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Immunopathology of the Pancreas in Type 1 Diabetes

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Abstract

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Type 1 diabetes (T1D) results from a loss of functional insulin-producing pancreatic beta cells. The etiology of T1D is poorly understood, but the detection of infiltrating inflammatory cells in the pancreas and circulating autoantibodies has led to the common notion that an autoimmune process plays a central role in the pathogenesis of the disease.

The aim of this doctoral thesis was to assess various aspects of the immunopathology of type 1 diabetes. To this purpose, studies have been conducted on pancreatic material from the Network for Pancreatic Organ Donors with Diabetes (nPOD) collection, the Nordic Network for Islet Transplantation, and the Diabetes Virus Detection (DiViD) study.

Paper I is a study on pancreatic tissue from organ donors with varying duration of T1D as well as non-diabetic donors and subjects with other types of diabetes, in which persistent expression of glucose transporters was shown on the beta cell membrane despite several years of T1D. Glucose transporter 1 was also confirmed as the predominant glucose transporter on human pancreatic islets. In **paper II**, we report on signs of inflammation in the exocrine but not in the endocrine pancreas in non-diabetic organ donors with diabetes-related autoantibodies, suggesting that diabetes-associated autoantibodies can occur in response to unspecific pancreatic lesions.

Paper III aimed to characterize the T cell-infiltration of pancreatic islets in material from recent-onset T1D patients. Insulinitis was shown in all subjects, but with distinct differences in expression analysis of T- and B cell activation to cell-mediated allogeneic kidney transplant. Also **Paper IV** was conducted on material from recent-onset cases and showed increased islet glucagon content, in combination with a reduced number of islets but sustained mean islet size.

Together, these results provide expansion of our knowledge of the immunopathology in T1D, and will hopefully assist in bringing us towards a deeper understanding of T1D aetiology and eventually an effective cure.

Keywords: Type 1 diabetes, glucagon, T cells, autoantibodies, glucose transporters, islets of Langerhans, pancreas

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Life would be so wonderful if we only knew what to do with it.

- Greta Garbo

List of Papers

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

- I Coppieters, K T., **Wiberg, A.**, von Herrath, M. (2011) Persistent glucose transporter expression on pancreatic beta cells from longstanding type 1 diabetic individuals. *Diabetes/Metabolism Research and Reviews*, 27(8):746–54.
- II **Wiberg, A.**, Granstam, A., Ingvast, S., Härkönen, T., Knip, M., Korsgren, O., Skog, O. (2015) Characterization of human organ donors testing positive for type 1 diabetes-associated autoantibodies. Clinical and Experimental Immunology. *Clinical and Experimental Immunology*, 182(3):278-88.
- III *Krogvold, L., ***Wiberg, A.**, Edwin, B., Buanes, T., Jahnsen, F.L., Hanssen, K.F., Larsson, E., Korsgren, O., Skog, O., Dahl-Jørgensen, K. (2016) Insulinitis and characterisation of infiltrating T cells in surgical pancreatic tail resections from patients at onset of type 1 diabetes. *Diabetologia*, 59(3):492–501. *Authors contributed equally.
- IV **Wiberg, A.**, Krogvold, L., Jahnsen, F.L., Korsgren, O., Dahl-Jørgensen, K., Skog, O. Increased glucagon content in the endocrine pancreas in recent onset type 1 diabetes. Results from the DiViD study. *Manuscript in preparation*.

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Abbreviations

aAb+	Autoantibody-positive
ADP	Adenosine diphosphate
APCs	Antigen-presenting cells
ATP	Adenosine triphosphate
BMI	Body mass index
C-peptide	Connecting peptide
CD	Cluster of differentiation
CTL	Cytotoxic T cell
DiViD	Diabetes Virus Detection
ELISA	Enzyme-linked immunosorbant assay
FACS	Flourescence-activated cell sorting
GAD	Glutamic acid decarboxylase antibodies
GLUT	Glucose transporter
HbA1c	Glycated haemoglobin
HLA	Human leukocyte antigen
IA-2A	Insulin antigen-2 antibodies
IAA	Insulin autoantibodies
ICA	Insulin autoantibodies
ICI	Insulin-containing islets
IDI	Insulin-deficient islets
IHC	Immunohistochemistry
Km	Michaelis constant
LADA	Late onset autoimmune diabetes of adults
LCM	Laser capture microdissection
MODY	Maturity Onset Diabetes in Youth
mRNA	messenger RNA
NOD	Non-obese diabetic
nPOD	Network for Pancreatic Organ Donors with Diabetes
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
T1D	Type 1 diabetes
T2D	Type 2 diabetes
WHO	World health organization
ZnT8A	Zinc transporter 8-antibody

Introduction

Type 1 Diabetes (T1D) is caused by insufficient insulin secretion from the beta cells in the pancreatic islets of Langerhans, which leads to inadequate blood glucose control in the afflicted patient. The impairment of beta cells is commonly regarded as a consequence of inflammatory cells infiltrating the islets and destroying the beta cells.

It is not yet established what causes the beta cell destruction and the auto-reactive immune response, but epidemiological studies strongly suggest a crucial role for one or more environmental factors. In support of this notion is the steep global rise in disease incidence that has been reported during the past decades, foremost among children younger than five years of age.

Mouse models have shown to be of insufficient significance to human T1D, rendering studies on human material important for the understanding of the disease.

Clinical perspective

T1D is one of the most common chronic diseases in children, with the highest incidence seen between 5-7 years of age and just before puberty (1). However, the disease can be diagnosed at any age, and up to 50% are 18 years or older at the onset of symptoms (2). The clinical onset is characterized by hyperglycemia, typically resulting in a triad of symptoms including polydipsia, polyuria and polyphagia. Untreated, T1D eventually leads to ketoacidosis and death.

According to the World Health Organization (WHO) guidelines, the diagnosis of diabetes mellitus can be based on repeatedly elevated plasma glucose levels during fasting or two hours after an oral glucose tolerance test (OGTT). Added recently, also glycated haemoglobin (HbA1c), which reflects average plasma glucose over the previous eight to 12 weeks, can be used as a diagnostic test for diabetes (3,4).

T1D represents 5-10% of the total cases of diabetes worldwide (5). The presence of diabetes-related autoantibodies, normal BMI, acute onset of symptoms, early insulin dependence and reduced or non-detectable connecting peptide (C-peptide) levels in a patient indicate T1D, as opposed to type 2 diabetes (T2D) which constitutes the majority of diabetes cases and is connected to higher age, obesity and peripheral insulin resistance (5). Late onset

autoimmune diabetes of adults (LADA) is characterized by slow adult onset diabetes with similarities to T1D, and is often seen as a continuum of T1D rather than a separate disease (6). More unusual forms of diabetes mellitus include secondary diabetes, gestational diabetes and rare monogenic forms such as Maturity Onset Diabetes in Youth (MODY). The most common form of secondary diabetes is pancreatogenic diabetes, often caused by alcohol-induced pancreatitis (7). T1D is connected to increased risk of developing other autoimmune diseases, primarily coeliac disease and autoimmune thyroiditis (8,9).

Up to 60% of T1D patients go through a partial remission phase, also called the 'honeymoon phase' after insulin treatment has been initiated (10). The occurrence of this phase increases with age at onset. The partial remission phase is characterized by reduced insulin requirement (< 0.5 units/kg), often lasts for three to six months, and has gained interest from a therapeutic perspective (11).

