The pathogenesis of type 1 diabetes - lessons from pancreatic biopsies in the Diabetes Virus Detection Study (DiViD)

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1 Preface

1.1 Acknowledgements

Albert Einstein allegedly said: “If we knew what we were doing it wouldn't be research”. Based on this, my work with this project actually deserves to be called research! My entry into the world of research has been a roller coaster of highs and lows, setbacks and advances. But when looking back, the simple and basic truth is; I have enjoyed the life as a researcher every single day! This project has opened doors into a completely new world for me. The research society consists of warm, open-minded, friendly and bright people, and I have been lucky to make friends with many of them. For me, this has definitively been the most valuable outcome of my efforts in recent years.

There are many people I want to express my gratitude to, who all made this project possible. First of all I will thank the six brave and altruistic persons who joined the study. Your effort were priceless, and can never be repaid. I hope I have earned your trust, and managed to make your participation worthy.

My supervisor, Knut Dahl-Jørgensen, had the idea for the DiViD-study already in 2000, and Knut has been working determined, focused and persevering with the project ever since. I am honoured by the trust given to me when I was recruited in 2009, and grateful for all supervision, support and practical help provided ever since. Knut’s ability to spot new opportunities by combining old and new knowledge is remarkable. In addition, he is a very pleasant man to be with, and I have really enjoyed our friendship.

My co-supervisor Kristian F. Hanssen is extremely analytic, and I am grateful he entered this project with all his strength and power. For me Kristian has been a reliable source for the recent articles. He is a very good discussion partner and has kindly pushed me in the right directions. Specialist nurse Trine Roald has been essential for the project, and made
recruitment of the patients possible. Trines capacity in establishing systems and procedures was essential, and I really enjoyed our close collaboration.

The very skilled surgeons, Bjørn Edwin and Trond Buanes, were of course essential for implementation of the project, as were the Intervention Centre and the surgical department at Oslo University Hospital. Bjørn Atle Bjørnbeth showed very competent leadership when that was needed, which never will be forgotten.

I am also grateful for the support from my employer, the Division of Paediatric and Adolescent Medicine at Oslo University Hospital, led by Terje Rootwelt. Oslo Diabetes Research Centre and the colleagues in this group have been good fellows for research inspiration and also for social events.

There are many collaborators I owe my deepest gratitude. The PEVNET society, and later the nPOD society, both with willingness to share material, ideas and thoughts, have shown that modern science is founded on cooperation and not competition. Some have to be mentioned by name; Oskar Skog in Uppsala for trying to teach me some basic laboratory techniques, and also for reading my thesis and providing lots of relevant comments and inputs; Noel Morgan in Exeter for proofreading the thesis and making the language fluent and readable; Olle Korsgren in Uppsala for questioning results and conclusions, thereby improving the quality of the work; Johnny Ludvigsson in Linköping for the important initial work with anti-GAD; Heikki Hyöty for his expertise into the life of enteroviruses.

Finally, the genuine support, interest, enthusiasm and patience from my beloved wife Stine have been endless. Without her everlasting faith and love, this project had never reached completion.

Oslo, February 2016

Lars Krogyvold
1.2 Summary of thesis

Type 1 diabetes (T1D) is a chronic, lifelong disease affecting children, adolescents and adults characterised with loss of insulin production due to progressive destruction of the beta cells in the pancreas. The disease leads to increased risk for several serious acute and chronic complications, and even when optimally treated the life expectancy of a patient with T1D is statistically significantly reduced. In spite of intense research since the pathogenesis of T1D was identified in 1924, the exact causes of T1D remain unknown. Even though the disease is associated with certain gene combinations, studies in genetically identical twins have clearly shown that there have to be one or several environmental factors involved when someone develops T1D. Several such factors have been proposed, among them nutritional factors (cow’s milk, gluten, vitamin D and others) and microbiological agents. Among the latter, special attention has been paid to enteroviruses, which presence in blood and stool have been shown to be associated with both prediabetes and diabetes. Still there has been a striking lack of studies of pancreatic tissue, especially collected close to onset of T1D, which has impeded clarification of the exact role of enterovirus in the development of T1D.

The overall aim of the Diabetes Virus detection study (DiViD) was to investigate and explore high quality pancreatic tissue collected from live adult individuals with recent onset T1D. In this thesis the search for possible presence of enterovirus in the pancreatic tissue by different methods is reported. In addition, a thorough description of the ongoing T-cell-mediated inflammatory process affecting the islets of Langerhans (insulitis) is given, as is functional analyses in respect of insulin production of live islets stimulated with glucose solution.
This pancreatic material was collected by laparoscopic tail resections performed by highly skilled pancreatic surgeons at the Intervention Centre at Oslo University Hospital. A total of 6 patients, age 24 to 35 years, were enrolled in the study. Due to the observation of transient leakage of pancreatic fluid into the abdominal cavity in case 5 and 6, we decided to stop recruitment of additional patients. The patients include three men and three women, all of them insulin dependent, with positive anti-GAD antibodies as typically seen in T1D. The pancreatic tail resection was performed from 3 to 9 weeks after the diagnosis was established, and the tissue was immediately snap-frozen at minus 80 degrees Celsius (2 to 4 minutes after sampling).

Functional analyses of live islets stimulated with glucose solution in a perifusion experiment showed that even though the insulin secretion in the islets from the DiViD-cases were impaired compared to the secretion from islets collected from otherwise healthy organ donors, most of the cases secreted some amounts of insulin. More important, this insulin secretion increased in several of the cases when the islets were kept in culture for 6 days, and in 2 of the cases the insulin secretion then resembled normal insulin secretion. In addition, whole transcriptome sequencing of the RNA from these islets showed that all the genes involved in the insulin secretion pathways were expressed in all cases. These results show that the potential for insulin production in patients with recent onset T1D is large, and that it increases when the islets are kept in a non-diabetogenic environment in vitro. These observations are encouraging in respect of the potential restoration of insulin production at onset of T1D, given that an effective intervention therapy is developed.

When examining the collected material regarding the presence of inflammation, consecutive sections from two different formalin fixated paraffin embedded blocks from each of the 6
cases were double stained for insulin/glucagon, insulin/CD3 and glucagon/CD3. All 6 cases fulfilled the criteria given for the diagnosis of insulitis. In total 11 % of all investigated islets where surrounded by or infiltrated with > 15 T-cells (CD3-positive leucocytes). A majority of the T-cells were located outside the islets, at the exocrine-endocrine interface (peri-insulitis). Most of the inflamed islets (82%) still contained insulin producing cells. A total of 36% of all investigated islet were containing insulin. The expression of 84 T and B-cell-genes were examined in laser captured islets, and compared with the expression of the same genes in kidney transplants undergoing T-cell mediated rejection. The results showed a marked difference in qualitative and quantitative difference in T and B cell activation. This observation, together with the finding of no significant differences in cytokine/chemokine expression in isolated islets from the DiViD cases when compared to non-diabetic organ donors, argue for a different role of the CD3 + T cells in T1D when compared to the CD3+ T cells observed in allograft rejection. Are they merely passive bystanders to an ongoing process within the islets, or is the observed per-insulitis an earlier event to the intra-insulitis, the latter being dependent of a degradation of the basal membrane surrounding the islets? Regardless of the answer, the exact role of the T-cells involved in T1D has to be further explored.

Viral investigations of the specimens showed that all six DiViD-patients were positive for enterovirus in the endocrine pancreas by at least one of the methods used to detect either viral protein or viral RNA. All 6 cases were immunopositive for VP1, whereas the protein was detected in two of nine controls (100% vs. 22%; p < 0.01). Viral genome was detected in RNA extracted from culture medium were the islets had been cultured in four of the six patients. A snap-frozen pancreas sample containing 30 mg of whole tissue was enterovirus positive in one of the patients. The results were confirmed in two different laboratories. The
amount of present virus was low, with 1.7% of all islets being positive for VP1, and with a low virus titre on PCR. Therefore the exact genotypes of the present viruses could not be identified, but the viral genome was partially sequenced in all four PCR positive cases and the sequence showed a perfect match with enterovirus sequences. High-throughput sequencing of total RNA extracted from whole frozen tissue from each patient did not detect any viral sequences. Hyperexpression of HLA class I molecules, as a possible marker for virus induced interferon secretion in the islets, was observed homogeneously in the islets of all six T1D patients but in only one of the nine controls (p < 0.01).

In conclusion, the results from the DiViD-study have shown there is a significant insulin reserve reservoir is present at the diagnosis of T1D. This reserve reservoir is due to both a loss of beta cells and a dysfunction in insulin secretion in remaining beta cells. Furthermore, a significant inflammation – insulitis - was present in all cases as defined by the consensus criteria. More T-cells were located outside the islets, than inside, and the role of these T-cells remain to be elucidated. Finally, DiViD is the first study of living newly diagnosed type 1 diabetic patients to demonstrate the presence of enterovirus in pancreatic islets using multiple techniques across several independent laboratories including the detection of enterovirus specific sequences in the islets. Even though these results support the hypothesis of viruses playing a role in the development of T1D, they do not formally prove any secure causation between viruses and T1D. Hopefully our results will lead to an intervention trial with anti-viral treatment to patients with recent onset T1D, which may clarify this very important question.
1.3 List of papers


1.4 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
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<tbody>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD11c</td>
<td>Cluster of differentiation 11, a defining marker for dendritic cells</td>
</tr>
<tr>
<td>CD20</td>
<td>Cluster of differentiation 20, a defining marker for B-lymphocytes</td>
</tr>
<tr>
<td>CD3</td>
<td>Cluster of differentiation 3, a defining marker for T cells</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 45, a defining marker for T helper cells</td>
</tr>
<tr>
<td>CD45</td>
<td>Cluster of differentiation 45, a defining marker for all leucocytes</td>
</tr>
<tr>
<td>CD68</td>
<td>Cluster of differentiation 68, a defining marker for macrophages</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8, a defining marker for cytotoxic T-cells</td>
</tr>
<tr>
<td>DASP</td>
<td>Diabetes Antibody Standardization Program</td>
</tr>
<tr>
<td>DiViD</td>
<td>Diabetes Virus Detection (study)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>EV</td>
<td>Enterovirus</td>
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<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GRO</td>
<td>CXC chemokines growth-regulated oncogene</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose-stimulated insulin secretion</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Human leukocyte antigen- antigen D related</td>
</tr>
<tr>
<td>IA2</td>
<td>Islet antigen-2</td>
</tr>
<tr>
<td>IC1</td>
<td>Insulin-containing islet</td>
</tr>
<tr>
<td>IDI</td>
<td>Insulin-deficient islet</td>
</tr>
<tr>
<td>ISH</td>
<td>In-situ hybridization</td>
</tr>
<tr>
<td>IEQ</td>
<td>Standard islet equivalent</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon y inducible protein 10</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto encyclopaedia of genes and genomes</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser-capture microdissection</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>nPOD</td>
<td>Network of pancreatic organ donors</td>
</tr>
<tr>
<td>NGS</td>
<td>Next-generation sequencing</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEVNET</td>
<td>Persistent virus infection in diabetes network</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPKM</td>
<td>Reads per kilobase per million mapped reads</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>VP1</td>
<td>Enterovirus capsid protein 1</td>
</tr>
<tr>
<td>WFS1</td>
<td>Wolfram syndrome 1</td>
</tr>
<tr>
<td>ZnT8</td>
<td>Zinc transporter 8</td>
</tr>
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2 Introduction

Type 1 diabetes (T1D) is one of the most common chronic diseases of childhood, with the incidence in Norway in 2013 in the age group 0-14 years from 2002-2009 being 34.4/100 000 (1) according to figures from The Norwegian Childhood Diabetes Registry. T1D is frequently diagnosed in childhood, but the disease can also develop during adolescence and in adulthood. Approximately the same or even a higher number of patients will develop T1D after the age of 18 years as before that age (2;3). The development and natural history of T1D was illustrated by the so-called Eisenbarth-model, originally published by George Eisenbarth in 1986 (4). Later the model has been modified and expanded by inclusion of information gained through an improved understanding of the roles for genetics, immunology, and environment (5). Both models are shown in figure 1 (on courtesy).

