaggregation of islets leads to β cells becoming glucagon producing while retaining the β cell-specific transcription factors PDX1 and NKX6.1[131]. Similarly, expression of PDX1 and NKX6.1 mRNA is maintained in T1D islets despite the apparent loss of β cells, instead the expression of α cell-specific transcription factors MAFA and ARX is decreased[132]. In long-standing type 1 diabetics, many islets have been found to produce very low amounts of insulin, while also containing PDX1 and NKX6.1[24].

We can confirm that PDX1 is expressed in glucagon-containing cells in both recent onset and longstanding T1D, however to a greater degree in the former.

β cell dedifferentiation due to deficiency in islet numbers and β cell mass?

Type 1 diabetes incidence is highest during the period of life when insulin demand increases the most. If the reduced islet numbers seen in this material is a result of a deficiency in islet neogenesis and inability to establish an adequate β cell mass, perhaps due to periductal fibrosis, it could constitute the necessary stressor to cause β cells to dedifferentiate. If β cells dedifferentiate rather than cease to exist, this would explain the maintained islet size seen in type 1 diabetics.

Paper IV – Transcriptional analysis of islets of Langerhans from Organ Donors of Different Ages

Comparison of islets of different ages

The islets compared came from organ donors between 1-81 years of age, divided into age groups: Children (1-12 years), Adolescents (13-18 years), Young adults (21-35 years), Middle-aged (50-63 years) and Elderly (72-81 years).

Isolated islets donors from Young adults, Middle-aged and Elderly groups exhibited similar absolute levels of insulin secretion when stimulated with 20mM of glucose, however due to higher basal insulin secretion at 1.67mM, the oldest donors exhibited a reduced dynamic stimulation index.

Whole transcriptome analysis of islets

14,794 genes were expressed at a high enough level (≥10 counts per million) in enough samples (≥5) to pass filtering and be included in the remaining analyses. Based on the expression of these genes there were no clustering according to donor age in a principal component analysis or a hierarchical clustering. Suggesting that interindividual differences outweigh any age-related global changes in the islet transcriptome.

However, using a generalised linear model with age as the continuous variable, 383 genes were found to co-vary with age, 276 were upregulated and 107 downregulated with increasing age (FDR<10%). A study by Arda et al found 567 genes that differed between children and adults[33]. 344 of those genes passed filtering in our analysis, 39 of which co-varied with age in our material, 36 that co-varied in the same direction. This discrepancy between the studies could be due to differences in study design. In this study laser capture microdissection was used, avoiding any changes in transcriptional profile during islet isolation, culture and dissociation. In a paper by Solimena et al it

was shown that samples cluster based on the isolation method. Such as that two samples from the same individual would cluster with the respective isolation method, LCM or enzymatic isolation, rather than together[133].

Pathway enrichment analysis unravels decreased cell cycle activity

Gene set enrichment analysis identified 318 gene sets that correlated significantly with age, 158 were upregulated and 161 downregulated with increasing age (FDR<10%). Downregulated gene sets primarily concerned different aspects of mitosis and the cell cycle. A functional enrichment analysis using only genes significantly altered by age mirrored these results. This was corroborated by age-related increase in cell-cycle inhibitors p16, p21 and p57 (*CDKN2A, CDKN1A* and *CDKN1C*, respectively). Especially expression of *CDKN2A* has been connected to the aging β cell[33,134].

The age-related genes and diabetes

As T2D incidence increases with age, it is highly interesting to ascertain if the aging islets resemble T2D. Data from six studies on the T2D transcriptome were used and genes overlapping at least two data sets were chosen to increase validity, resulting in 216 genes downregulated and 60 upregulated in type 2 diabetes. Seven of the downregulated genes and five of the upregulated genes co-varied in our material. Notably, 2 genes that were seen to be downregulated in T2D were upregulated with age. Only 12 T2D-associated genes were differentially expressed in this material. This would suggest that the general age-related changes to the islet transcriptome is not an underlying driving force of T2D.

Senescence-associated secretory phenotype (SASP) has been suggested as a possible cause of loss of β cells in both T1D and T2D[34,135]. Apart from expression of senescence markers *CDKN2A* and *CDKN1A*, it is also associated with secretion of *SERPINE1, MMP2, IL6, CCL4, IL1A, CXCL10*. While aging islets expressed the senescence markers, only *SERPINE1* expression was positively correlated to increasing age. The remaining genes were not correlated to age, indeed, apart from *MMP2* they did not even pass filtering.