Despite intensive research, there is to this day no curative treatment for T1D. Insulin was detected and introduced as a therapy for diabetes in 1922 by Banting, Best and Collip, and daily administrations of insulin is still today the only available therapy for T1D. Even with well-controlled insulin administration complications including retinopathy, nephropathy and neuropathy are common, and the average quality of life and life expectancy is still significantly decreased (12–15).

Epidemiology

The prevalence of T1D in Sweden is 0.5%, with women and men affected equally. There is a great variation in T1D incidence and prevalence between countries, where Finland followed by Sweden and Sardinia have the highest incidences, while China and South America have the lowest (16). Interestingly, the discrepancy in T1D incidence may be large even between countries with geographical proximity and similar population genetics, like Finland and Estonia (17). Furthermore, migration studies have reported on increased T1D risk when children move to a country with higher T1D incidence (18). A seasonal variation has been noted, with highest incidence in autumn and winter, but also related to the month of birth (19,20).

Of yet unknown reasons, the incidence of T1D has increased worldwide during the last decades, foremost among children under the age of five (21). In addition, the proportion of T1D patients with high-risk susceptibility genotypes have decreased (22,23). During the 2000's, a plateau in incidence has been reported both in studies from Sweden and other countries (24,25). Other studies, however, continue to show an increase in incidence (1), and the significance of shorter periods of time when T1D incidence seems to be leveling off has been debated (26).

The Pancreas

The pancreas is an unpaired gland of about 15 cm of length located posteriorly in the abdominal cavity, with an average weight of 68 g (27). It is anatomically divided into three parts; caput, corpus and cauda (head, body and tail), which are further composed of small lobules measuring 1-10 mm (28). Histologically and functionally, the pancreas is instead divided into exocrine and endocrine tissue. The endocrine tissue is made up by hormone-producing cells forming islet-like clusters that are scattered throughout the organ. The islets have an average diameter of 150 μm (29,30) and are estimated to constitute approximately 1-2 % of the pancreatic mass. The beta cell mass does, however, vary greatly between subjects, ranging from 0.4 to 4% of the pancreatic weight (31). The exocrine pancreas consists of acinar, centroacinar and ductal cells which together comprise 90% of the pancreatic mass, while blood vessels, connective tissue stroma and nerves make up the remaining 8-9%. A thin layer of loose connective tissue forms a septa that divides the gland into lobules (32). The acinar cells produce digestive enzymes and bicarbonate (the 'pancreatic juice') that flow via the pancreatic duct, mixes with bile and is distributed through the papilla of Vateri into the duodenum. While some studies report that the islet volume density is similar between the different parts of the pancreas (33,34), other studies show an uneven distribution of islets, with a more than two-fold higher islet count in the tail as compared to the pancreatic head and body (35-37).

The islets of Langerhans

The endocrine cells form so called 'pancreatic islets' or 'islets of Langerhans', named after Paul Langerhans who in 1869 was first to describe them. Each islet is surrounded by an incomplete fibrous capsule (38). The islets consist of five major endocrine cell types: insulin-producing beta cells (50-60%), glucagon-producing alpha cells (30-40%), and to a lesser extent somatostatin-producing delta cells, pancreatic polypeptide-secreting PP cells and ghrelin-producing epsilon cells. There has been some debate regarding the arrangement of different cell types in the islets, which differs significantly from rodents (39). A recent morphological study on the human pancreas proposed that the islet cells are organized into a trilaminar plate, where one layer of beta cells is sandwiched between two alpha cell layers. The structure is folded with vessels circulating along both sides (30). The cellular composition remains similar throughout the pancreas, with the exception of the PP cell rich uncinata process (40). Efferent venules from the islets are emptying into exocrine capillary networks or collecting venules (28). Both the innervation and vascularization of human islets has a density similar to the exocrine tissue, and significantly less than mouse islets (41,42). Replication of beta

cells peaks at two years of age, and is only seen rarely after the age of ten (43).

Insulin has a strong hypoglycemic action and is essential for glucose uptake. After ingestion of a carbohydrate meal, glucose is likely transported into the beta cells via glucose transporter 1 (GLUT1) (44). Inside the beta cell, the enzyme glucokinase phosphorylates glucose to glucose-6-phosphatase. Glucokinase has a relatively low affinity (high K_m) for glucose and is thereby the rate-limiting step in the glucose-stimulated insulin secretion. This and following steps in the glycolysis lead to increased ATP:ADP ratio, and thereby elevated cytoplasmic potassium- and subsequently calcium levels. The elevated calcium levels cause insulin granules to be transported to the cell membrane and release insulin into the blood stream in a pulsatile manner (45). Insulin is derived from its precursor proinsulin, which splits into the insulin A- and B-chain and the C-chain (connecting peptide) (46).

Once released, insulin binds to the insulin receptor which is present on the outer membrane of e.g. muscle cells and adipose tissue. This interaction upregulates the GLUT4 expression on the cell membrane, which facilitates the uptake of circulating glucose into the cell.

In the fasting state on the other hand, counterregulatory hyperglycemic hormones are secreted in order to evade hypoglycemia. Glucagon plays a key role in this group that also contains epinephrine, norepinephrine, cortisol and growth hormone. Glucagon increases during hypoglycemia and decreases during hyperglycemia (47), but whether the regulation is paracrine or intrinsic is still not completely elucidated (48).

The pancreas in T1D

The endocrine pancreas in T1D is characterized by partial or complete loss of insulin-producing beta cells. While it has often been claimed that 90% of the beta cells are lost at T1D onset, a meta-analysis has shown that a 40% reduction in beta cell mass can be sufficient to develop clinical symptoms in adults (49). The beta cell loss is greater with younger age at onset (50).

Immune cell infiltration of the pancreatic islets (insulinitis) has been called a hallmark in T1D and pointed out as a culprit in the beta cell destruction (51). Also hyperexpression of HLA class I and expression of interferon alpha in beta cells has been reported (52–54).

It is commonly suggested that a triggering event (e.g. virus infection) leads to beta cell damage and subsequent recruitment of antigen-presenting cells (APCs). When the APCs reach the lymph node, beta cell antigens are thought to be presented to autoreactive T cells with low avidity, which have escaped thymic deletion. The T cells supposedly then migrate to the islets and promote killing of the beta cells as well as inflammation (55). Due to

recent discoveries, this notion is gradually being challenged and adapted (56).

Insulin-containing beta cells, as well as detectable amounts of insulin secretion, have been noted up to several years after diabetes onset (57) and islets from patients with T1D are able to regain some, or all, insulin-secreting function after some days of in vitro culturing (58). This implies that some beta cell function can be retained despite the pathological process.

Increased cell proliferation in the pancreas has been reported in some but not other studies on recent-onset T1D, and not in cases with longer duration (59,60). However, replication is still rare in these cases (found in approximately 11% of islets) and no correlation to beta cells or immune cell infiltration has been reported (61). Low levels of beta cell apoptosis have been noted in long-standing cases, suggesting a certain beta cell turnover (57,62).

Immune cell infiltration

Insulitic lesions in T1D were first described in 1902 by the pathologist MB Schmidt, and then systematically studied and described in a larger material by Willy Gepts in 1965 (51). The current definition of insulitis requires that a minimum of 15 immune cells are present in three or more islets (63). When present, insulitis usually affects ~10% of the islets. While CD8+ T-cells comprise the majority of the cells in insulitic lesions, CD4+ T-cells, CD20+ B-cells and CD68+ macrophages are present to a lesser extent (64). Insulitis is primarily seen in children with recent or rapid T1D onset, but is reported as more uncommon in adults irrespective of disease duration (61,65,66). The islet inflammation is heavier and affects more islets in 0-14 year old children with T1D duration <1 year, than in patients with onset at 15-39 years of age (67).