In T1D, the insulin-producing beta-cells located in the islets of Langerhans are gradually destroyed until the amount of insulin produced is insufficient to maintain a normal level of blood glucose. This happens in individuals at genetic risk, and is believed to be triggered by one or several environmental factors. Before the threshold for insufficient insulin is reached, specific auto antibodies against beta-cell proteins (insulin, GAD, Zink transporter 8 and tyrosine phosphatase-like protein IA2) can be found circulating in the blood. This period, called prediabetes in the model, can last for weeks, months or even years (6). There is a marked gap between the onset of autoimmunity and the onset of diabetes, and it has been presumed that clinical diabetes does not develop until >80-90% of the beta cells have been destroyed (7). Based on the observation of specific auto-antibodies being present, T1D is considered to be an autoimmune disease (5). No interventions have so far proven able to halt the development of T1D, and T1D always leads to a lifelong need for exogenous insulin therapy unless the beta cells are replaced by either islet or pancreas transplantation. The
treatment of T1D is comprehensive and the disease is associated with an increased risk of serious acute and chronic complications. Even when treatment is optimised, the mortality of patients with T1D is three to four times higher than among the general population (8).

Figure 1: Traditional and more modern model of pathogenesis and natural history of type 1 diabetes
Reproduced with kind permission from Atkinson (5) and the Lancet.

2.1 Islet function
The pathology and functionality of islets of Langerhans at onset of T1D in human remains surprisingly poorly characterized. Previous in vitro studies (9;10) have shown remaining functionality of islet cells obtained several months after diagnosis but so far there has been a lack of in vivo access to islets obtained from subjects at onset of T1D. Studies characterizing the gene expression profiles in human pancreas and in purified islets in T1D have been
published, providing interesting data supporting the view that T1D is caused by a chronic inflammatory process with participation of innate immunity (11;12). In the purified islets the predominant change was a reduction in the expression of endocrine and neural function genes, while in whole pancreas most changes were increases in immune system genes (11). The human islets express and release cytokines and chemokines, providing a link between the islet cells and the immune system in T1D which reinforces the concept of a dialog between pancreatic islets and the immune system in T1D (13). However, these studies are based on a few diseased cases, mainly including tissue from subjects with long-standing T1D (11;12).

Insulin secretion from islets obtained from non-diabetic human subjects exhibits a typical bi-phasic pattern when the islets are stimulated with glucose (14). At diagnosis most patients with T1D have significant, but insufficient insulin secretion, losing the bi-phasic pattern. The degree of beta-cell destruction required for symptomatic onset of T1D has been of growing question, with recent studies suggesting that 40%–50% b-cell viability may be present at the onset of hyperglycaemia (15). This high percentage suggests that beta cell dysfunction in addition to loss of beta-cells is present at diagnosis of T1D (16;17).

2.2 Islet inflammation - Insulitis

Histologically, a discrete predominantly lymphocytic infiltration specifically targeting the islets of Langerhans is found in about 68 % of children and 29 % of young adults when examined within 1 month after onset of T1D (18). This lesion, first described in 1902 (19), was named insulitis in 1940 (20). Insulitis has, together with the presence of circulating autoantibodies directed against islet cell antigens, been regarded as evidence for an immune-mediated pathogenesis of T1D (4). The infiltrating cells may be found in the islet-exocrine interface, called peri-insulitis, or be present within the islet parenchyma, called intra-insulitis.
The lesions affect mainly islets containing insulin-producing cells and, according to a recent consensus report, it should be present in a minimum of three islets, with a threshold level of \( \geq 15 \) CD45+ cells/islet, to be classified as insulitis (21). Surprisingly, since 1902 only a total of 166 cases with insulitis have been described in the literature (18). Most of these cases are from two different cohorts collected by Gepts in the 1960s and by Foulis in the 1980s. These cases are heterogeneous, and often lack important supporting clinical data such as disease duration, age at onset, insulin treatment and the extent of complications. Most of these samples are formalin fixated in paraffin, precluding thorough studies using more modern techniques. In addition, their immunological status and estimates of beta cell mass are rarely available. In recent years initiatives like the network of pancreatic organ donors (nPOD) and the Belgian Diabetes Registry have identified additional cases, but still the total number is below 200 (18). These initiatives are making it possible to analyse pancreatic tissue recovered from individuals defined as having prediabetes or established, often long-standing T1D. These materials undoubtedly provide a valuable resource for several kinds of studies, including a very recent publication that demonstrates the presence of \( \beta \)-cells as well as insulitis several years after diagnosis in children and young adults (22). Nevertheless, it is important to note that most of these patients have been treated in intensive care units, causing stress reactions with associated leucocytosis and increased cortisol secretion, thereby perhaps influencing insulin secretion and insulin sensitivity. In humans, major endocrine alterations occur after brain death (23), and in rats the level of several cytokines in serum is increased, in parallel with an upregulation of mRNA species encoding these same cytokines in the pancreas (24) shortly after induction of brain death. This might, therefore, lead to artificial alterations in the tissue that are not due to the disease process associated with T1D. Maybe even more importantly, it is evident that at diagnosis individual islets can be identified within the pancreas which have either no insulitis or are clearly inflammation, and that some islets are
entirely devoid of insulin while others retain insulin immunopositivity. Hence, this suggests that the immediate period at diagnosis provides a perfect window of opportunity in which to study the pancreatic lesions, but so far nPOD and BDR have collected very few samples from newly diagnosed T1D.

2.3 Triggers of disease – genes and viruses

Despite extensive research, the environmental trigger(s) that causes T1D is still unknown. It is well documented that heredity plays an important role, especially the HLA class II genes, were the haplotype HLA-DR3 and DR4 confers the greatest risk (25). In addition, genome wide association studies have revealed more than 40 different genes that influence T1D risk (26). The genes known to affect T1D susceptibility can be grouped into three general categories: immune function, insulin expression, and β-cell function (27). However, based on parameters such as the relatively low concordance rate in twin studies (28;29), the fast changing prevalence in migrating populations (30) and a rising incidence in many countries (31), it is clear that T1D cannot solely be explained by patterns of inheritance. Therefore, several environmental factors have been suggested to contribute to development of the disease, including various viruses (32), early introduction of bovine milk proteins into the diet (33), a depletion of vitamin D (34), daily intake of nitrite and nitrate (35) and sources of drinking water (36). The possible influence of viral infections was first postulated by Harris as long ago as 1899, who described a case of diabetes quickly following mumps (37). In 1927, Gundersen observed a marked increase in the incidence of T1D following epidemics of mumps in Norway, in a paper called “Is diabetes of infectious origin?” (38). Since then, numerous studies have addressed the possible role of viruses as causative agents in T1D and it has emerged that, in particular, infection with enteroviruses provides a strong association with disease development. A metanalysis of this evidence has concluded that a clinically
significant association exists between enterovirus infection (detected with molecular methods in blood samples) and the occurrence of islet autoantibodies (odds ratio 3.7) as well as between enterovirus infection and the onset of clinical T1D (odds ratio 9.8) (39). Enteroviruses have been isolated from the pancreas of dead patients with T1D on two occasions, the serotypes was Coxsackievirus B4 and B5 respectively (40;41). In one case the potential diabetogenicity of the virus strain was emphasized by the demonstration that inoculation of the isolated virus into mice also caused diabetes (40). Enteroviral-RNA has also been detected in blood samples taken from patients with newly diagnosed diabetes (42;43) and prospective studies have suggested that the islet cell damaging process may be initiated and/or the progression to clinical T1D accelerated by an enterovirus infection (44;45). Nevertheless the question of causality remains open and additional data which confirm the presence of enterovirus in the pancreases of type 1 diabetic patients would significantly strengthen the conclusions.

2.4 Methods for enterovirus detection in pancreas

Enterovirus present in pancreas can be detected with different techniques, each of them having different strength and weaknesses. The method most commonly reported in studies of pancreatic autopsy material is immunohistochemistry (IHC) with the use of a mouse monoclonal antibody (mAb), clone 5-D8/1 (46) directed against the capsid protein VP1. This antibody reacts with multiple enterovirus serotypes due to its conserved immunodominant region (47). It has also been shown that, under some conditions, the antibody may label certain human proteins (48). This led to further evaluation of the fidelity of this antibody, with the conclusion that when used under carefully optimised conditions, the immunolabelling pattern detected in sections of human pancreas with clone 5D8/1 is likely to be representative of enteroviral antigen expression.
Techniques showing the presence of enteroviral genome in pancreas include in situ hybridization (ISH) and reverse transcriptase – polymerase chain reaction (RT-PCR). In ISH, an enterovirus-specific oligonucleotide probe is designed to hybridize with a known sequence of the enteroviral genome. ISH will, as IHC, not only confirm presence of EV, but also show the exact location of the virus within the tissue. The method has been used to show the presence of EV in the intestine of T1D patients (49;50), but so far only rarely the method has been able to convincingly show the presence of EV in pancreas (51). The challenges posed with detecting viruses using ISH, especially RNA viruses such as EVs, include the lack of a common nucleotide sequence in all viruses, high sequence variation and genomic recombination (52). ISH is dependent on the sensitivity and the specificity of the oligonucleotide probes in use, and especially the specificity has been a challenge in the development of the probes. A recent publication shows that in-house designed ISH probe sets were able to detect enteroviruses from formalin-fixed tissue samples in cell cultures and animal models (52). The results will need to be confirmed on samples collected in vivo.

PCR is the most sensitive method for virus detection, with a theoretical possibility to detect one single copy of viral RNA (53;54). An evaluation of RT-PCR and IHC as tools for detection of EV in human pancreas and islets of Langerhans clearly showed that PCR was far more sensitive than IHC in detecting EV in in vitro infected samples (55). However, PCR does not work optimally in formalin-fixed paraffin embedded tissue samples and is not able to determine the localization of EV within the specimen. The sensitivity of the method may also be reduced by presence of slowly replicating (genomically modified?) virus in only a tiny proportion of human islet cells (56).
In a very recent study, a comparison of the sensitivity of different methodologies to detect enterovirus (Coxsackievirus B1) in in vitro infected human A549 alveolar basal epithelial cells was performed (57). A549 cells were infected with CVB1 and diluted with uninfected A549 cells to produce dilution series in which the proportion of infected cells ranged from $10^{-1}$ to $10^{-8}$. The results showed clearly that RT-PCR was the most sensitive method for EV detection yielding positive signals in the most diluted sample ($10^{-8}$) (57). The sensitivity of IHC was depending on the antibody used, and the most sensitive antibody was clone 5-D8/1, which detected virus proteins at a dilution of $10^{-6}$ (57). ISH detected the virus at dilutions of $10^{-4}$ (57).

Next-generation sequencing (NGS) has revolutionized many fields of science, including molecular virology. Still, application of NGS to detect viruses may be challenging, as you need to know a specific viral sequence to actually be able to detect the virus. In a recent study exploring the stool virome in 19 children shortly before they developed auto-antibodies and comparing them with 19 matched controls, NGS was found to be less sensitive and specific than RT-PCR in identifying viral sequences (58). The study clearly showed that the existing NGS protocols does not allow complete characterization of all viruses present in the sample, which is leading to a failure to detect low virus titres (amplification threshold cycle of 30 and later). The authors conclude that NGS should be complemented with virus-specific PCR as the gold standard of molecular detection of viruses; at least until more effective NGS protocols are available (58).

In conclusion, the different methods for virus detection complement each other, rather than outperform each other. The preservation of tissue morphology is a clear advantage of IHC and ISH, thereby making it possible to localize the virus in individual cells. The main advantage
of RT-PCR is its high sensitivity and the possibility to derive sequence information from the viral genome. Based on the different methods strengths and weaknesses, detection of EV in pancreas should be based on a set of complementary detection techniques (59).

2.5 Studies of the pancreas

There are some obvious reasons why optimal studies of pancreatic tissue recovered from patients with newly diagnosed T1D have rarely been undertaken. In particular, the pancreas is located deep within the abdomen and exists in close proximity to vital organs such as the duodenum, bile ducts, transverse colon, the spleen and vital blood vessels, making biopsy sampling a challenging procedure. Furthermore, the insulitis in T1D is both focal and segmental, which means that biopsies of a certain size are needed to ensure the recovery of representative material. Accordingly, needle biopsies are deemed unsuitable (60) and a surgical method is mandatory to obtain sufficient biopsy material from living patients. With the advent of improved laparoscopic surgical techniques, pancreas biopsy has become more feasible and attractive since the risks associated with a laparoscopic pancreatic tail resection have decreased dramatically (61;62). More than 10 years ago, a research group from Osaka, Japan, published results from a biopsy study in 35 patients where pancreatic biopsies were taken 3 (0-13) months after diagnosis of T1D. (60). The authors did not observe any severe complications of the procedure as haemorrhages, peritonitis, leakage of pancreatic fluid or pancreatitis in any of the patients. Two patients had minor complications such as abdominal pain two days after the procedure, and two patients had some subcutaneous air at the site of incision. In a clinical setting, pancreatic biopsies or tail resections are used in routine diagnostic workup of patients with other pancreas diseases, and the safety is good. In a study of 215 patients with serious diseases such as cancer, other tumours, acute and chronic pancreatitis, complications were observed in 7 patients (3.3%): 3 had haemorrhages, 2 leakage
of pancreatic fluid, one acute pancreatitis and one isolation of necrotic tissue (63). These patients were older and more ill than patients with stabilized newly diagnosed T1D. This study was published in 1983, and with more modern optical and surgery equipment specialized for laparoscopy of the pancreas, and more experienced surgeons, the results have been substantially improved (64;65).