T1D incidence is highest in childhood and adolescence. In adulthood, the diabetes onset can come slowly, often initially misinterpreted as T2D. Curiously, among the highest differentially expressed genes were *SPP1*, coding for osteopontin, a protein that has been linked both to protection from IL1β-mediated cytotoxicity as well as hyperglycemia[136,137].

Conclusions

Paper I

- The current consensus definition of insulitis (≥15 CD45+ in ≥3 islets) cannot sufficiently discriminate between the immune cell infiltration around islets in type 1 and type 2 diabetes.
- Pancreatic inflammation is common in type 2 diabetic pancreata and made up predominantly of macrophages.
- As exocrine inflammation is also prevalent in type 1 diabetes, we suggest a new definition of insulitis ≥15 CD3+ in ≥3% of islets which has now been validated to not include type 2 diabetics.

Paper II

- Tissue resident memory T cells are present in association with islets in both type 1 diabetes and in the non-diabetic pancreas. They make up a substantial proportion of T cells in the insulitic lesion in type 1 diabetes. Their presence in the pancreas suggests a possible role in protecting against pathogens.
- Expression of genes associated with an acute cytotoxic response are not detected in the insulitic islets. Instead genes associated with an innate immune response were upregulated.

Paper III

- Islets do not decrease in size in type 1 diabetes, despite a presumed loss of a majority of the cells. The insulin-deficient islets are mostly made up of alpha-cells.
- Islet density is markedly reduced already at type 1 diabetes onset. This in conjunction with lower pancreatic weight suggest a significantly lower β cell mass prior to disease onset.
- α cells in both recent-onset and to a lesser degree in long standing type 1 diabetes express the β cell marker PDX1. This could indicate a transdifferentiation of β cells into glucagon-producing cells.

Paper IV

- As seen in previous studies of islets from enzymatic digestion, markers of senescence such as *CDKN2A* were upregulated and pathways concerning the cell cycle machinery were downregulated with increasing age.
- There was only a small overlap between our study of laser capture microdissected islets and a study on dissociated islet cells.
- Among the novel genes found to increase significantly by age was *SPP1*, coding for Osteopontin. A protein that has been previously linked to protection from cytotoxicity and hyperglycaemia.

Comments and Future perspectives

Insulitis – the importance of getting it right

Insulitis, the inflammation of islets, is seen as an important morphological hallmark of type 1 diabetes. In **paper I** we showed that the consensus definition for insulitis cannot reassuringly differentiate between the immune cell infiltration in type 1 and type 2 diabetes. In a comment on our paper, the original authors of the consensus definition stress the importance of pseudoatrophic islets, in order to differentiate between immune cell infiltrate in T1D from the general inflammation seen in chronic pancreatitis or T2D[138]. However, as exocrine inflammation is also seen in T1D[16,104], this feature becomes useless if one needs to discern islet specific inflammation from general inflammation in the type 1 diabetic pancreata.

While the insulitic immune cell infiltrate is present at the islet, it is most often present outside the islet in the form of peri-insulitis. No clear proof of T-cell mediated killing of β cells has been seen. We do not yet know the role of insulitis in T1D. As automated image analysis becomes more powerful and prevalent, it will be important to remember, that not all islets surrounded by immune cells necessarily have insulitis.

The alternative history of T1D

The so-called *precipitating event* initiating islet autoimmunity often happens early in life, as seen in at-risk children who seroconvert before 2 years of age[50]. This is an important period in the postnatal development of the endocrine pancreas[30]. As patients with T1D also exhibit autoantibodies against the exocrine tissue it is possible that this *precipitating event* also affects the exocrine pancreas[79,80]. This could be the origin of the lower pancreatic weight seen in type 1 diabetics[7]. As well as the cause of lower islet density as seen in **paper III** and by others[78]. Lower islet density must also be further regarded in the context of reduction in pancreatic weight seen in T1D, further reducing the total number of islets.

The nature of this *precipitating event* is unknown. However, both viruses and bacteria have been suggested[127,128]. Tissue-resident memory T cells are usually present in barrier tissues such as skin or airway epithelium, as gatekeepers prepared to respond immediately to re-infection[139]. In **paper II** we

found these T_{RM} cells in association with islets in non-diabetic pancreata, but to a greater degree in islets of the T1D pancreata. Recently, it was shown that bacteria reside inside the pancreatic duct, with inter-individual differences in bacterial profiles but with similarities with the duodenal microbiome in the same individual, often containing bacteria commonly recognised as part of the oral flora[140]. Curiously, HLA allele combinations that confer susceptibility to T1D, the greatest genetic risk factor by far, are associated with a significant difference in the bacterial profile of the microbiome in stool samples[141]. Disruption of the pancreatic microbiome could lead to ductal inflammation and subsequently give rise to the periductal fibrosis seen in T1D.