Four categories of islets can be seen in the pancreas of subjects with T1D: insulin-containing islets (ICI) with insulitis, ICI without insulitis, insulin-deficient islets (IDI) with insulitis and IDI without insulitis (68). Insulitis is primarily present in ICI but rarely in IDI, which has led to the conclusion that insulin-containing beta cells are a driving factor in the inflammation (51,68,69).

The pathological lesions are distributed in a heterogenic 'patchy' pattern throughout the pancreatic tissue in T1D subjects. Seemingly unaffected islets may be located next to insulin-deficient islets and islets with marked insulitis, both in adjacent lobes and within the same lobe (50). The cause for this heterogeneity has not yet been addressed, but virus infection and pancreatic duct reflux have been suggested as possible explanations (70,71). Interestingly, a similar pattern of patchiness has also been noted in other autoimmune diseases such as vitiligo and multiple sclerosis (72).

An interesting observation, hitherto without explanation, is that immune cells tend to be located in the periphery of the islet rather than inside the islet parenchyma (73).

Alpha cells in T1D

Since clinical symptoms of T1D arise as a result of insulin deficiency, the pathological process is commonly thought to be beta cell specific. However, the role of alpha cells and glucagon production in T1D may have been underestimated. A paradoxical finding has been that people with T1D suffer a risk of postprandial glucagon increase and ensuing hyperglycemia, but also have a diminished glucagon response to hypoglycemia (74,75). While some studies report on increased alpha cell numbers in T1D subjects (76,77), it has not been verified by others (37,69,78,79).

The exocrine pancreas in T1D

Changes in the exocrine pancreas were reported already in early morphological observations (51), and both pancreatic size and weight is decreased in patients with T1D already at onset and in potential pre-diabetic subjects (80–81), which argues against a loss of paracrine effect by insulin on acinar cells as the sole cause. In addition, autoantibodies targeting exocrine tissue as well as impaired exocrine secretory function is overrepresented in T1D patients (83,84). While diabetes is a known complication to pancreatitis, an etiological connection between the two has also been suggested (82). Increased immune cell numbers in the exocrine tissue of T1D subjects as well as in non-diabetic autoantibody-positive (aAb+) subjects have been reported, further supporting a pathological process also in the exocrine tissue in T1D (85).

Autoimmunity in T1D

Observations of insulinitis and the presence of autoantibodies directed towards beta cell epitopes in serum from patients with T1D have formed the basis for the notion that T1D is caused by a T-cell mediated autoimmune attack, with a subsequent development of autoantibodies (86). Whether autoimmunity is the cause or merely the consequence of beta cell destruction has however been debated (87).

Innate immune system

Signs of an active innate immune response through cytokine secretion in islets, and in blood even before clinical onset (88–90), as well as observations of increased HLA-staining in islets (53), together suggest a potential

role of the innate immune system as a key player in initiating and/or maintaining an adaptive immune response in T1D.

Virus infection is commonly suspected as a trigger of the innate immune system, and may upregulate HLA class I expression and thereby theoretically make the beta cells accessible to the adaptive autoimmune response.

Hyperexpression of HLA class I has been described in pancreatic islets from T1D subjects, but is not specific to beta cells (52). However, the validity of IHC-based findings of HLA hyperexpression in beta cells has lately been debated (91).

T cells

The most frequently identified cells in the insulitic lesions in T1D are CD8⁺ T cells, commonly assumed to be cytotoxic T cells (CTLs) which normally function in protection against viruses and in elimination of tumor cells. However, the phenotypes of the T cells present in insulitic lesions are poorly sub-characterized.

In support of a critical role for CD8 T cells in T1D pathogenesis is the genetic predisposition of HLA class I loci for T1D risk (92). Studies in humans have reported on both CD8⁺ and CD4⁺ T cells with specificity towards beta cell epitopes in peripheral blood of patients with T1D (93–97) and in some insulitic lesions, but the majority of T cells in the lesions are of unknown specificity (98). T cells have also shown to be able to kill human beta cells *in vitro* (93). Additional support is largely based on studies in mouse models, including transfer of diabetes via T cells (99) and diabetes prevention by genetic modifications abrogating HLA class I expression (100). The insulitic lesions observed in the commonly used mouse models are generally massive and differ significantly from infiltrations seen in human islets at T1D onset, and should therefore be interpreted with caution (101).

The relatively low fraction of islets affected by insulitis at onset of T1D (<10%), and the mild and transient effect of intense immunosuppressive therapy has raised doubts on whether T cell-mediated beta cell destruction is actually a decisive event in T1D pathogenesis (87,102,103). Furthermore, it was recently shown that islet autoreactive CD8 T cells are as common in peripheral blood from healthy subjects as in recent onset T1D patients (104). When compared to other human autoimmune diseases, the degree of insulitis seen at T1D onset is markedly lower than the immune cell infiltration seen in e.g. rheumatoid arthritis, psoriasis, arteritis and colitis (87). High levels of immune cell infiltration of the pancreas due to prolonged life support and use of steroids also complicate the interpretation of T1D-associated immune infiltration in studies of organ donors (105).

Autoantibodies

Autoantibodies directed towards islet cell antigens are frequently detected in patient serum several years before T1D onset and can be used to identify patients with high risk of developing the disease. The risk of developing T1D increases with the number of autoantibodies in serum.

Though the overrepresentation of autoantibodies targeting beta cell proteins was one of the initial observations leading to the conclusion that T1D is an autoimmune disease (87,106), autoantibodies are now generally seen as a consequence of tissue damage with subsequent exposure of intracellular epitopes, rather than as a primary culprit in the disease process.

The first test for the presence of serum-autoantibodies in T1D consisted of an analysis for unspecific detection of islet cell antibodies (ICA) (106). More specific autoantibodies were later detected and are now in clinical use. Insulin autoantibodies (IAA) targeting secretory granules in beta cells are found in virtually 100% of children under the age of five before onset of T1D. Interestingly, first-degree relatives with multiple diabetes-related autoantibodies have an earlier T1D onset if IAA are detected at repeated time points, than if IAA reactivity is lost (107). Glutamic acid decarboxylase antibodies (GADA) are present in 70-80% of newly diagnosed subjects (108). GAD can be found in synaptic-like microvesicles of neuroendocrine cells. Islet antigen-2 antibodies (IA-2A) target an inactive member of the tyrosine phosphatase family. Both GADA and IA-2A are associated with an older age at diagnosis (109). More recently described is the zinc transporter 8-antibody (ZnT8a). Zinc transporters are important in the beta cells, as zinc is needed for insulin storage. A number of newly described diabetes-related autoantibodies may also become important in future T1D prediction (110).

Noteworthy, most of the hitherto used diabetes-associated autoantibodies are not specific to beta cells. For example, GAD-antibodies are commonly found in serum from patients suffering from the Stiff Man-syndrome. Furthermore, also patients with T2D more frequently carry diabetes-associated autoantibodies more frequently than healthy control subjects (111). It should also be noted that subjects with a single diabetes-related autoantibody rarely develop T1D (112).