In the Diabetes Virus Detection Study (DiViD), the overall aim was to collect pancreatic tissue of optimal quality by laparoscopic pancreatic tail resection from adult patients newly diagnosed with T1D. The pancreatic tissues were immediately processed for storage and isolation of living cells, making it possible to design comprehensive laboratory analyses addressing various aspects of the pathogenesis of T1D.

The specific objectives were formulated as follows in the original protocol:

- Evaluate the intensity of insulitis and related immunological process in pancreas, regional lymph nodes and small intestine of newly-diagnosed T1D patients, and the influence of GAD-Alum treatment on this process.
- Detect viruses and virus receptors in the islets and in insulin producing beta cells of the pancreas in patients with newly diagnosed T1D mellitus.
- Describe the influence of GAD-Alum treatment on insulin secretion in newly-diagnosed T1D.
3 Outline of the thesis

In the following, to give an overview of the study, I will firstly describe the clinical experience and discuss some ethical issues arising during the implementation of the study (paper 1). Then the methods and results regarding residual beta cell function, the extent of inflammation/insulitis and analysis of the possible presence of viruses will be reported (papers 2-4). These results will be summarized and discussed. The implications for our understanding of the aetiology and development of T1D, possible clinical implications and future perspectives will be put into a new and updated understanding of T1D. Future studies, both on the collected material and possible intervention studies will be discussed, as will the opportunities arising of finally solving the puzzle of the pathogenesis of T1D.
4 Clinical experience and ethical issues (Paper 1)

The paper «Pancreatic biopsy by minimal tail resection in live adult patients at the onset of type 1 diabetes - Experiences from the DiViD study” (66) focuses on recruitment of the patients, the surgical procedure, the collected clinical data and the complications. In addition, some ethical issues regarding the project are discussed in light of the experiences made during the study.

4.1 DiViD 1

In the DiViD I protocol, adults with newly diagnosed T1D were invited to participate in a randomized prospective placebo-controlled trial using GAD-alum (DiAmyd®) in an effort to preserve beta cell function, based on existing evidence at that time (67). Resection of the tail of the pancreas was to be performed as soon as possible after inclusion in the trial and, at the latest, three months after the diagnosis of T1D. Patients were recruited from hospitals all over the Southern parts of Norway, after provision of thorough information to both the patients and the local health personal (medical doctors and nurses).

4.1.1 Inclusion criteria

The patients should be adults, with an age between 18 to 40 years, have elevated GAD auto antibodies at the time of diagnosis, be insulin dependent (insulin dosage > 0.1 IU/kg/24 hours) and have a fasting c-peptide level > 0.1 mmol/L. In addition, females should agree to avoid pregnancy and have a negative urine pregnancy test, and all patients should agree to use adequate contraception, if sexually active, until 1 year after the last study drug administration. They had to be willing to comply with intensive diabetes management. All patients of course gave their written informed consent according to Norwegian regulations after oral and written information from the diabetologist and the surgeon separately.
4.1.2 Exclusion criteria

The exclusion criteria included pregnancy, alcohol and drug abuse, previous or current treatment with immunosuppressant therapy, treatment with any oral or injected anti-diabetes medication and significantly abnormal haematological values at the time of screening.

4.2 DiViD II

After recruitment of the first two patients, the design of the trial was slightly modified in the DiViD II protocol due to lack of efficacy of GAD, shown in the European GAD Phase III-trial (68). The major changes were that intervention with GAD-alum was discontinued and that the planned pancreatic biopsy after three years was removed. The information provided to the patients was changed accordingly, but besides that, the recruitment procedures and the inclusion and exclusion criteria remained unchanged.

4.3 Ethical approval

The study was approved by the Government’s Regional Ethics Committee. This committee is composed of a wide spectrum of authorities from the areas of ethics, genetics, law, medicine, psychology, and nursing. The committee also includes lay people and representatives from patient organizations. Due to the invasive procedures in the protocol, the processing of the application by the Regional Ethics Committee was very thorough. In a meeting with the committee in January 2010, the project was explained in detail, with special attention paid to questions regarding potential risks for the participating patients. After the described modifications of the protocol (DiViD II), a renewed approval by the Government’s Regional Ethics Committee was given upon application.
4.4 Surgical procedure

Surgery was performed under general anaesthesia. The laparoscopic distal pancreatic resection, performed by two skilled surgeons, was a standard spleen preserving procedure, with dissection of the pancreatic tail and the splenic vein before dividing the tail from the pancreatic body with an endoscopic GIA-stapler. At the end of the procedure each patient was thoroughly examined for bleeding, and suction drainage was left at the resection margin, according to present surgical guidelines. They were postoperatively closely followed by the operating surgeons, to ensure prompt discovery of potential complications. Picture 1 shows the operation theatre during surgery.

![Operation Theatre](image)

Picture 1. The operation theatre.
The biopsy was processed under sterile conditions and immediately divided into multiple smaller pieces (Figure 2). The use of ViaSpan® (Bristol-Myers Squibb Pharmaceutical Limited) made it possible to send fresh, live tissue to Uppsala, Sweden by express air-courier for isolation and culturing of islets for functional analyses and ultrastructural studies. Biopsies from different parts of the tail of the pancreas were fixed in formalin or frozen in liquid nitrogen, to ensure the preservation of tissue composition, structure and morphology for subsequent analyses. Part of the tissue was placed in RNAlater® (Life Technologies) to ensure better preservation of high quality RNA over time.

Figure 2. Division of the biopsy.

4.4.1 Sampling of other materials

Duodenal biopsies were collected endoscopically during the same period of general anaesthesia and were also immediately snap-frozen in liquid nitrogen or fixed in formalin. According to the original protocol, regional lymph nodes would be sampled only if easily accessible, but none was sampled. In addition, sampling of serum and plasma for PBMC extraction, stools and urine were undertaken, to ensure as much information as possible would be available to inform later analyses.
4.5 The recruited patients

A total of six patients (three men, three women), age 24-35 years (median 28 years) were recruited into the DiViD-studies, patient 1 and 2 in DiViD I, the remaining four in DiViD II. The biopsies were taken between three to nine weeks after diagnosis of T1D (median five weeks). According to the inclusion criteria, all patients were insulin dependent and treated with daily insulin injections at the time of biopsy operation (daily dose ranged from 0.16 to 0.52 U/kg, mean 0.37 U/kg). They were all positive for at least one of the four measured diabetes-related autoantibodies and all had at least one high risk HLA serotype (Table 1).

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>BMI</th>
<th>Weeks from diagnosis until biopsy</th>
<th>HbA1c at biopsy % (mmol/mol)</th>
<th>Insulin U/kg/da</th>
<th>Anti GAD &lt;0.08 ai</th>
<th>Anti-insulin &lt;0.08 ai</th>
<th>anti-ZnT8 &lt;0.12 ai</th>
<th>Anti-IA2(&lt;0.1 ai*)</th>
<th>HLA risk alleles†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>F</td>
<td>21.0</td>
<td>4</td>
<td>6.7 (50)</td>
<td>0.5</td>
<td>1.76</td>
<td>0.7</td>
<td>0.28</td>
<td>0.16</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>M</td>
<td>20.9</td>
<td>3</td>
<td>10.3 (89)</td>
<td>0.35</td>
<td>0.79</td>
<td>&lt;0.01</td>
<td>0.44</td>
<td>&gt;3</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>F</td>
<td>23.7</td>
<td>9</td>
<td>7.1 (54)</td>
<td>0.17</td>
<td>1.77</td>
<td>&lt;0.05</td>
<td>1.45</td>
<td>&gt;3</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>M</td>
<td>25.6</td>
<td>5</td>
<td>7.4 (57)</td>
<td>0.4</td>
<td>0.77</td>
<td>&lt;0.01</td>
<td>2.54</td>
<td>&gt;3</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>F</td>
<td>28.6</td>
<td>5</td>
<td>7.4 (57)</td>
<td>0.36</td>
<td>0.46</td>
<td>0.1</td>
<td>0.06</td>
<td>&gt;3</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>M</td>
<td>26.7</td>
<td>5</td>
<td>7.1 (54)</td>
<td>0.52</td>
<td>1.85</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.04</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 1. Clinical data of the DiViD-cases.
*Arbitrary units according to Diabetes Antibody Standardization Program (DASP) (69).

The patients were hospitalized for between four and 16 days (median 4 days). The duration of the procedure varied between 55-110 minutes (median 94 min). Parts of the biopsies were frozen in liquid nitrogen within 110-240 seconds (median 155) of intraperitoneal sampling (Table 2).
### Table 2. Details regarding the hospital stay.

<table>
<thead>
<tr>
<th>Case</th>
<th>Days in hospital</th>
<th>Length of operation, minutes</th>
<th>Seconds until snap-freezing of pancreatic tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>77</td>
<td>110</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>96</td>
<td>150</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>93</td>
<td>150</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>94</td>
<td>160</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>55</td>
<td>240</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>110</td>
<td>190</td>
</tr>
<tr>
<td>Median</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>88</td>
<td>167</td>
</tr>
</tbody>
</table>

### 4.6 Complications

#### 4.6.1 Postoperative bleeding

One patient (case 1) developed postoperative bleeding on the first postoperative day, as discovered by the presence of blood in the drainage fluid. Therefore this patient underwent re-laparoscopy and the bleeding was localised to the lateral surface of the spleen (adhesion to the abdominal wall had generated a tear in the splenic capsule). After complete haemostasis, the rest of the postoperative course was uneventful (i.e. the bleeding was of severity grade B (70)).

#### 4.6.2 Leakage of pancreatic fluid

Two patients (cases 5 and 6) suffered from a post-operative leakage of amylase-rich pancreatic juice from the margin of the resection. This resulted in pain, a moderate rise in temperature and a steep rise in the level of serum C-reactive protein (peak 43 and 418 mg/L respectively). Both patients were treated with antibiotics, however without any evidence of infection as defined by lack of growth of bacteria in subsequent cultures. Ultrasound of the
abdomen demonstrated a small accumulation of fluid at the resection border in both cases. The secretion ceased spontaneously after a few days, and subsequent percutaneous ultrasonic examination showed complete regression of the fluid accumulation. None of the patients had a sustained secretion or developed pseudo-cysts (i.e. the leakage had severity grade B (71)). The postoperative course was uncomplicated in three patients, who all were discharged three days after the procedure.

4.7 Discussion
DiViD was the first study that systematically collected surgical pancreatic biopsies from adult patients newly diagnosed with T1D, although there were published core needle biopsy studies of patients with fulminant diabetes and T1D from Japan at planning of the project (60;72;73). Previously the risk of serious perioperative complications had been considered too high to accept pancreatic resections solely for scientific research. However, after systematic refinement of the laparoscopic surgical techniques our centre collected a large series of pancreatic resections with only a very low incidence of complications (74). In DiViD, a limited tail resection distal to the main pancreatic duct was planned. We therefore regarded the protocol as safe and justifiable on ethical grounds, and as previously described, the Regional Ethical committee approved the procedure.