If the *precipitating event* leads to pancreatic damage and an inability to establish an adequate β cell mass, as the individual grows and insulin demand increases, this would eventually reach the point where β cells struggle to maintain glucose homeostasis. This would lead to β cell stress, which in turn can lead to production of cytokines and immune cell-recruiting chemokines such as IL-1β and CXCL9, CXCL10 and CXCL11[100,101]. In this scenario the β cells themselves would be responsible for initiation of insulitis. Alternatively, reactivation of T_{RM} could lead to the recruitment of an immune cell infiltrate. However, in **paper II** we found no CD8+CD69+ cells that were not classified as tissue resident memory cells in subjects recently diagnosed with T1D, suggesting that the resulting infiltrate did not consist of cytotoxic T cells activated by recognition of cognate antigen. This was corroborated by the lack of expression of genes associated with a cytotoxic response in the insulitic lesion of these subjects.

Later in the disease process, islets are found to be insulin-deficient. In **paper III** we could not detect any reduction in islet size corresponding to a loss of ~60% of the cells within, in line with data by Rodriguez-Calvo et al[78]. While a modest increase in β-cell apoptosis could be seen in a study of recent-onset T1D there was no correlation to the presence of insulitis[97]. However, when attempting to measure cell death by detection of cell-free DNA in blood, as can be done in islet transplantation or multiple sclerosis, no β cell DNA can be detected[142]. If the β cells do die, a maintained islet size can only be explained by either a significantly larger islet size to begin with, or a substantial expansion of α cells. None of which has been reported in the literature.

The remaining alternative is that β cells do not die, as much as simply cease being β cells. During T1D progression, β cells lose insulin expression while maintaining PDX1 and NKX6.1, markers of β cell identity[16]. Similarly, NKX6.1 has been found in α cells of subjects with T1D in conjunction with lower levels of ARX, an α cell transcription factor[132]. β cell markers in glucagon-producing cells has been implied to be due to a partial α-to-β transition in an attempt to compensate for β cell loss. However, dissociation of human islets can cause β cell degranulation and dedifferentiation into glucagon-producing cells expressing PDX1 and NKX6.1[131]. Our findings in **paper III** of

PDX1 expression inside α cells in recent-onset and long-standing T1D could represent loss of β cells through transdifferentiation.

If β cell stress is the cause of insulitis and the insulitis exacerbates this β cell stress, this would explain the findings in the recent clinical trial of teplizumab, an anti-CD3 antibody. Nondiabetic relatives of T1D patients, with at least two autoantibodies and dysglycaemia were included into the study. A delay in diagnosis was only seen in the participants with under the median levels of C-peptide production in an oral glucose tolerance test[143]. In other words, those with the highest levels of β cell stress, and hypothetically, with the highest frequency of insulitis. This would also explain the honeymoon phase after diagnosis and commencement of insulin therapy, another avenue by which to decrease ongoing β cell stress. Indeed, presence of IL-15 and IL-18 in the insulitic islet as seen in **paper II** could lead to bystander activation of T cells without recognition of cognate antigen.

Older age at T1D onset is associated with a slower decline in C-peptide after diagnosis but also to higher residual C-peptide levels five decades after diagnosis[59,144]. If T1D is a β cell-centric disease, then there could be two explanations for this. Either due to that the relative increase in insulin demand slows down with age or due to factors in the β cells themselves. In **paper IV** we identified an increased expression of *SPP1* coding for osteopontin with increasing age. Osteopontin has *in vitro* been shown to protect β cells from both hyperglycaemia as well as cytotoxicity[136,137]. And to be increased in islets from hyperglycaemic T2D donors[137]. Factors such as osteopontin could be an explanation as to how β cells do not succumb to stress in the same manner in T2D as T1D, despite sometimes significant hyperglycaemia.

Moving forward

All of the recently diagnosed T1D subjects analysed throughout this thesis contracted the disease in adulthood. It is possible that they represent a subgroup separate from those that contract the disease in childhood. The findings presented herein must therefore be confirmed in younger donors. This also highlights the greatest weakness in T1D research, the scarcity of human samples representing the progression of the disease. Luckily, most patients with T1D survive their diagnosis and as such, not many pancreases are available to teach us more about what happens at T1D onset. Technological innovations such as multiplex stainings, allowing for simultaneous visualisation of tens of markers and three dimensional stainings, allowing for morphologic description of entire islets and other structures, will enable us to extract more information from these rare specimens. Not only interrogating immune cells or β cells, but the entire pancreas and findings such as periductal fibrosis or islet volume. It will also allow us to re-examine old truths.

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