Natural course

In 1986, George Eisenbarth suggested a model for the natural history of T1D, which has later on been modified and remains the most referenced (113,114). The model describes a possible course where individuals are born with various degrees of genetic predisposition for T1D, and environmental events during life lead to a gradually decreased beta cell mass until the critical insulin level cannot be reached and clinical symptoms appear. This mod-

el is supported by reports on diabetes-associated autoantibodies being detected in serum several years before disease (115), and a noticeable decrease in stimulated C-peptide up to two years prior to clinical onset (116,117). The relatively few immune cells present in insulitic lesions and the low degree of apoptosis are often interpreted as signs of a slow autoimmune process, driven by low-affinity T cells (118). Furthermore, observations of ongoing insulitis have been reported several years after clinical onset (73).

T1D has been proposed to be a relapsing-remitting disease, with a cyclicity similar to that of other autoimmune diseases, like systemic lupus erythematosus and multiple sclerosis. This hypothesis would explain the partial remission phase, and suggests that similar remission episodes occur also before onset (119).

Heredity and genetics

The risk of developing T1D is 0.4% in the general population, but is increased 15-fold in people with a sibling or a parent with the disease (120). However, only 13-15% of children with newly diagnosed T1D have a first-degree relative with the disease (121,122).

Around 50 genetic regions are hitherto known to be involved in the genetic susceptibility for T1D. The HLA region (IDDM1 locus) on chromosome 6 accounts for about 50% of the genetic risk, with the strongest association to HLA class II (123). This region is also connected to almost all disorders classified as autoimmune (92). However, the HLA region is expected to contain more than 100 expressed genes but only some are known to be involved in the immune response.

The vast majority of T1D patients in Sweden carry the HLA-DR3-DQ2 and/or the HLA-DR4-DQ8 allele (124). The heterozygous form of these haplotypes confers a much higher risk than for either of the homozygotes. These haplotypes are however not uncommon in the general population. HLA class II risk alleles are not only related to the risk of developing T1D, but also to the development of specific autoantibodies. HLA-DR3-DQ2 is connected to GADA as the first antibody in children, while children with HLA-DR4-DQ8 more commonly have IAA as their first autoantibody (125,126).

HLA-DP is a genetic determinant for T1D, but to a lesser extent than DR and DQ. Additional susceptibility regions have been found using genome-wide mapping (92,127,128). Of the non-HLA class II risk loci, alleles of the class I *HLA-B* gene accounts for the strongest connection to T1D. Risk loci located outside the HLA region include e.g. the insulin gene (*INS*), *PTPN22*, *IFIH1* and *CTSH*. 40-50% of these genes have been reported to be expressed in the human islets (129,130), which could be interpreted as evidence that genetic susceptibility to T1D affects also the beta cell dysfunction.

Environmental factors

Though genetics are known to play a part in T1D incidence, there are a number of observations strongly indicating a decisive role for one or more environmental factors. Among these are the increased T1D risk in populations moving from a low-risk to a high-risk area and the difference in incidence between countries (131,132). The concordance of T1D between monozygotic twins has been reported to be as low as 30%, and suggested as an additional factor indicating environmental influence on T1D incidence. The cumulative incidence however reaches 65% with extended analysis (133).

A number of explanatory models have been presented, including dietary components, infectious agents and vitamin D insufficiency (71,134–136). Virus infection, in particular by enteroviruses, is the most commonly studied environmental factor. Several studies have supported this theory, but none has so far been able to provide unambiguous evidence (137).

The hygiene hypothesis suggests that a lack of exposure to environmental agents early in life contributes to the development of T1D (138), and has been supported by studies on mouse models and epidemiological studies (139–141).

Aims

General aims

To study aspects of pancreas pathology and immunopathology in T1D, in order to approach an understanding of T1D etiology and ultimately how the pathological process can be modulated or prevented.

Specific aims

Paper I

To evaluate potential alterations in the expression of glucose transporter 1, 2 or 3 on the protein- or messenger ribonucleic acid (mRNA) level in the endocrine pancreas from organ donors with long-standing type 1 diabetes, as compared to non-diabetic controls and to the NOD mouse model.

Paper II

To characterize endocrine and exocrine pancreatic tissue in non-diabetic organ donors with diabetes-associated autoantibodies in serum, with focus on immune cell infiltration and fibrosis. In addition, we aimed to address if the rate of autoantibodies targeting exocrine epitopes as well as noted alcohol overconsumption was higher in aAb+ organ donors than in controls.

Paper III

To describe the insulitic lesion and remaining insulin reservoir in patients with recent onset T1D, and to compare T cells present in infiltrated islets in T1D to cell-mediated allograft rejection.

Paper IV

To assess the glucagon content in pancreatic islets from patients with recent-onset T1D, and to compare islet numbers and size distribution to non-diabetic control subjects.

Materials and Methods

Some of the material and methods are described and discussed below. Detailed information can be found in the respective papers.

Biobanking (paper I-IV)

Pathological and morphological studies have been of great importance to our present knowledge in the field of T1D. Because of the relative inaccessibility of the pancreas due to anatomical conditions, tissue from heart beating organ donors and autopsy material from deceased subjects with T1D and non-diabetic subjects are crucial for these studies. Fortunately, the mortality in ketoacidosis is today low in both children and adults, but the access to pancreatic material from recent onset cases is therefore very scarce.

All biobanks from which material for the studies included in this thesis are collected maintain a high standard of tissue quality. A quick recovery of the organ is especially important for pancreatic tissue, since autolysis of the pancreas starts closely after death and quickly degrades the tissue quality. The DiViD study stands out in this regard, as the biopsies were obtained from living patients and tissue was snap-frozen within minutes.

Network for Pancreatic Organ Donors with Diabetes (paper I)

The Network for Pancreatic Organ Donors with Diabetes (nPOD) was started in 2007 by the charitable organization JDRF in order to address the needs of researchers for high quality pancreas and immune cell samples. nPOD has recovered tissue from more than 400 cadaveric organ donors and is one of the world's largest biobanks of pancreata and related tissue from cadaveric organ donors with T1D and donors with islet autoantibodies (142). The specimens are available for investigators worldwide for studies aimed at addressing T1D-related questions. Tissue samples from T1D patients with new onset to long-term disease, non-diabetic aAb⁺ subjects, non-diabetic controls and individuals with disorders relevant to beta cell function are continuously included in the nPOD biobank. Recovery and transport of the pancreas meet transplant-grade criteria, and biospecimens from the entire pancreas are directly processed for cryopreserved cells, fresh frozen blocks and fixed in formalin and paraffin-embedded (143). In addition, tissue samples from

spleen, duodenum and lymph nodes are collected if available. T1D-related autoantibodies (GADA, IA-2A, ZnT8A), C-peptide levels and HLA genotyping for risk alleles are determined (66,144).

As of September 2016, the nPOD collection contained tissue from 115 organ donors with T1D, whereof one donor had a duration of <1 week and three had a duration of 4-12 months. The low access to pancreatic tissue from T1D patients with recent onset disease thus constitutes a limitation to the nPOD collection.

Nordic Network for Islet Transplantation (paper II-III)

An important source for pancreatic research material in Uppsala, Sweden is the islet isolation center at Uppsala Akademiska Hospital, a part of the Nordic Network for Islet Transplantation.

Pancreatic islets are isolated from the pancreas of brain-dead organ donors for islet transplantation purpose, using a protocol approved by the local ethnics committee. The islets are used for research purpose if the islet volume after isolation is too low for transplantation, and if consent for research has been left by the potential donor or from the relatives of the deceased donor.