Despite the earlier experience of our surgical team, the complication rates in the present series were higher than expected. In the risk assessment before the study, we had considered the risk of leakage in young diabetic, but otherwise healthy, individuals to be lower than in patients undergoing pancreatic resection for neoplastic disease and/or pancreatitis. As we instead recorded higher complication rates in diabetic patients than in our previous series, that may lead to speculation that the soft, presumably normal exocrine pancreatic tissue presents a
higher risk of leakage. This is precisely opposite to our pre-study expectations, and questions the external validity of our previous data from a patient population of older patients with neoplastic lesions and/or pancreatitis. The reasons for the complications are probably coincidental, but it may be speculated that the on-going inflammation in the islets of the pancreas renders the tissue more prone to leakage. Later, an increased immune cell infiltration of the exocrine pancreas has been reported (75), possibly contributing to increased risk for leakage. Also in the samples from the DiViD-cases, CD3-positive T-cells were found located in the exocrine tissue (see paper 2), although a significantly higher density of CD3-positive T-cells were found within or in the periphery of the islets than in the exocrine tissue (76). Recently the pancreas in newly discovered T1D has been shown to be reduced in volume by 26% compared to healthy control subjects (77). This indicates atrophic changes in the pancreas at onset of T1D, making a surgical procedure more risky in these individuals. The bleeding from the splenic surface was probably caused by the subsequent gastroscopy, with pressure on the spleen from the endoscope (78;79), leading to tearing of the adhesion between the abdominal wall and the splenic capsule. The complications were perceived as unpleasant for the patients, mainly since they were accompanied by pain, fever and prolonged hospital stays. Although none of our patients experienced permanent injuries after participating in the study, we concluded that it would be unethical to continue to recruit participants to the study.

4.8 Ethical considerations

It is beyond any doubts that participation in a project such as DiViD required considerable personal effort from the subjects involved. Otherwise healthy people with T1D were required to undergo general anaesthesia and surgery. It was very important for us to ensure that the patients had full confidence that the study was as safe as possible and that it would provide valuable new knowledge. The patients must also have full confidence that the information
provided to them about the risks and potential outcomes was complete, truthful and fully up-to-date. For anyone undertaking clinical research, it is important that an on-going assessment of the project’s benefits and risks is undertaken continuously, and that the approach must be reconsidered if the incidence of complications proves to be higher than anticipated. As in all clinical research, the patients must be able to give informed consent prior to their participation in the study, and this must be based on the provision of thorough oral and written information.

Approximately 25% of patients who were invited to participate ended up giving written informed consent. The dominant cause of non-participation was the time-consuming procedure, but also a fear of complications/pain/discomfort was a major cause of refusal to participate. It remains a big challenge to provide such information in a way that is both accessible and fully informative. The provider must, of course take responsibility for ensuring that all aspects of participation in the project are understood. One of the recruited patients argued retrospectively that the information given in advance did not provide a satisfactory description of all possible eventualities experienced as a result of the procedure. We find this very regrettable and it represents an important reminder of the difficulty associated with provision of thorough, accessible and detailed information. On reflection in retrospect, we would suggest that when undertaking a project which is as invasive and innovative as DiViD, it is important to compile an assessment of how the patient will tackle the development of an unexpected complication prior to their inclusion. This might have been better addressed in the project, so that people who are perceived to display a low tolerance for unforeseen events were to be excluded, even if expressing a desire to take part.
5 Materials and methods

The materials and methods used in papers II-IV describing function of pancreatic islets (80), insulitis (76) and detection of enterovirus (81) will be described in the following. A more thorough description can be found in the papers, including then online supplemental materials (see appendix).

5.1 Non-diabetic controls

In paper II (80), in addition to the previously described DiViD patients, the pancreases from two organ donors (named case 7-8) who died at the onset of T1D and from three organ donors (controls 1-3) without pancreatic disease were included in this study. The two T1D donors died of brain oedema and total brain infarction (82). Clinical data regarding the DiViD cases can be found in table 1, while the data regarding cases 7 and 8 and the controls are shown in Table 3. In addition to the approval from the Government’s Regional Ethics Committee in Norway, the Regional Ethics Committee in Uppsala also approved the study.
<table>
<thead>
<tr>
<th>Category</th>
<th>Case 7</th>
<th>Age, years</th>
<th>Sex, M/F</th>
<th>Time from T1D diagnosis weeks</th>
<th>HbA1c at biopsy, % mmol/mol</th>
<th>Insulin U/kg/day</th>
<th>Anti GAD, &lt;0.08ai*</th>
<th>Anti-insulin, &lt;0.08ai*</th>
<th>anti-ZnT8, &lt;0.12ai*</th>
<th>Anti IA2, &lt;0.10ai*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ-donors died at onset of T1D</td>
<td>Case 7</td>
<td>40</td>
<td>M</td>
<td>0</td>
<td>Not available</td>
<td>-</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
</tr>
<tr>
<td>Organ-donors without T1D</td>
<td>Control 1</td>
<td>22</td>
<td>M</td>
<td>-</td>
<td>5.5 (37)</td>
<td>-</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
</tr>
</tbody>
</table>

Table 3. Demographic data, case 7 and 8 and control 1-3.

*Arbitrary units according to DASP (69).

In paper III (76), pancreatic tissue from three organ donors without T1D matched for age (20, 22, and 25 years) and kidney graft biopsies from two patients with ongoing cell-mediated allo-rejection were used as controls for the gene expression analysis only. The kidney transplant rejections were classified as Banff 1A and 1B respectively, and occurred after immunosuppression with cyclosporine A and prednisolone switched to tacrolimus, mycophenolate mofetil and prednisolone (patient with Banff 1A), and tacrolimus and prednisolone (patient with Banff 1B). Islets isolated from 6 donors (mean age 58, range 54-61) were used as controls for the analysis of cytokines and chemokines. In order to run the case and control islets in parallel, islets that were available for research at the time were used and thus they do not match for age or other factors. Ethical permission for using kidney biopsies for research was approved by the Regional Ethics Committee in Uppsala (Dnr 2011/349/2). Consent for using isolated islets and pancreatic tissue for research was obtained verbally from the deceased’s next of kin by the attending physician and documented in the medical records of the deceased in accordance with Swedish law and as approved by the Regional Ethics Committee (Dnr 2009/043 and 2009/371).
In paper IV (81), nine otherwise healthy, non-diabetic, cadaver Caucasian organ-donors (two women, seven men, age range 18-38, mean 25.2) collected by the network for pancreatic organ donors (nPOD) were used as controls for immunohistochemistry (IHC) analyses, all being negative for anti-GAD, IA2, insulin and ZnT8 autoantibodies. In addition, six non-diabetic organ-donors from Uppsala (age range 55-70, median 67) also negative for anti-GAD and IA2 were used as methodological controls in virus detection from isolated islets using PCR. Demographic details regarding age, sex, body mass index (BMI) and auto-antibodies and HLA of the controls are shown in Table 5.
<table>
<thead>
<tr>
<th>Control (nPOD-number)</th>
<th>Age</th>
<th>Sex</th>
<th>BMI</th>
<th>Anti GAD</th>
<th>Anti-insulin</th>
<th>anti-ZnT8</th>
<th>Anti IA2</th>
<th>HLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>nPOD 1 (6001)</td>
<td>22</td>
<td>M</td>
<td>21.9</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>A<em>02/02, B</em>07/62, DR<em>04/15, DQ</em>07/02</td>
</tr>
<tr>
<td>nPOD 2 (6024)</td>
<td>21</td>
<td>M</td>
<td>27.8</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>A<em>01/31, B</em>07/08, DR<em>13/17, DQ</em>07/02</td>
</tr>
<tr>
<td>nPOD 3 (6030)</td>
<td>30.1</td>
<td>M</td>
<td>27.1</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>A<em>02/31, B</em>07/44, DR<em>15/07, DQ</em>06/02</td>
</tr>
<tr>
<td>nPOD 4 (6034)</td>
<td>32</td>
<td>F</td>
<td>25.2</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>A<em>03/03, B</em>07/62, DR<em>01/08, DQ</em>05/04</td>
</tr>
<tr>
<td>nPOD 5 (6073)</td>
<td>19.2</td>
<td>M</td>
<td>36.0</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>A<em>29/01, B</em>37/44, DR<em>13/16, DQ</em>05/06</td>
</tr>
<tr>
<td>nPOD 6 (6098)</td>
<td>17.8</td>
<td>M</td>
<td>22.8</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>A<em>01/32, B</em>08/27, DR<em>17/08, DQ</em>02/04</td>
</tr>
<tr>
<td>nPOD 7 (6140)</td>
<td>38</td>
<td>M</td>
<td>21.7</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>A<em>01/03, B</em>35/35, DR<em>01/17, DQ</em>05/02</td>
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<tr>
<td>nPOD 8 (6160)</td>
<td>22.1</td>
<td>M</td>
<td>23.9</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>A<em>11/29, B</em>35/44, DR<em>10/30, DQ</em>02/05</td>
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<tr>
<td>nPOD 9 (6178)</td>
<td>24.5</td>
<td>F</td>
<td>27.5</td>
<td>Negative</td>
<td>Negative</td>
<td>Not tested</td>
<td>Negative</td>
<td>A<em>02/24, B</em>27/44, DR<em>04/15, DQ</em>06/03</td>
</tr>
<tr>
<td>Uppsala1</td>
<td>54</td>
<td>F</td>
<td>25.7</td>
<td>Negative</td>
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<td>Not tested</td>
<td>Negative</td>
<td>A<em>02/03, B</em>35/35, DR<em>01/01, DQ</em>05/05</td>
</tr>
<tr>
<td>Uppsala2</td>
<td>64</td>
<td>M</td>
<td>24.7</td>
<td>Negative</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Negative</td>
<td>A<em>01/24, B</em>27/27, DR*01/01</td>
</tr>
<tr>
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<td>69</td>
<td>F</td>
<td>19.5</td>
<td>Negative</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Negative</td>
<td>A<em>02/26, B</em>62/64, DR*04/16</td>
</tr>
<tr>
<td>Uppsala4</td>
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<td>Not tested</td>
<td>Negative</td>
<td>A<em>01/02, B</em>08/40, DR<em>03/12, DQ</em>02/03</td>
</tr>
<tr>
<td>Uppsala5</td>
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<td>M</td>
<td>25.7</td>
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<td>Not tested</td>
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<tr>
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<td>Not tested</td>
<td>Negative</td>
<td>A<em>01/02, B</em>08/62, DR<em>04/17, DQ</em>02/03</td>
</tr>
</tbody>
</table>

Table 4. Demographic data, controls, paper IV
5.2 Specific methods, paper II

5.2.1 Islet isolation

The most distal part (0.5-1 cm) of the laparoscopic pancreatic tail resections (Figure 1) was immediately shipped by air courier in cold organ preservation solution to Uppsala University for islet isolation. The islets were isolated by a method based on the procedure used for clinical islet isolation that has been described by Goto et al (83). 300-700 islets from each patient were handpicked from the digested tissue under a microscope by skilled islet technicians with multiple years of experience. Islets from the brain-dead organ donors were isolated and cultured as described previously (82;83).

5.2.2 Islet function

Glucose-stimulated insulin-secretion (GSIS) was assessed in a dynamic perfusion system. Twenty handpicked islets cultured for 1, 3, or 6 days were perifused with low glucose (1.67 mM) for 42 min, high glucose (20 mM) for 48 min, and then low glucose again. Fractions were collected at 6 min intervals and the secreted insulin was measured by ELISA.

5.2.3 Whole transcriptome sequencing and analyses

RNA was extracted from 50-100 islets per subject, immediately after handpicking from the digested pancreatic tail resections, or after storage of isolated islets from multi-organ donors (case 7 and 8, and controls 1-3) on day 1 after isolation at -80°C in RNAlater (Qiagen). The extracted RNA was of good quality (RIN values between 7.1 and 9.5) and sufficient quantity (>1 µg) for performing whole transcriptome sequencing.

Total RNA samples were depleted from rRNA and used to prepare a fragment library. The read length was 50 base pairs for all samples and directionality of RNA molecules was
preserved in the sequencing. The neighbour-joining method, based on the normalized Euclidean distance of reads per kilobase of exon per million fragments mapped (RPKM) between samples was used to analyse differences in expression between all samples, using uniform gene expression to root the tree. Gene lists for the insulin secretion pathway and the complement system pathway were extracted from the KEGG database and relevant literature. RPKM values for all genes in the pathways were calculated (84) and each case was compared to the mean of the three controls in order to identify up- and down-regulated genes. Genes with RPKM values below 0.1 were considered non-expressed.