During the infusion of the digestion enzyme blend, a clamp is used to compress the pancreatic duct at the head of the pancreas. The tissue adjacent to the clamp is taken as a biopsy if consent for research has been obtained. The pancreatic tissue is divided and processed for fresh frozen blocks and fixated in formalin. A piece of the biopsy is also saved in RNAlater for future RNA extraction. The biopsies collected this far include tissue from non-diabetic subjects with and without diabetes-associated autoantibodies, as well as 51 subjects with T2D and 12 with T1D, whereof two with acute onset T1D. In addition to pancreatic tissue, pancreatic lymph nodes and tissue specimens from spleen, duodenum and liver are collected when available.

Testing of islet function in response to glucose stimulation is carried out as a part of the standard quality testing in islet isolation. Information on cytokine expression as well as HLA typing, C-peptide, HbA1c, age, and sex is collected. Since 2007, serum from all donors considered for research is screened for the autoantibodies GADA and IA-2A.

Diabetes Virus Detection Study (paper III-IV)

In the Diabetes Virus Detection (DiViD) study laparoscopy with pancreatic tail resection was performed on living T1D patients within in the first weeks of diagnosis (145). Patients newly diagnosed with T1D were recruited from hospitals in the south of Norway. The following inclusion criteria were applied: 18-40 years of age, elevated GAD-antibodies at the time of diagnosis, insulin-dependence (insulin dosage >0.1 IU/kg/24 hours) and fasting C-

peptide levels >0.1 mmol/L. The patient also had to be willing to comply with intensive diabetes management in order to be considered. Patients matching the criteria were asked to join the study after oral and written information from the diabetologist and the surgeon was given separately. All patients gave written informed consent according to Norwegian regulations.

The study was originally designed as a randomized prospective placebo-controlled trial using GAD-alum (Diamyd®). The GAD-alum treatment was discontinued after inclusion of the two first patients, due to lack of efficiency in the European GAD phase III-study (146). An additional pancreatic biopsy from the same patients had been planned three years after initiated GAD-alum treatment, but was now removed from the protocol.

The pancreatic tail was resected laparoscopically during a standard spleen preserving procedure performed under general anesthesia, during which also duodenal biopsies were collected endoscopically. Each biopsy was divided into smaller pieces for use in histopathology, electronmicroscopy and gene expression analysis. Fresh tissue was sent to Uppsala, Sweden for islet isolation.

The DiViD study was discontinued after six patients had been included, due to complications including postoperative bleeding and leakage of pancreatic fluid (145).

Pre-diabetic subjects and auto-antibody testing (paper I-II)

Genetic predisposition, family history of T1D and the presence of serum-autoantibodies can provide information on a non-diabetic patient's risk of developing T1D (119). As the pathologic process in T1D is thought to begin up to several years prior to clinical onset, pancreatic tissue obtained at onset may be collected too late to show the crucial steps in the beta cell demise. Pancreatic tissue from potential pre-diabetic subjects, where this process is thought to have begun already, is therefore a valuable resource for research in T1D etiology. Some studies have reported on insulinitis, but not beta cell loss, in subjects with multiple autoantibodies (61,147).

As there are no preventative actions to hinder the T1D development in high-risk patients, testing of autoantibodies in non-diabetic subjects is only done for research purposes. Islet cell antibodies (ICA) were discovered in 1974 and are visualized through cytoplasmic fluorescence when serum is applied to pancreatic tissue (86,106). Later on, additional diabetes-related autoantibodies were discovered and are commonly analyzed with the more specific method enzyme-linked immunosorbant assay (ELISA) or radiobinding assay. When ELISA is used for antibody-detection (paper I and II), the relevant antigen (GAD or IA-2) is attached to the bottom of a well. The pa-

tient serum is then added to the well and let to incubate. After repeated washing, a secondary antibody conjugated to an enzyme is added. A signal proportional to the antibody concentration in serum can then be detected by letting the enzyme convert its substrate. Radiobinding assay was used for detection of ZnT8a and IAA in paper II. In radiolabeling, a radiolabeled antigen is mixed with serum and form a precipitation collected by centrifugation into pellets, which has a radioactivity proportional to the amount of autoantibodies.

The majority of T1D cases do however not have a family history of diabetes, and the T1D risk alleles are relatively common also in subjects that will never develop T1D (148). Thus while some subjects, with high-risk HLA alleles, family history of T1D and multiple autoantibodies, are relatively easy to define as pre-diabetic, the future T1D patients lacking those risk-factors are harder to predict.

Immunohistochemistry and immunofluorescence (paper I-IV)

Immunohistochemistry and immunofluorescence are used for detection of antigens on cells in tissue sections and are based on the detection of specific antigens by using antibodies targeting an epitope on the antigen in question. The antibody used for detection is either directly or via a secondary antibody conjugated to either an enzyme such as peroxidase, which catalyzes a color-producing reaction, or a fluorophore. If an enzyme is used, the method is called immunohistochemistry and can be analyzed under a light microscope (paper II-IV). If a fluorophore is used, the method is called immunofluorescence and can be detected using a fluorescence microscope (paper I).

There are some limitations to immunohistochemistry and immunofluorescence in T1D research: only a relatively small two-dimensional piece of the pancreas can be analyzed at a time, which is unsatisfying considering the heterogeneous pathology of T1D. Furthermore, the ongoing process in the tissue can only be visualized at a single time point, making the distinction between cause and consequence in the pathology a challenge. Also, these methods are not quantitative and can only be used to determine the localization of the antigen in question, but not its expression level.

Insulinitis has in many reports been defined as a minimum of five cells infiltrating a pancreatic islet, but several different definitions have been applied. In response to this, a consensus article defined insulinitis as a minimum of 15 cells infiltrating three or more islets. However, this definition does not take into account the different probability of small islets being infiltrated with a certain number of immune cells, as compared to larger islets. The presence of e.g. ten immune cells infiltrating a small islet should reasonably

be of greater significance than the same number of cells found in a larger islet. With the current definition of insulinitis, there is thus a risk of underestimating the immune cell infiltration in small islets and overestimate the infiltration in large islets. Furthermore, the limit of three infiltrated islets is not related to the number of islets examined. It may be more adequate to decide on a limit for the proportion of infiltrated islets.

Also a definition of pancreatic islets in immunohistochemistry would be desirable. Islets of small size make up a large part of the endocrine pancreas, but are easily left out during analysis.

Islet isolation (paper II, IV)

Islet isolation is routinely performed at Uppsala University Hospital as a part of the islet transplantation program, in a procedure described previously (149). In the DiViD study, 0.5-1 cm of the distal part of the resected pancreatic tail was immediately shipped by air courier in cold organ preservation solution to Uppsala University for islet isolation. Subsequent to enzymatic digestion of the tissue, 300-700 islets were handpicked under a light microscope.

Islet isolation makes it possible to analyze cytokine secretion from the islets, and to measure the insulin-producing function in response to e.g. glucose stimulation.

During islet isolation, there is however a risk of inducing stress in the islets. It should also be noted that handpicking entails a risk of selection bias, where mainly the larger islets are selected. Furthermore, when islets are analyzed *in vitro* it is not possible to distinguish between ICI, IDI and islets with or without insulinitis. Isolating the islets from the rest of the pancreas also leads to a risk of simplification, as the islets are not observed in their natural environment or with their normal interactions with adjacent tissue.

Quantitative polymerase chain reaction and laser capture microdissection (paper I, III)

Quantitative polymerase chain reaction (qPCR) was used to verify the immunofluorescence findings of GLUT in paper I. A limitation to this study was the use of the entire tissue section for RNA extraction and qPCR analysis. As the islets compose only a few percent of the tissue, the concentration of GLUT mRNA derived from the islets was low, and could not be distinguished from GLUT mRNA present in other pancreatic tissue.

Laser capture microdissection (LCM) is a method where specific areas in a tissue section can be selected and extracted. RNA can then be purified

from the extracted tissue, and analyzed with PCR-techniques (150). LCM thus allows for combined analysis of protein expression using immunohistochemistry, with gene expression analysis from the same cells. The feasibility of performing qPCR analysis of RNA extracted from human pancreatic islets by LCM has been validated in a previous study (151).

LCM was used in paper III, where it enabled expression analysis of genes associated with T- and B cell activation specifically in infiltrated islets. The method was however not available when the study described in paper I was conducted, but could potentially have contributed to the study by providing more reliable results on relative GLUT expression.

Results and discussion

Paper I: Persistent glucose transporter expression on pancreatic beta cells from longstanding type 1 diabetic individuals

Sustained expression of glucose transporters in beta cells

The main finding in this study was that glucose transporters in insulin-producing beta cells persist in type 1 diabetic subjects even after several years of disease. Though many steps are required in order for the beta cells to sense and respond to elevated glucose levels, our results suggest the possibility that the surviving beta cells in these subjects have a sustained insulin-sensing function. As discussed in the paper, this finding may be important to the possibilities of developing therapies also for long-standing T1D patients.

In clinical support of our findings are the results from the Joslin Medalist study, where a majority of long-standing type 1 diabetic patients still had detectable random serum C-peptide levels despite ≥ 50 years of duration (57). Furthermore, even long-standing T1D patients with low random C-peptide levels have been reported as responders to mixed meal tolerance test with increased serum C-peptide concentration. This implies that glucose sensing and insulin secretion is preserved in these patients, although to a small degree (152).

In 2013, our group presented a gene expression analysis on laser capture microdissected pancreatic islets from tissue obtained from an organ donor who died at onset of T1D, as well as from a non-diabetic control donor (151). No differences in GLUT expression were seen between these subjects.

Discrepancy between predominating glucose transporters in human and NOD-mouse

We confirm the findings by de Vos et al, who in 1995 showed that human islets differ from mouse islets in that they express very low levels of GLUT2, but instead express GLUT1 and to a lesser extent GLUT3 (44). A substantial amount of studies on T1D and islet biology are conducted on the NOD-mouse, and understanding the differences between the species is essential when interpreting and applying results from animal studies on human.

In support of our results was a study published during the same period of time, which reported on GLUT3 and GLUT1 as predominating glucose transporters in isolated pancreatic islets as well as in beta cells sorted with fluorescence-activated cell sorting (FACS). GLUT2 expression was barely detectable also in this study (153).

The Fanconi-Bickel syndrome is caused by biallelic mutations in the GLUT2-encoding gene *SLC2A2*, and rarely causes diabetes. However, a study published in 2012 reported on five cases of neonatal transient diabetes with homozygous *SLC2A2* mutations (154). These cases though only represented 4% of neonatal patients with GLUT2 defects, and the vast majority did thus not develop diabetes. An interesting point is also that patients suffering from GLUT1-deficiency only show mild or no impairment of insulin secretion (155). Hence, GLUT2 may after all be an important glucose transporter in beta cells at least during the neonatal period (156), and GLUT1 does not appear to be an indispensable glucose transporter in beta cells. Together, these results suggest that also other glucose transporters (e.g. GLUT3) and hitherto unknown interplays between glucose transporters may be important to the beta cell glucose transport.

One could hypothesize on the possibilities of a discrepancy between the most predominant and the functionally most important glucose transporter. Yet, as the K_m of GLUT1 is much lower than the K_m of GLUT2, the limited amount of GLUT2 detected in beta cells should not reasonably be of significance to the limitation of glucose influx rate in the beta cells.

Three of the organ donors included in paper I tested positive for diabetes-associated autoantibodies; one for three aAbs, and two for one aAbs. Interestingly, the donor with three autoantibodies had normal islets but mild chronic pancreatitis. Three of the T1D donors also had morphological features consistent with chronic pancreatitis, whereof one tested positive for insulin autoantibodies (IAA). None of the non-diabetic donors without autoantibodies had signs of pancreatitis. This is of interest for paper II in this thesis, in which we studied the exocrine pancreatic tissue in aAb⁺ organ donors.

Paper II: Characterization of human organ donors testing positive for type 1 diabetes-associated autoantibodies.

This is one of the largest studies conducted on pancreatic tissue from organ donors carrying diabetes-associated autoantibodies. We report on an increased number of immune cells in the exocrine pancreatic tissue from donors with high autoantibody titers as compared to controls. However, no

insulinitis or other signs of a pre-diabetic process or damage were present in the endocrine tissue from aAb⁺ donors.

Insulinitis

Two studies on aAb⁺ subjects have together reported on insulinitis in a total of four aAb⁺ subjects. In't Veld found insulinitis in two out of 62 aAb⁺ organ donors, of which five donors had multiple autoantibodies (147). The other two aAb⁺ donors with insulinitis were reported in a recent study by Campbell-Thompson et al where 18 aAb⁺ donors were screened, whereof seven had multiple autoantibodies (61). It should be noted that several sections were screened for insulinitis in the latter study, as compared to our study where only one tissue block was available from each donor. This obviously increases the likelihood of observing islets with insulinitis, especially since it has been found in only 1.4-9 % of the islets (61,147).

Both insulinitic donors in the study by In't Veld et al were positive for three or more autoantibodies and carried susceptible HLA-DQ genotypes. In that regard, these donors were statistically more likely to be pre-diabetic than the donors in our study. However, the donors were of 46 and 52.5 years of age, an age in which T1D incidence is low.

None of the aAb⁺ donors in the study by Campbell-Thomson et al had high-risk HLA alleles, but both donors with insulinitis were considerably younger (21 and 22 years of age) than in our study and the study by In't Veld et al.

Taking these studies together, we can conclude that insulinitis has thus far been found in 30-40% of donors with multiple autoantibodies screened for insulinitic lesions. However, like the donors in our study, these subjects are not obviously pre-diabetic when taking age and/or absence of risk HLA-alleles into account. It should also be noted that no beta cell reduction or diabetes-related pathology, as would be expected in early phases of the diabetes process, were noted in any of these studies. The significance of these findings to T1D pathology is thus uncertain.

Exocrine pathology

Interestingly, the area constituted by collagen was increased in pancreatic tissue from aAb⁺ donors as compared to controls. In addition, there was a trend towards a higher frequency of organ donors with known alcohol overconsumption in the aAb⁺ group, and increased occurrence of serum-autoantibodies targeting exocrine epitopes.

Together, these results point towards a possible connection between aAb-positivity and general pancreatic damage, due to e.g. alcohol overconsumption, rather than specific beta cell damage indicating a pre-diabetic subject.