5.3 Specific methods, paper III

Several formalin-fixed, paraffin embedded tissue blocks were prepared simultaneously from different parts of the pancreatic tail. Three dual immunostaining with anti-CD3/insulin, anti-CD3/glucagon and insulin/glucagon were performed on consecutive sections. Four μm sections from two different paraffin blocks from each of the six cases (in total 36 sections from 12 blocks) were processed and labelled using a standard immunoperoxidase technique for formalin-fixed paraffin-embedded sections. Slides were then counterstained with haematoxylin and analysed by light microscopy. The total number of islets was registered by counting glucagon-containing islets. Only islets containing at least 5-10 endocrine cells were registered, while single glucagon or insulin positive cells scattered in the exocrine area were not. All islets that contained at least one insulin-positive cell were counted and their endocrine area estimated using an ocular grid consisting of one hundred 0.0016 mm² squares at a magnification of 200x. For each insulin-containing islet (ICI) the number of CD3-positive cells within the islet area (intra-insulitis), and in the endocrine-exocrine interface in close contact with the islet boundary (peri-insulitis) was counted and registered. The new definition for insulitis was used, meaning that the islet should contain or be closely surrounded by ≥15
leukocytes (CD45+ cells) to be considered insulitic (21), but with the modification that only T cells (CD3+) were counted. By combining these counts, the percentage of four different categories of islets (85), indicating the progress of disease, could be calculated:

- Category A: Insulin containing islets (ICIs) without insulitis
- Category B: ICIs with insulitis
- Category C: Insulin deficient islets (IDIs) with insulitis
- Category D: IDIs without insulitis

To normalize the number of CD3-positive cells per islet to a Standard Islet Equivalent (IEQ), the islet area was divided by the area of an average standard cross-section of an IEQ. In the field of islet isolation and in the consensus statement on insulitis (21) an IEQ is defined as an islet of 150 µm diameter, giving an average standard cross section area of 0.010936 mm² ($D_{\text{cross-section}} = (\pi/4)D_{\text{Sphere}}$). To estimate insulin area and the area of connective tissue and number of CD3+ cells in exocrine tissue, 15 randomly placed grids, constituting a total area of 2.4 mm², in each section were analysed by registering the content in each of (121x15) 1815 intersections.

5.3.1 Data Analysis

Differences between group pairs were analysed by Wilcoxon matched-pairs signed rank test, or between groups by Mann-Whitney test, using GraphPad Prism version 6. Findings were considered statistically significant at p<0.05.
5.3.2 Laser-capture microdissection

Frozen tissue samples from the cases with recent onset T1D, from three organ donors without pancreatic disease and needle biopsies from rejected kidneys were prepared for laser capture microdissection (LCM), essentially as described in (86). Sections stained for CD3 were used to identify and localize infiltrating T cells in the T1D islets and in the rejected kidneys. Sections consecutive to those stained for CD3 were used to microdissect islets with insulitis and inflamed renal cortex. Islets without insulitis from the three non-diabetic organ donors were used as control.

5.3.3 RNA isolation and expression analysis from microdissected tissue

Total RNA was isolated from the microdissected tissue and synthesis and preamplification of cDNA was performed. Expression analysis was performed on the preamplified cDNA with an array containing a panel of 84 T and B cell genes and five reference genes. ACTB and GAPDH were chosen for normalization since they are commonly used reference genes in human islets and displayed little variation between samples. To compare expression levels in immune cells between infiltrated islets and infiltrated kidney, PTPRC (CD45) was used for normalization.

5.3.4 Islet isolation

Islets from the T1D cases and non-diabetic controls were isolated as described.

5.3.5 Cytokine/Chemokine analysis

Islets (100 handpicked) from the six T1D patients and six non-diabetic controls were analysed for 41 different cytokines and chemokines.

5.3.6 Whole transcriptome sequencing and analyses

RNA was extracted from isolated islets from the T1D cases and from three non-diabetic controls and whole transcriptome sequencing was performed and analysed as described (87).
5.4 Specific methods, paper IV

5.4.1 Islet isolation

Pancreatic islets were isolated in Uppsala as described. Isolated islets were cultured for a few days and aliquots of the culture medium were collected 1, 3, and 6 days post isolation for enterovirus PCR analyses. For practical reasons, the PCR-studies were not blinded, but both the cases and controls were studied in parallel in the same PCR runs in two laboratories (Uppsala and Tampere).

5.4.2 Detection of enterovirus

Immunostaining with clone 5D8/1 (Dako, Glostrup, Denmark) to detect enterovirus capsid protein 1 (VP1) was performed in two laboratories (Tampere and Exeter). Consecutive four μm sections from two different paraffin blocks from each of the cases and from one block from each of the controls were processed and labelled using a standard immunoperoxidase technique for formalin-fixed paraffin-embedded sections.

5.4.3 Auto-antibody analyses

Diabetes-related auto-antibodies (Glutamic acid decarboxylase autoantibodies (anti-GAD), insulin autoantibodies (IAA), autoantibodies targeting the phosphatase-related IA2 molecule (anti-IA2) and zinc transporter autoantibodies (anti-ZnT8)) where measured at the Hormone Laboratory, Oslo University Hospital, Aker, Oslo, Norway.

5.4.4 Virus isolation

Virus isolation was undertaken in Uppsala. Culture medium from purified islets and exocrine cell clusters at different time points post isolation (days 1, 3, and 6) were inoculated onto monolayers of permissive (HeLa, RD, GMK and EndoC-βH1) cells. Primary human islets
were also cultured and inoculated with culture medium as described above. Cells were observed daily for evidence of a visible cytopathic effect and EV PCR was performed on cultures that demonstrated a change in cell morphology.

5.4.5 RNA extraction, RT-PCR and sequence analysis

RNA was extracted for analysis of viral RNA by RT-PCR in two laboratories (Tampere and Uppsala). The Uppsala laboratory extracted viral RNA from islet culture medium recovered from islets isolated from all six cases and six controls. The Tampere laboratory extracted RNA from culture media harvested after islet and exocrine cell culture (cultured in Uppsala), and from homogenized biopsies, PBMC samples, serum and stool samples. The presence of several different viruses was analysed using RT-PCR. Enterovirus and rhinovirus RNA was analysed using two independent methods, a qualitative RT-PCR method and a real-time RT-PCR method, both including a probe for the detection of virus specific amplification. In addition parechovirus, norovirus and rotavirus were analysed in pancreas extracts. A sample was considered positive if it tested positive in at least one of three parallel tests.

The Uppsala laboratory detected enterovirus RNA using a nested RT-PCR.

5.4.6 Sequencing of virus positive samples

Enterovirus positive PCRs from culture media and biopsies were sequenced at the core facility at Uppsala University.
5.4.7  **High-throughput RNA-sequencing**

In Oslo, total RNA from approximately 30 mg frozen (-80°C) whole tissue from patients 1-6 was isolated. Quality of the RNA isolates was sufficient for sequencing. Sequencing was done on a HiSeq 2000 instrument (Illumina).

5.4.8  **Analysis of RNA-sequencing results**

Raw sequences were generated and demultiplexed, and the quality of sequences was evaluated. Estimates using 100k random single reads from each sample showed that the rRNA content in generated sequences ranged from approximately 4-47%, while the total number of reads not mapping to human RNA or DNA ranged from 6-12%. Sequences were filtered for low quality reads and sequencing artefacts. RNA-sequencing data was searched for viral sequences.

5.5  **My specific contributions**

As being apparent from the comprehensive list of methods used in analysing the collected specimens, the work presented in this thesis is obviously based on extensive cooperation with a long list of researchers at home and abroad. The results in DiViD have been created because clinicians and basic scientists have met with a common goal, which have been to reveal some of the mysteries of the pathogenesis of T1D. Therefore, DiViD fits well with the term “translational research”, which is defined by The National Institutes of Health (NIH) as the movement of discoveries in basic research to application at the clinical level. In all translational research, the main contributions from the clinicians will be to facilitate possible research from the basic researchers simply by connecting the world of science with the world of clinics.
In more details, my main contributions in DiViD have been:

- Planning the study in all details: writing the protocols, apply for approvals, establish agreements with collaborators
- Implementation of recruitment of the patients: provide information to doctors, nurses and patients at different hospitals, examination before and follow up of the patients after the procedure
- Handling the samples in the operation theatre.
- Coordinating and planning all the investigations: shipment of samples, arranging collaborative meetings, collecting and interpretation of the results
- Specific laboratory analyses: counting all sections stained for insulin, glucagon and CD3 (paper III)
- Authoring all the manuscripts
6 Results, summarized

6.1 Paper II

All human transcripts involved in the insulin pathway were present in the islets of Langerhans collected from the DiViD-cases. Glucose-induced insulin secretion was present in some of the cases, and a normalized biphasic insulin release was obtained after some days in a non diabetogenic environment in vitro for two of the cases. This shows a dysfunction in insulin secretion at onset of T1D and indicates that the potential for endogenous insulin production is good, which could be taken advantage of if the disease process was reversed at diagnosis.

6.2 Paper III

All six DiViD-cases fulfilled the criteria for insulitis, and a total of 11% of all islets were inflamed. Of all the islets, 36% contained insulin, and 27% resembled completely normal islets. The majority (61–83%) of T cells were found as peri-insulitis rather than within the islet parenchyma. The expression pattern of T cell genes was found to be markedly different in islets compared with the rejected kidneys. The islet-infiltrating T cells showed only background levels of cytokine/chemokine release in vitro.

6.3 Paper IV

VP1 was detected in the islets of all type 1 diabetic patients (two of nine controls). Hyperexpression of class I HLA molecules was found in the islets of all patients (one of nine controls). Enterovirus-specific RNA sequences were detected in four of six patients (zero of six controls). The results were confirmed in various laboratories. Only 1.7% of the islets contained VP1+ cells, and the amount of enterovirus RNA was low. The results provide evidence for the presence of enterovirus in pancreatic islets of type 1 diabetic patients, which
is consistent with the possibility that a low-grade enteroviral infection in the pancreatic islets contributes to disease progression in humans.
7 Results in details

7.1 Paper II

7.1.1 Islet function

The mean glucose-stimulated insulin secretion (GSIS) was reduced in islets from T1D subjects (Fig. 1A). GSIS was lowest when islets were examined one day after isolation but seemed to increase after 3 and 6 days of in vitro culture (Fig. 1A). Islets from individual subjects with T1D had varying levels of GSIS (Fig. 1B). When examined on day 1 after isolation, islets from all subjects except case 6 had a very low or undetectable GSIS (Fig. 1B). After 3 and 6 days of culture, islets from two of the subjects (Case 1 and 2) secreted slightly increased amounts of insulin upon glucose stimulation, but did not display biphasic insulin secretion, whereas islets from case 4 which displayed a poor GSIS one day after isolation, recovered to a normal biphasic secretion after 3 and 6 days of culture. Case 6 displayed a close-to-normal GSIS already one day after isolation, and the insulin secretion levels increased further after 3 and 6 days of culture (Fig. 1B). For the rest of the cases (3, 5, 7 and 8) the insulin secretion remained low or undetectable after culturing. Islets from non-diabetic subjects responded with a bi-phasic insulin release.
Figure 3. Glucose-stimulated insulin secretion by isolated islets

Twenty handpicked islets were perifused with low glucose (1.67 mmol/L) for 42 min, high glucose (20 mmol/L) for 48 min, and then low glucose again as indicated. Fractions were collected at 6 min intervals and the secreted insulin was measured by ELISA. A: Mean insulin secretion from islets isolated from six T1D patients (case 1-6) and cultured for 1 (open circles), 3 (black squares) or 6 days (open squares), and from islets isolated from 15 organ donors without pancreatic disease (open triangles). B: Individual insulin secretion from islets from two organ donors with T1D (case 7-8) and from six live patients with T1D (case 1-6), cultured for 1 (open circles), 3 (black squares), or 6 days (open squares). Observational data.
7.1.2 Whole transcriptome sequencing

We generated a total of 362 million reads and mapped those to the human genome. Both cases and controls generated approximately the same number of mapable reads.

When comparing expression similarities, using RPKM values, across all genes, islets from 5 of the 6 live subjects (Case 1-6) were grouped together and separated from the brain dead donors (case 7 and 8 and control 1-3), regardless of whether the islets were from T1D or non-diabetic subjects (Fig. 4A), reflecting the major impact of brain death on the results obtained from whole transcriptome analysis. Similarly, genes that are part of the complement system pathway also reflected the differences between islets from live and brain dead subjects (Fig. 4B). By contrast, genes in the insulin secretion pathway grouped islet samples irrespective of T1D, resulting in longer branch lengths compared to the complement system pathway (Fig. 4C).