Paper III: Insulinitis and characterisation of infiltrating T cells in surgical pancreatic tail resections from patients at onset of type 1 diabetes

T cell infiltration of endocrine and exocrine tissue

The criteria for insulinitis were fulfilled in all six recent onset T1D patients in the DiViD study, with a majority (82%) of the infiltrated islets containing insulin. Consistent with the earlier described inter- and intra-individual variations, insulinitis as well as insulin content in islets differed greatly between donors and even within the same pancreatic lobe. Interestingly, only 11% of all islets (26% of all ICIs) were infiltrated with ≥ 15 CD3⁺ cells, despite the short time since diagnosis. This differs from earlier studies that have showed considerably lower insulinitis-infiltration in adults than in children (65), but is in concordance with another recent study (73). The results are also in line with the results from a recent study on a large number of cases from the nPOD collection, showing no correlation between age at onset and insulinitis rate (61). However, in this study we stained for CD3, and not CD45, which is used for the definition of insulinitis (63). Though CD3 T cells are known to be the most common immune cell in the insulitic lesions, there is thus a risk of underestimating of the insulinitis in this study. The application of a standard islet equivalent as a mean to normalize the definition of insulinitis to islet size offers an attractive way to compare immune cell infiltration between different studies.

As reported before (73), CD3⁺ cells were more commonly observed in the periphery of the islets than within the islet area. While the reason for this 'peri-insulinitis' is still unknown, Korpos et al have suggested that penetration through the basal membrane surrounding the islets is a crucial step in immune cell infiltration into the islet (157). The double membrane surrounding human pancreatic islets could perhaps partly explain that the peri-insulinitis in human does not seem to develop into intra-islet infiltration when the islets are destroyed, as opposed to what has is commonly seen in NOD mice, which only have single peri-islet basal membrane (73,158).

In a recently published study it is suggested that the T cell-recruiting chemokine CXCL10 is expressed in the periphery but not within the islet core of insulitic islets in T1D patients (89).

The infiltration of the exocrine pancreas consisted of 51 ± 27 CD3⁺ cells per mm² in the DiViD patients, which was considerably lower than reported in T1D brain-dead organ donors (85), and could result from the tissue being collected from living patients.

T cell characterization

Existing therapies directed towards T cells are drugs used to prevent allograft rejection. These drugs have therefore often been used in T1D intervention trials. However, the expression analysis of T- and B cell activation in laser dissected infiltrated islets from the patients with T1D showed an expression pattern that was distinctly different from the expression pattern seen in rejected kidney allograft. Though the material in this study is limited (six T1D patients and two patients with rejected kidney allograft), these results point towards a different role for T cells in insulinitis than in allograft rejection, which is in concordance with the results from studies on immunosuppressing agents targeting T cells in T1D (159,160). The subtraction of genes expressed in islets from non-diabetic patients from further analysis should reasonably have excluded gene expression not related to immune cells.

Further interesting controls for T cell expression would have been T cells from non-diabetic pancreas. A resemblance in gene expression to such cells would imply that T cells in insulinitis are merely passive bystanders. In addition, a comparison to gene expression in T cells from pancreatitis lesions could add information on whether there is an unspecific inflammatory process. Finally, as there is a connection in incidence between T1D and certain other autoimmune diseases, it would be interesting to compare expression analysis of T- and B cell activation in insulinitis to immune cells in lesions found in other autoimmune diseases. It is plausible that T cells indeed do have an active role in the beta cell-attack, but through another mechanism of action.

No difference in cytokine expression in isolated islets cultured in vitro were seen on the protein- or RNA level when comparing patients with T1D to controls. One should take into account however that a majority of islets were not heavily infiltrated by lymphocytes and the results may have looked different if it would have been possible to analyze cytokine expression from infiltrated islets only.

Persisting insulin content

An interesting observation is that insulin was seen in 36% of all screened islets and to some extent in all donors. ICI often morphologically resembled normal islets not affected by disease. As mentioned in the introduction, this has also been seen in other studies. The extensive presence of insulin despite clinical T1D symptoms suggests that there may be a functional deficiency of the beta cells and also offers hope of successful recovery of insulin production.

Paper IV: Increased glucagon content in the endocrine pancreas in recent onset type 1 diabetes. Results from the DiViD study.

We found a more than doubled glucagon containing area per pancreatic islet from patients with recent onset T1D as compared to controls, both in absolute numbers and when calculated in proportion to the entire islet area. However, the ratio between the glucagon area and the exocrine area did not differ significantly from controls, due to a 40% decrease in pancreatic islet count in the T1D patients. Furthermore, our morphological analyses on biopsies are likely to overestimate the total number of pancreatic islets in the T1D patients, as the volume of the pancreas in T1D patients generally is significantly decreased at clinical onset (80,81). Putting the biopsy findings in relation to the entire pancreatic volume would prevent such overestimation, but information on the pancreatic volume was unfortunately not available in the DiViD study. It would have been necessary to count the endocrine cell numbers in order to determine if the increased glucagon-containing area was due to hyperplasia or hypertrophy, but was not feasible with the methods used.

Alpha cell hyperplasia is a common feature in mouse models of T1D, and has been linked to beta cell stress, beta cell injuries and impaired glucagon signaling, all of which have been noted in human T1D. The reason for, or possible advantage of, alpha cell hyperplasia in these conditions is however not known. Past studies in subjects with T1D have presented no difference (61,69,78,161) or increased alpha cell numbers or area (76,77). Whether increased glucagon content in islets could be causing the disrupted glucagon response to hyper- and hypoglycemia often seen in T1D patients, is yet unknown.

Despite the absence of insulin in the majority of the islets from T1D patients, the mean islet area remained similar to control islets. As expected, the islet areas of IDI were in average smaller than non-diabetic control islets. In contrast, all other islet categories were larger than controls, including IDIs with insulinitis. To our knowledge, the islet size in different categories of islets (ICI, IDI with or without insulinitis) in T1D pancreas has not been assessed earlier. A similar analysis in long-standing T1D subjects would be interesting for comparison. As we lack the possibility to follow the diabetic process over time, we cannot draw any conclusions as to whether the increased size of islets with insulinitis could be a result of islets affected by insulinitis increasing in size, or if the larger islets more often become affected by insulinitis.

The maintained mean islet size argues against a general reduction in islet size as the explanation for the decreased islet number counted in the two-dimensional immunohistological analysis. In this context however, it should be noted that islets were identified using staining for glucagon. Islets devoid of glucagon, but still containing delta cells, PP cells and/or epsilon cells

would not be detected. Staining for an endocrine marker, such as the neuro-endocrine synaptic vesicle protein synaptophysin would have clarified if other islet cells were still present.

General discussion and future perspectives

What happens to the beta cells?

The lack of insulin-producing beta cells in T1D subjects has led to the conclusion that beta cells are destroyed and insulin production is irreversibly lost. However, stimulated C-peptide can be detected despite decades of disease (152), and it was recently shown that beta cell function could be regained after some days of in vitro culturing of islets from patients with recent onset T1D (58). In line with this, we reported on persistent glucose transporters, which form part of the prerequisites for glucose sensing, despite several years of T1D (paper I) and a substantial amount of remaining insulin-containing islets in recent onset T1D patients (paper III-IV). An explanation for these findings could be a functional block of beta cells, either as a result of glucotoxicity, or through other mechanisms. An interesting finding in this context is that islets in the pre-diabetic and diabetic state have a close to normal response to other stimuli than glucose, i.e. the insulinotropic amino acid arginine (162).

If the insulin-deficiency in T1D would be a result of blocked insulin-production, insulin-deficient beta cells could hypothetically be present in the pancreas. It is not known whether such cells exist, as they would not be detectable with the methods available today. A beta cell marker separate from insulin would be needed in order to clarify this. In addition, it may be of value to characterize islets not affected by insulinitis or insulin loss, in order to address why these islets seemingly have been able to avoid disease and whether they have a completely normal insulin secretion in response to blood glucose variations.