In all cases, except case 6, the genes involved in the secretion of insulin and the insulin biosynthetic pathway, were less expressed when compared to the controls (Fig. 5). This included all the genes in the insulin pathway, both the insulin gene INS itself, and the genes involved in production and release of insulin. In case 6, all the genes in the pathway were up-regulated compared to the non-diabetic controls. INS was expressed at about ten-fold lower levels in all other diabetic samples, together with low expression of two upstream regulators, the pancreatic and duodenal homeobox 1 (PDX1), and MAFA, a transcription factor that binds RIPE3b, a conserved enhancer element that regulates pancreatic beta cell-specific expression of INS (88). Additional genes that were consistently lower in expression include (a) the adenylate cyclase activating polypeptide (PACAP) and its receptor PACAPR; (b) FFAR1, a member of the GPR40 family of G protein-coupled receptors which may be involved in the
metabolic regulation of insulin secretion; (c) G-protein controlled integral membrane protein and inward-rectifier type potassium channel (KCNJ11); (d) ATP-binding cassette transporter of the MRP subfamily (ABCC8), which is involved in multi-drug resistance but also functions as a modulator of ATP-sensitive potassium channels and insulin release; (e) calcium-activated nonselective ion channel that mediates transport of monovalent cations across membranes (TRPM4); and (f) three major glucose transporters in the mammalian blood-brain barrier, which are found primarily in the cell membrane and on the cell surface, where they also function as receptors for human T-cell leukaemia virus I and II (GLUT1/2/3). By contrast, the muscarinic cholinergic receptor CHRM3, and the G-protein coupled receptor CCKAR, which binds non-sulphated members of the cholecystokinin family of peptide hormones and acts as a major physiologic mediator of pancreatic enzyme secretion, were unchanged in expression, or were slightly more highly expressed.
Figure 4. Neighbour-joining trees based on expression similarity.
Shown are groupings for all genes (a), the complement system pathway (b), and the insulin secretion pathway (c). With the exception of sample case 6, live and brain dead donors cluster together in (a) and (b), while the insulin secretion pathway (c) splits diabetic from non-diabetic samples.
Figure 5. Insulin secretion pathway for all diabetic samples

We normalized expression by three brain dead controls and showed lower expression in blue and higher expression in red. With the exception of case 6, the majority of genes in the pathway were reduced in expression in the diabetic samples.
7.2 Paper III

7.2.1 Insulin content

Figure 6 shows the distribution of islets in the different categories in the 12 sections examined. On average, 36% of all islets investigated (n=1596) contained insulin, either with or without signs of insulitis. 74.2% of these insulin containing islets did not seem to be affected by the disease, resembling completely normal islets. In some areas of the pancreatic biopsies the insulin-positive area was completely normal. Large variations were observed regarding the percentage of ICI, both between individuals (18 to 66%) but also between sections from different formalin-fixed blocks from the same case (0 to 94% in case 6). The insulin area in the T1D cases comprised on average 0.58% of the pancreatic tissue (0.44 - 1.2%).

![Figure 6](image_url)

Figure 6. Histological analysis of all islets in two pancreatic sections (columns a and b) from each of the 6 cases with recent onset T1D. The islets were categorized as insulin-containing (ICI) or insulin-deficient (IDI) with (+) or without (-) insulitis. Insulitis was defined as >15 CD3+ cells in or in close association with the islet. Black bars: ICI/insulitis-; light grey: ICI/insulitis+; dark grey: IDI/insulitis+; white: IDI/insulitis-.
7.2.2 Insulitis and infiltrating T cells

All six cases fulfilled the criteria for insulitis (with at least three affected islets) (21). The variation between the cases was large. Only 6 of 130 (5%) ICIs were affected in case 6 (≥15 CD3 positive cells), versus 42 of 73 (58%) in case 1 (Table 4). There was also a large variation in the mean number of CD3 positive cells per ICI (peri- and intra-), ranging from 2.2 in case 6 to 31.6 in case 1 (Table 4). Although normalization of the number of CD3+ cells to the size of an IEQ (150 μm of diameter) changed the number of CD3+ cells in individual islets, the mean number of CD3+ cells per islet and the fraction of insulitic islets per case remained essentially the same (Table 4).

<table>
<thead>
<tr>
<th>Subject</th>
<th>ICI (n)</th>
<th>Area of ICIs, Mean mm²±SD, (median)</th>
<th>Number of CD3, Mean±SD, (median)</th>
<th>Number of Intra-islet CD3, mean±SD (median)</th>
<th>Number of Peri-islet CD3, mean±SD (median)</th>
<th>Intra CD3, % of all</th>
<th>ICI with insulitis, n (%)</th>
<th>≥15 CD3 per islet</th>
<th>≥15 CD3 per IEQ*</th>
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<tbody>
<tr>
<td>1</td>
<td>73</td>
<td>0.016±0.017 (0.008)</td>
<td>8.16±15.48 (2)</td>
<td>3.75±4.34 (2.3)</td>
<td>23.42±27.78 (16)</td>
<td>Per islet</td>
<td>Per IEQ*</td>
<td>29.63±115 (11.8)</td>
<td>6 (5%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Per islet</td>
<td>Per IEQ*</td>
<td>26%</td>
<td>42 (58%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Per islet</td>
<td>Per IEQ*</td>
<td>29.63±115 (11.8)</td>
<td>40 (55%)</td>
</tr>
<tr>
<td>2</td>
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<td>1.16±2.10 (0)</td>
<td>4.92±5.86 (3)</td>
<td>7.70±17.1 (2.9)</td>
<td>22%</td>
<td>9 (12%)</td>
<td>12 (15%)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Per islet</td>
<td>Per IEQ*</td>
<td>22%</td>
<td>9 (12%)</td>
</tr>
<tr>
<td>3</td>
<td>206</td>
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<td>3.24±5.14 (1.17)</td>
<td>18.70±21.55 (12)</td>
<td>22.20±26.07 (14.5)</td>
<td>17%</td>
<td>107 (52%)</td>
<td>114 (55%)</td>
</tr>
<tr>
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<td>55</td>
<td>0.016±0.014 (0.011)</td>
<td>7.36±11.53 (3)</td>
<td>5.05±6.68 (2.9)</td>
<td>11.35±14.11 (7)</td>
<td>11.24±15.87 (6.08)</td>
<td>39%</td>
<td>18 (33%)</td>
<td>21 (38%)</td>
</tr>
<tr>
<td>5</td>
<td>73</td>
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<td>3.71±5.65 (2)</td>
<td>2.71±2.98 (1.95)</td>
<td>6.56±6.04 (5)</td>
<td>8.45±13.35 (4.88)</td>
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<td>17 (23%)</td>
<td>18 (25%)</td>
</tr>
<tr>
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<td>130</td>
<td>0.009±0.010 (0.0064)</td>
<td>0.56±2.11 (0)</td>
<td>0.46±1.94 (0)</td>
<td>1.65±3.92 (0)</td>
<td>2.30±5.76 (0)</td>
<td>25%</td>
<td>6 (5%)</td>
<td>6 (5%)</td>
</tr>
</tbody>
</table>

Table 5. Peri- and intra-islet T cells in insulin-containing islets from the six cases with recent onset T1D.
*Each islet is normalized to the area of a Standard Islet Equivalent (IEQ) defined as an islet with a diameter of 150 μm or an average cross section area of 0.010936 mm2.
ICI; insulin-containing islet.

In total, 11% of all islets were infiltrated with ≥15 CD3-positive cells. Most of the inflamed islets, 82%, were ICIs. Again, large intra-individual (4 to 26%) and inter-individual (2 to 20 %
in case 5) variations were observed (Figure 6). Among the insulin-containing islets, 25.8 % had insulitis. In contrast, insulitis affected only 2.9 % of insulin deficient islets.

Significantly more CD3-positive cells were found in ICIs (328±232 per mm$^2$) than in exocrine tissue (51±27 per mm$^2$) (p<0.001, Figure 7). They were more frequently found at the periphery of the islet (peri-insulitis) than within the islet area (intra-insulitis) (Table 4, Fig. 8 and picture 2). This difference was significant (p<0.001) in all cases, but the fraction of total islet-associated CD3+ cells located inside the islets varied, from only 17% in case 3, to 39 % in case 4 (Table 4).

Figure 7. CD3 positive cells per islet and exocrine area (excluding peri-islet CD3) in sections from recent onset T1D.

Case 1 (solid circles), case 2 (open circles), case 3 (solid squares), case 4 (open squares), case 5 (solid triangles), and case 6 (open triangle). *; p<0.05 calculated on the mean of the two sections per patient
Figure 8. Peri-insulitis (Grey) vs Intra-insulitis (White)
Number of peri- (grey) and intra- (white) CD3-positive cells per insulin-containing islet (ICI) in two pancreatic sections from each of the recent onset T1D cases.

The total number of ICI, the percentage ICI of all islets, and the number and percentage of ICI with insulitis are shown. The boxes represent the inter-quartile range, the horizontal line the median, the plus sign the mean, and the whiskers the maximum/minimum.
Great variations in close neighbourhood. Pancreatic tissue from T1D cases stained for insulin, CD3 and glucagon.

(A) An islet with peri-insulitis where the majority of CD3+ T cells are found in the islet-exocrine interface and (B) an apparently healthy islet (bottom) located next to an islet with intense peri-insulitis (top). Examples of the three different double stains of the same area are shown in (C) CD3 red, glucagon brown, (D) CD3 red, insulin brown and (E) Glucagon red, insulin brown. The scale bar in the pictures (----) equals 100 µm.

7.2.3  Expression analysis of infiltrating cells

Of the 84 T and B cell genes studied, all except TLR1, CXCR3, TNFSF14, CD5 and TLR9 were expressed (Ct<35) in the infiltrated islets from at least two of the six cases. Islets from non-diabetic control donors had no or low expression of all genes analysed, except for CD47, DPP4, CD81, CD276, TGFB1, MAP3K7, and NCK1, which displayed significant islet expression and therefore were excluded from further analysis (Fig.10).
When compared with the expression pattern in cell-mediated kidney allograft rejection, the immune infiltrates in type 1 diabetic islets revealed a markedly different expression pattern (Fig. 11).

Figure 9. Expression profile of genes involved in T- and B-cell activation
Islets infiltrated with CD3+ T cells from 6 patients with recent onset T1D (A) and in uninflamed islets from three organ donors without pancreatic disease (B).
Figure 10 Expression of T and B cell genes
(A) In infiltrated pancreatic islets from six cases with recent onset T1D. (B) In kidney biopsies from two cases with ongoing cell-mediated allo-rejection. (C) Expression ratio islets/kidney. The expression of each gene is normalized to the expression of PTPRC (CD45)

7.2.4 Cytokines and chemokines in isolated islets
Luminex analysis of 41 cytokines and chemokines in isolated, handpicked islets from the six T1D patients showed signs of moderate inflammation, i.e. moderate levels of MCP-1, IL-8, IP-10 and GRO, that were not different from those in isolated islets from brain dead organ donors (Fig. 12A and B). 25 of the 41 analytes were above the detection limit in islets from at least one patient. Transcriptome data from the six T1D patients and three non-diabetic
controls confirmed that the expression of these 41 cytokines and chemokines were similar in the two groups (Fig. 12C and D).

Figure 11. Expression of cytokines and chemokines
Expression of cytokines and chemokines in isolated islets; proteins analysed by Luminex (a, b) and mRNA analysed by RNAseq (c, d). Data were collected from the DiViD-cases (a, c) and from non-diabetic controls (b, d).
7.3 Paper IV

All six type 1 diabetic patients were positive for enterovirus in the endocrine pancreas by at least one of the three methods used to detect either viral protein or viral RNA (Table 6). Four patients were enterovirus positive in the pancreas by two methods, one with three methods.

7.3.1 Detection of enterovirus genome

Enterovirus RNA was detected in the culture medium of the enriched islet preparation in three of the six T1D patients using RT-PCR in both the Uppsala and Tampere laboratories. Virus was detected in the medium harvested from islet cultures on days one and/or three, but not on day six (Table 6). In addition, islet culture medium from one patient was positive on day three in Tampere, and this patient was also positive in the remaining enriched exocrine cells, also containing some islets, in Uppsala. None of the islet cultures from six non-diabetic controls were enterovirus positive. A snap-frozen pancreas sample containing 30 mg of whole tissue was enterovirus positive in one of the diabetics in both laboratories. The same patient was also virus positive in the enriched islets and exocrine cell fractions as described above (Table 6). The amount of enterovirus RNA was low in all positive samples. The viral genome was partially sequenced in all four virus positive cases and the sequence showed a perfect match with enterovirus sequences (Table 7). Due to the low virus titre we were able to sequence only the conserved region of the genome. Therefore the exact genotype of the virus could not be identified. All cases and controls were PCR negative for rhinovirus, norovirus, rotavirus, and parechovirus in both islet enriched islets and exocrine cells as well as in snap-frozen whole tissue samples. High-throughput sequencing of total RNA extracted from whole frozen tissue from each patient did not detect any viral sequences.
7.3.2 Detection of enterovirus protein

Pancreatic islets from all six T1D patients were immunopositive for VP1 (Table 2), whereas this protein was detected in two of nine controls (100% vs. 22%; p < 0.01). Only 1.7% of the patient islets contained intense VP1+ cells (42 islets out of a total number of 2492 islets; immunostaining of consecutive sections for insulin and glucagon and for VP1). Altogether 60 intense VP1+ cells were identified in the islets.