Speculatively, a transdifferentiation of beta cells to glucagon producing cells could be a conceivable explanation for the findings of increased glucagon content in recent onset T1D patients in paper IV. A large degree of plasticity has been demonstrated in both alpha and beta cells in mouse models, with the possibility of transdifferentiation between the two cell types (163,164). The relevance of these findings to human is however not known. Further studies would be needed to elucidate if increased numbers of glucagon-producing cells are present in all or a subset of T1D donors, and whether transdifferentiation or dedifferentiation is indeed a feature of T1D pathogenesis.

Another possible explanation could be that people who develop T1D are born with a lesser number and/or altered composition of beta cells or islets, or have an abnormal pancreatic development during infancy and childhood. Such prerequisites would then lead to a higher susceptibility to developing hyperglycemia. This would explain the finding of a reduced number of islets in T1D patients in paper IV, as well as the documented decreased pancreatic weight already at T1D onset. The number of beta cells is varying up to ten-fold between individuals (31), but it is yet not known whether individuals with lower islet beta cell count more commonly develop diabetes. Techniques for repeated visualization of beta cell numbers would be needed in order to address this.

If a reversible blocking or differentiation of beta cells appears to be part of the insulin deficiency in T1D, it opens up for the possibility of developing therapies acting on these mechanisms.

Is T1D beta cell-specific?

In paper II, we propose that a general inflammatory process of the pancreas is connected to the development of diabetes-related autoantibodies. These donors did not have other risk factors for developing T1D (high-risk HLA alleles, age, known relatives with T1D), and the majority carried only one diabetes-related autoantibody. This indicates that not only diabetes-related or beta cell specific damage to the pancreas can give rise to autoantibodies, and suggests that also antibody-development in T1D may be a result of a general pancreatic insult. The finding of an increased frequency of exocrine autoantibodies in T1D patients, exocrine immune cell infiltration and decreased pancreas size are in support of this notion.

The significantly fewer islets seen in the pancreas of recent onset T1D patients as compared to non-diabetic organ donors (paper IV) could also be a result of the T1D process affecting not only the beta cells but the entire islets.

Thusly, the pathological process resulting in T1D seems to be affecting several different cell types in the endocrine and exocrine pancreas, though clinical symptoms arise as a result of insulin deficiency. Further studies focusing also on other pancreatic cells than beta cells are needed to evaluate this impact further.

The role of T cells

There may be reason to question the role of T cells in T1D pathogenesis. From the collected results on T cell infiltration in T1D present so far, it is not possible to determine whether the infiltrating T cells have an active role

in the pathogenesis or, like autoantibodies are passive bystanders. Future studies with further sub-characterization and information on T cell reactivity and means of action in T1D are needed.

In line with the comparison of expression analysis of T cell activation between the pancreas in T1D and allorjected kidney in paper IV, it would be interesting to further compare T cell activation with other autoimmune diseases, as well as to T cell response in virus infection and cancer, in order to evaluate potential similarities.

One or several diseases?

The remarkably high amount of remaining insulin noted in young adults with recent onset T1D (paper III-IV) has been also been reported in other studies on adults (49), but contrasts with the pathology usually seen in children. The noted disparities between children and adults, regarding T1D pathology, clinic and genetic susceptibility (165), has led to speculations on whether T1D may in fact represent two or more etiologies. Children often present with a more aggressive disease, including a more rapid loss of C-peptide and higher frequency of ketoacidosis at onset (166). Beta cell loss is more pronounced at onset in children, and a different insulinitic immune cell profile has been suggested (49,167).

Interestingly, the frequency of HLA susceptibility haplotypes is lower in adults, and 33% of patients aged 34 years at clinical onset are expected to be negative for all antibodies (168). This implies that there may be a larger impact from environmental factors in adult-onset T1D than in children. One could speculate that T1D with onset in childhood might owe largely to a hereditary autoimmune response, which gives rise to beta cell loss. In patients with adult T1D onset, environmental factors may play a more decisive role while the immune response is secondary. An overlapping etiology between the two models, and subgroups within these models, are however not excluded.

It should be noted that with the exception of two donors included in paper I, all donors and patients included in the studies of this thesis are 18 years of age or older. The subjects in the DiViD study form a genetically and clinically homogenous group. Due to the above-discussed heterogeneity between groups, caution should thus be taken before these results are applied to e.g. children. Furthermore, the relatively limited number of subjects in all studies included in this thesis did not allow for meaningful correlation to ethnicity, age, disease duration, and HLA polymorphisms, and information on family history of T1D was not available in any of the studies.

Visualizing the pancreas in T1D

Only a cross section of a small part of the pancreas can be visualized at a time using immunohistochemistry or immunofluorescence, and pancreatic tail biopsies may not be representative for the other parts of the pancreas (paper III and IV). In addition, biopsy studies only represent a snap shot of the pathogenesis, and the results are difficult to place on a time line.

Methods enabling three-dimensional visualization of the entire pancreas, where the lesions can be correlated to e.g. pancreatic innervation, the duct system or the lymphatic system in the pancreas could potentially add information on the origin of the pancreatic damage. It would also be desirable to follow the immunopathology and beta cell function over an extended period of time, ultimately starting before T1D onset. In order to achieve this, in vivo monitoring of the islets using safe minimal-invasive or non-invasive methods, such as e.g. positron emission tomography-magnetic resonance imaging (PET-MRI) would be needed.

Summary

Paper I

The mRNA and protein expression of glucose transporter 1, 2 and 3 was not decreased in pancreatic islets from T1D as compared to non-diabetic subjects. However, more sophisticated methods including removal of exocrine tissue would be needed in order to ensure the results.

Furthermore, we confirm that the predominant form of glucose transporter in human pancreatic islets is GLUT1, with GLUT3 present to a lesser degree. GLUT2 was not completely absent, but present to a much lesser degree.

Paper II

Organ donors testing positive for one or more diabetes-associated autoantibodies did not show any signs of tissue damage or increased inflammation of the endocrine tissue, as compared to controls. Thus, no pre-diabetic morphological findings were seen in the islets.

In contrast, an increased number of inflammatory cells were present in the exocrine tissue of donors with high GADA-titers, and the proportion of collagen in the pancreas was increased in the aAb⁺ group. Alcohol overconsumption and exocrine autoantibodies was also noted more frequently in aAb⁺ organ donors than in controls, though without statistical significance. Collectively, these results signal that diabetes-associated autoantibodies can occur in response to unspecific pancreatic lesions.

Paper III

Insulinitis was noted in all patients in the DiViD study, mostly located in the periphery of the islets, and with significant variations both between patients and within the patient biopsies. Expression analyses of T- and B cell activation showed distinct differences to T cells collected from cell-mediated rejection of kidney transplant, which points towards a different role for T cells in insulinitis than in allorejection. Furthermore, cytokine secretion from isolated islets did not differ significantly from the secretion of non-diabetic islets.

Insulin was detected in a considerable proportion of all islets, which would be of clinical importance if the disease could be halted.

Paper IV

Glucagon content in pancreatic islets was increased in biopsies from six patients with recent-onset T1D, as compared to non-diabetic organ donors. Significant differences in glucagon content were seen between insulin-containing and insulin-deficient islets as well as in islets with and without insulinitis.

Furthermore, though islet count was reduced in T1D patients, the mean islet size remained similar to controls. Further studies, including evaluation of replication and transdifferentiation, would help in understanding these findings.

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