7.3.3 Expression of class I HLA molecules

Hyperexpression of HLA class I molecules was observed homogeneously in the islets of all six T1D patients but in only one of the nine controls (p < 0.01) (Table 6). Among the six patients, all insulin containing islets showed HLA-class I hyperexpression irrespective of the presence of enterovirus VP1 protein. Picture 2 shows an insulin-containing islet with hyperexpression of HLA class I and the presence of enterovirus VP1 protein.
Table 6. Detection of enterovirus protein, enterovirus RNA and expression of class 1 HLA molecules in the pancreas of newly diagnosed type 1 diabetic patients.

<table>
<thead>
<tr>
<th>Case</th>
<th>Methodology</th>
<th>Tissues</th>
<th>Laboratory</th>
<th>Enterovirus specific RT-PCR</th>
<th>RNA sequencing</th>
<th>Class I HLA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IHC</td>
<td></td>
<td></td>
<td>Supernatant from cultured purified pancreatic islets</td>
<td>Snap-frozen pancreas 30 mg</td>
<td>Snap-frozen pancreas 30 mg</td>
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<td></td>
<td></td>
<td>Pancreatic islets</td>
<td></td>
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<td>Uppsala</td>
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</tr>
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</tr>
</tbody>
</table>

* Positive day 1 and 3
† Positive day 1
‡ Positive day 3
§ Positive in RNA extracted from culturing of the remaining cells, containing both exocrine and unknown number of islets

7.3.4 Detection of enterovirus genome in other materials (to be published)

Five out of six patients were EV positive by RT-PCR in at least one of the tested materials (Table 8). EVs were most frequently detected in the islet culture medium (four out of six patients). Case 6 was also EV positive in PBMCs, pancreatic tissue, duodenal biopsy and stools. Based on sequence analysis the detected virus was the same EV strain in stool, duodenal and pancreas samples and it was typed as echovirus 30 using the partial VP1 sequence (Fig. 2). However, the virus detected in cultured islets of the same patient was a different virus strain showing a variation in six nucleotides within the 91 long fragment of the 5NCR of the viral genome compared to the virus in stool.
In addition, EV was found in the stool samples from case 1 and 3. Case 1 was also EV positive in PBMCs while case 3 was EV negative in all other sample types. In case 3, the virus could be typed from stools by sequencing the VP1 region of the viral genome (Coxsackievirus A22, Fig. 14). Case 4 was EV positive in PBMC and islet cell culture supernatant. Serum samples were EV negative in all six patients (Table 1). In stool samples the virus loads were high, but in other sample types virus was present at very low titres. All patients were negative for rhino-, parecho-, rota-, and noroviruses in all tested sample types.
Table 7. Sequence alignment of PCR-products
Variable nucleotide sites between different cases are highlighted with yellow.

A. B. C. VP1-positive cells.

Picture 3. One single pancreatic islet from one of type 1 diabetic patient
A: Stained for insulin (brown) and glucagon (red). B: stained for HLA class 1 molecules. C: stained for enterovirus protein VP1. Cells positive for enterovirus protein are marked by arrows.
<table>
<thead>
<tr>
<th>Case</th>
<th>Purified islets</th>
<th>Snap frozen tissue</th>
<th>RNAlater tissue</th>
<th>PBMC</th>
<th>Serum</th>
<th>Duodenal biopsy</th>
<th>Stool</th>
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<td>10^3</td>
<td>10^5</td>
</tr>
</tbody>
</table>

Table 8. Summary of enterovirus RT-PCR results. The positivity is indicated as copy number (N) of the enteroviral RNA (real-time RT-PCR) or as the presence of enterovirus specific sequence in semi-nested PCR (POS).

Figure 12. Genotyping of enteroviruses detected in the stool samples. Viruses obtained from diabetic cases are marked in red and prototype enteroviruses with black font according to acronyms: CA = Coxsackievirus A, CB = Coxsackievirus B, E = Echovirus and EV = Enterovirus. Phylogenetic analysis is based on the partial aminoacid sequence of VP1 region. Bootstrap values over 75 are marked in the phylogenetic tree.
8 Discussion

8.1 Paper II

It is well-known that residual insulin production (as measured by C-peptide secretion) is correlated with a favourable long-term clinical outcome and with reduced risk of developing both micro and macrovascular complications, as recently reviewed by VanBuecken and Greenbaum (89). The original Eisenbarth model of T1D postulated an immune-mediated linear loss of β cells, with clinical diagnosis occurring when there was insufficient insulin secretion to meet glycaemic demand. However, recent studies have shown that mostly low level residual C-peptide levels can be present long after the onset of T1D (90). These observations do not allow conclusions as to whether the observed insulin production is from beta-cells surviving the immune attack, or from newly generated beta-cells.

As expected, the results in Paper II show that the expression of key regulatory elements of the insulin secretion pathway and GSIS were reduced in islets isolated from 5 of the 6 DiViD cases compared to islets from non-diabetic organ donors. However, in 1 of the 6 cases, the insulin pathway and GSIS were remarkably preserved on the day of islet isolation. Also, the observed GSIS improvement after three and six days of culture in 4 of the 8 cases suggests that when the islets are removed from the ‘diabetic milieu’, any remaining beta cells can recover. Soluble factors released within the vicinity of the islets could induce a functional impairment of the beta cells, which could be reversed after islet isolation and in vitro culture. However, a screening of 41 cytokines and chemokines in isolated islets from the six T1D patients showed no differences from those in isolated islets from brain dead organ donors (data not shown). It should be noted that both the procedure of islet isolation and the brain dead condition of the controls most likely increases the secretion of cytokines and chemokines (24;91), which might hide a possible difference between the DiViD-cases and the controls.
It is known that prolonged exposure of human islets to high glucose impairs beta cell function (92) and since even short episodes of hyperglycaemia dramatically affect glucose tolerance (93) it is possible that glucotoxicity could play a role in the reduced GSIS observed in islets from these patients. In 1940 Jackson described that intensive insulin therapy at diagnosis of T1D lead to a long partial remission of the disease (94). Later these findings were confirmed using C-peptide determinations (95). However, in an experimental transplantation model, where human islets were exposed to hyperglycaemia for 4-6 weeks in vivo followed by normoglycemia for 2 weeks (96) it was found that the deleterious effects of the diabetic state on human islet insulin release remained. This was true even if the hyperglycaemia induced impairment in glucose metabolism, depletion of insulin mRNA, decreased (pro) insulin biosynthesis, increased glycogen accumulation and depletion of insulin content was reversed by the 2 week period of normoglycemia.

Endoplasmic reticulum (ER) stress has been proposed as an important contributor to beta cell dysfunction in T1D (97). In this study, the gene expression of ER stress markers (BiP, CHOP, ATF4, and XBP1) was at the same level in islets from all subjects with T1D as in islets from the brain dead non-diabetic controls. The process of brain death has been shown to induce ER stress (98) and the finding of a similar level of expression of these genes in islets isolated from subjects with recent onset T1D might imply that the ER stress in these islets is a feature of T1D. The expression of the protein Wolfram syndrome 1 (WFS1) was lower in islets from subjects with T1D (both brain-dead and live subjects) than in islets from the control donors, with the exception of case 6 from whom the islets with almost preserved GSIS were isolated. It may be speculated that ER stress contribute to reduced beta cell function, possibly by
entrapment of and low expression of WFS1 thereby inhibiting the synthesis of cAMP and the secretion of insulin (99).

The level of transcription of the genes in the insulin pathway was reduced in most of the diabetic cases, compared to the non-diabetic controls. However, although reduced, all genes in the insulin pathway were expressed also in islets from the subjects with no or very low GSIS. This is in line with earlier studies reporting that insulin synthesis and storage were preserved in islets even many years after T1D onset (11,90), and show that the destruction of the β-cells is a slow ongoing process (90). A limitation in all studies of transcriptome profiling of an unfractioned tissue is that any changes observed could be due to differences in either modulation of gene expression or to changes in its cell composition. The current analysis was conducted with handpicked islets, thereby avoiding to a large extent the problem of analysing pancreatic biopsies in which the islets constitute only about 1% of the tissue. However, the insulin-producing beta cells constitute only about 60% of the total cell number in human islets. A reduction in the number of insulin producing cells in the islets isolated from subjects with recent onset T1D could be in agreement with the observation of a reduction in the insulin pathway.

Recently we have, in close collaboration with Professor Ivan Gerling, University of Tennessee, performed gene expression profiles of laser-captured islets from the DiViD patients and compared them with profiles from islets from non-diabetic organ donors collected through the network of pancreatic donors (nPOD). The results, published at American Diabetes Association 75th scientific sessions June 2015 (reference) showed enrichment of several gene ontologies (i.e. cellular respiration, respiratory electron transport chain, oxidoreductase activity) and pathways (i.e. oxidative phosphorylation, mitochondrial
dysfunction) in the diabetic patients, suggesting mitochondrial dysfunction and cellular stress in islets from the diabetic patients. These data, although still arriving from complete islets and not purely from beta cells as would have been ideally, may help to better explain the beta cell dysfunction observed in the perifusion experiments.

The most obvious limitation of the present study is the small number of cases examined. A further important consideration is the degree of matching between the tissues obtained from the patients with T1D and those used as controls. The DiViD study did not include pancreatic biopsies from healthy individuals and we therefore chose to employ tissue harvested by alternative methods. Non-diabetic organ donors were chosen as their clinical characteristics are well defined, all having normal HbA1c and being negative for 4 auto antibodies. The islets from the controls were not cultured and stimulated with glucose on day 3 and 6, but it has been shown previously that culturing of normal beta cells does not improve their insulin secretion ability (100).

We are also aware that the agglomerative clustering algorithm we have used was designed to study large data sets and assumes that the distance between clusters is additive. This assumption is rarely satisfied, particularly when there are only a small number of samples (101). Small sample sizes tend to show substantial variation in P values (102). Due to lack of material, we were not able to test whether beta-cell gene expression in the islets was restored after three or six days in euglycemic culture.
8.2 Paper III

All six patients showed substantial intra- and peri-islet infiltration of CD3+ T cells. A majority of these islets contained insulin, whereas insulitis was seldom observed in insulin deficient islets. The number of CD3 cells per islet varied greatly between cases despite the fact that the patients in this study were clinically homogenous (66). The causes of this variation are most likely the lobular pattern of disease progression with large variations between different pancreatic regions.

The consensus definition of insulitis, which requires at least 15 CD45+ cells in at least three islets for a positive diagnosis, does not take into account the huge variation in islet size of the tissue sections, leading to underestimation of inflammation in small islets and vice versa. In the field of clinical islet transplantation, a standard sized islet is defined as having a diameter of 150 μm (103). Based on this we propose that the number of inflammatory cells in an islet should be normalized to the average cross section of a 150 μm standard islet equivalent (150 μm * pi/4 ≈ 118 μm) to allow accurate comparisons between different studies.

Notably, 36% of all islets contained insulin and of these, 74.2% did not seem to be affected by the disease, resembling completely normal islets (27% of all islets); indeed, in some areas of the pancreatic biopsies the insulin-positive area was completely visually normal. This demonstrates a relatively large reservoir of insulin-producing cells at diagnosis of T1D. These findings also highlight the focal and temporal distribution of the injurious inflammatory processes ongoing in subjects diagnosed with T1D. The research field has, for several decades, communicated the message that most β-cells were already destroyed at onset, leaving no time and option to intervene during further disease progression. Recent publications have shown that most patients with a long-duration of T1D continue to secret low levels of endogenous
insulin (90;104), and our findings demonstrate that large numbers of insulin positive, albeit possibly dysfunctional, cells remain in subjects at onset of T1D. If disease progression could be halted, this beta cell volume would be of significant clinical importance.

All cases enrolled in this study had one or more auto-antibodies present at onset, and carried HLA DR3 and/or HLA DR4 haplotypes (66). The biopsies were collected in a laparoscopic procedure in the patients shortly after diagnosis and when near-normoglycemia had been reinstated by exogenous insulin administration, thereby excluding superimposed metabolic phenomenon such as ongoing ketoacidosis or post-mortem alterations potentially influencing the local conditions in the pancreas. The size of the resected specimens from the pancreatic tail allowed us to investigate several formalin-fixed blocks from each patient, which is important given the great variation in the presence of insulitis and other disease-related processes across the pancreas (105). One obvious weakness of our study is the limited number of cases examined; even so the study presented here is the largest study on well-preserved pancreatic tissue from cases examined at onset of T1D undertaken anywhere.

The prevalence of insulitis in the age group between 15-39 years with a duration of disease of less than 1 month has been reported to be only 29% (10 of 35 cases) (18). The previously reported higher prevalence of insulitis in children than in young adults has led to speculation that the aetiology of T1D is more heterogeneous in adults (106). Our results indicate that the difference between children and adults is possibly overestimated, and that insulitis might be as frequent in adults, as in children (107). This corresponds with the results in a thoroughly conducted study recently published that found insulitis in 11 out of 11 organ donors with type 1 diabetes, of which 5 had disease duration between 0.25 and 1.5 years (108).
A striking finding in this study is that the vast majority of islet-associated T cells were located outside the islet parenchyma at the exocrine-endocrine interface (peri-insulitis). The pathogenic role of these T cells is unclear, but since they are not in direct contact with the endocrine cells they are unlikely to bind to islet auto-antigens presented on HLA molecules on the beta cells. Previous studies using tetramer staining to detect T cells with reactivity to distinct beta cell antigens showed the presence of only occasional T cells in the periphery of the islets (109). Notably, the frequency of beta cell-specific CD8+ T cells in peripheral blood is similar between newly diagnosed T1D cases and HLA matched healthy controls (110). The failure of the CD8+ T cells to invade the islet and mediate efficient beta cell lysis could potentially find its explanation in the extraordinarily weak affinity between the T-cell receptor (TCR) and the HLA class I presenting the postulated T1D auto-antigen (111;112). TCR promiscuity is abundant at such low levels of affinity and it was recently reported that these TCRs recognize more than a million different peptides presented on the appropriate HLA class I allele (113). The qualitative and quantitative difference in T and B cell activation in the insulitic lesions in T1D by comparison with that observed during on-going allograft rejection (Fig. 10), as well as the baseline cytokine/chemokine expression in isolated islets from T1D cases when compared to non-diabetic organ donors (Fig. 11), are in agreement with previous observations (109-113) and argue for a different role of the CD3 + T cells in T1D when compared to the CD3+ T cells observed in allograft rejection. However, when compared to non-diabetic controls, cases with recent onset T1D showed the presence of an increased number of T cells in the peri-islet area, and their role in T1D remains to be clarified. For example, they may be merely passive bystanders located in the inflamed peri-islet area and unable to access the beta cells within the islets? Peri-insulitis may also be an earlier event than intra-insulitis, the latter being dependent of a degradation of the basal membrane surrounding the islets. Elucidating the role of these T cells is urgently important to obtain an
in-depth knowledge of the immunopathology of T1D in order to develop more efficient intervention therapies, cf. previously reported intervention trials in T1D focusing on blocking T effector cells using drugs highly efficient in protecting an allograft have shown suboptimal protection of beta-cell function. In the best situations, C-peptide production could be preserved temporarily and insulin requirements reduced, yet no durable effects could be achieved (114-117).

Isolated islets from the T1D patients expressed a number of chemokines (i.e. MCP-1/CCL2, IL-8, IP-10/CXCL10, and GRO/CXCL1) involved in leukocyte recruitment, but the levels were not different from those expressed in islets from brain-dead non-diabetic control donors, and were significantly lower and with a much narrower repertoire than was demonstrated by Eizirik et al (13) in islets stimulated with IL-1β and IFN-γ (Fig. 13). The low or absent expression of most of these chemokines and cytokines in islets from the T1D cases described here fits well with the relatively low frequency of intra-islet T cells and lack of signatures in the transcriptome analysis of activation (Fig. 12).
Figure 13. Data extracted from whole transcriptome analysis performed by Eizirik et al. Islets isolated from non-diabetic organ donors, untreated (A) or cultured with IL-1β and IFN-α (B).
8.3 Paper IV

This study of fresh pancreatic tissue collected close to the time of diagnosis of T1D shows that a low-grade enteroviral infection is sustained within the islets of Langerhans. Thus, enterovirus RNA was amplified successfully by RT-PCR from four of the six patients in two independent laboratories. The presence of enterovirus sequences was also confirmed by sequencing of the PCR products. Moreover, the enterovirus capsid protein VP1 was detected immunohistochemically in islet cells of all six patients. The expression of VP1 is known to be most intense during the acute phase of an enterovirus infection (118) while it is reduced during persistent infection (119). This is because enteroviral persistence is characterized by naturally-occurring deletions at the 5′ terminus of the genome (120;121), which reduce the replication rate of the virus. It was recently shown that terminally deleted viruses also can persist after inoculation of murine pancreas in the absence of cytopathic virus, for weeks beyond the acute infectious period (120). Hence, these results are consistent with the possibility that a low-grade enterovirus infection had been established and maintained in the islet cells of the patients with T1D. The biopsies were taken from the pancreatic tail, not overlooking the possibility that the infection could be affecting other parts first and is then slowly disseminated to the rest of tissue. The lack of virus induced cytopathic effects in islets and exocrine cell clusters during 3-5 days of culture, indicate that the virus is not powerfully cytolytic. This implies that the virus might be rendered replication deficient during the development of a persistent infection.

It is well understood that the amplification achieved by PCR allows for the detection of even very small quantities of target RNA and it is thus significant that four of the T1D patients were positive for viral RNA when analysed by RT-PCR in two different laboratories. The detection of positive signals required as many as 40 cycles of amplification or the use of a
nested RT-PCR method, indicating that only very small amounts of viral RNA were present. Sequencing confirmed that amplified sequences originated from enteroviruses.

Case 6 is particularly interesting because two different enterovirus strains were identified. Echovirus 30 was detected at multiple anatomical sites including pancreas, stools, duodenal mucosa and blood (PBMCs). The viral copy numbers were high in stools and duodenal mucosa suggesting that the virus was actively replicating in the gut, causing an acute systemic infection that had spread to the pancreas. As the patient had neither clinical symptoms nor supportive laboratory corroboration, this infection must be considered subclinical. Sequencing of the viral genome confirmed the presence of the same virus strain in stools and in the pancreas. The virus was also isolated from stools and could be replicated in A549 cells confirming that it was infective. As the viral titres were very low in PBMCs and pancreas, and serum was completely negative, the peak of virus replication and possible viremia may have occurred earlier.

Another enterovirus strain was present in case 6 in the culture medium of purified islets, but could not be detected in other tissues. This virus could not be genotyped due to its low titre, but nucleotide alignment of part of the 5’UTR of the viral genome clearly shows that the two strains are different (see below). This strain might represent a chronic, low virulence infection located solely in smaller regions of the pancreas, for which it could not be discovered in the frozen pancreatic specimen from a slightly different region of the pancreatic tail.
Figure 14. Sequence alignments of viruses detected in different samples of T1D patients. Number in the codes indicates the patient number. Various sample types were sequenced: “PBMC” refers to peripheral blood mononuclear cell sample, “cultured” is media of cultured islets, “panc” is snap frozen sample from pancreas and “stool” refers stool sample.

The high sensitivity of PCR makes it susceptible to false positivity due to viruses which may contaminate the samples during the analysis. Several actions were taken to avoid such contamination and to detect them if they occurred. First, the two virus laboratories obtained the same results even though they carried out all RNA extraction and RT-PCR steps independently and used different primers and PCR protocols. Secondly, the amplified enterovirus sequences in the different patients differed from each other suggesting that they had originated from different enterovirus strains, thus excluding a common contaminating virus. Thirdly, culture medium from exocrine cells which were isolated from the same pancreases at the same time and place were all, except case 6, PCR negative. As also the isolated exocrine cells contain an unknown, but not negligible endocrine fraction, this positivity is not a surprise. Virus negative internal control samples included in each test run were also negative. In addition, all pancreas samples were PCR negative for all other tested viruses. Finally, control pancreases from non-diabetic individuals were PCR negative. Although the control pancreases were processed in a laboratory dedicated to clinical isolation, after isolation they were cultured in the same laboratory as the DiViD islets and processed for virus PCR using exactly the same procedures and in the same laboratory as the samples from
diabetic patients. It should be noted however, that the control donors were not matched to the DiViD patients and the islets were cultured and analysed several days after isolation, at a time point where also the DiViD islets were negative for Enterovirus. Thus, they serve mainly as a control to the PCR procedure.

The antibody used to detect VP1 is known to recognize this protein from multiple different enteroviruses in formalin fixed samples (122). However, it has also been shown that, under some conditions, the antibody may label certain human proteins (48). We were careful to employ the antibody under conditions optimized to avoid such interactions without compromise of virus-specific binding (123). Thus, we are confident that the immunolabelling achieved in human pancreas sections is likely to represent the presence of viral protein.

It should be noted that only 1.7% of the islets contained some of the overall 60 intensely VP1+ cells detected. Considering these relatively low numbers, one might think that 60 VP1+ cells in 2,492 islets are not many. However, the importance of this finding should not be downplayed. It has recently been proven in NOD mice that enteroviruses and, more specifically, coxsackie viruses can persist in the pancreas as terminally deleted forms with slower replication and no evident cytopathic effect for at least 35 days after inoculation (16). If this were also to be shown for human pancreata, we might be able to detect persistent viral forms and in some cases prove that an enterovirus infection took place in the pancreas and islets before the onset of disease. The low levels of viral protein in the biopsied samples could be a reflection of one or several previous acute infections, but many terminally deleted forms could also be present. The disparity between the fact that 6 of 6 cases were positive for VP1 while 4 of 6 cases were positive for EV-RNA have previously been observed also in other tissues like heart muscle (124) and stomach (125), and might be due to methodological
challenges regarding the PCR-analyses. In collaboration with Antonio Toniolo, we are attempting to overcome these challenges by conducting amplification of the possible viruses in an endothelial cell culture followed by extraction of RNA, gene amplification and sequencing. These results are still pending, but preliminary data suggest enterovirus genome positivity in all 6 cases. The results must be quality assured before they are to be published.

The detection of two different enteroviruses in cases 6 discussed above support a modification of the Eisenbarth-model suggested by Rodriguez-Calvo and von Herrath (126), suggesting that several infections causes several waves of beta-cell destruction, as illustrated below (by courtesy).

![Timeline of mass decline](image)

Figure 15. Timeline of mass decline
Reproduced with kind permission from Rodriguez-Calvo and von Herrath (126)
In addition to virus specific markers, the expression of class I HLA molecules was upregulated in the islets of all T1D patients. This may be considered a “viral footprint” and fits with previous observations showing that pancreatic islets of T1D patients hyperexpress class I HLA molecules and interferon-alpha (127;128). This might indicate ongoing virus-induced interferon secretion in the islets. It has previously been shown that enterovirus infection in human pancreatic islets leads to such HLA class I hyperexpression in vitro, partly mediated by secretion of type 1 interferons (129).

This study did not include pancreatic biopsies from healthy living individuals. Although not ideal, the non-diabetic organ donors from nPOD are clinically well defined and age matched to the cases. The mean age of the subjects from whom islets were isolated for the culture studies was higher than the cases, but we would emphasize that these served mainly as methodological controls in PCR analyses being negative for all tested viruses in PCR.
9 **Overall conclusions**

1. A significant insulin reserve reservoir is present at the diagnosis of T1D. In 36% of all islets, insulin-containing beta-cells were identified.

2. At onset of T1D, both a loss of beta cells and a dysfunction in insulin secretion in remaining beta cells contributes the observed insulin deficiency in patients.

3. Significant inflammation – insulitis - was present in all cases as defined by the consensus criteria. Most of the observed T-cells were located outside the islets in the exocrine/endocrine interface (peri-insulitis). Expression analyses of T and B-cell-genes in the islets revealed a marked qualitative and quantitative difference in activation compared to T-cells collected from T-cell mediated kidney transplant rejection.

4. DiViD is the first study of living newly diagnosed type 1 diabetic patients to demonstrate the presence of enterovirus in pancreatic islets using multiple techniques across several independent laboratories including the detection of enterovirus specific sequences in the culture medium of isolated islets.
10 Concluding remarks and general discussion

A significant insulin reserve reservoir is present at the diagnosis of T1D. The findings illustrate the importance of β-cell dysfunction, and not only a loss of insulin-producing cells. The restoration of specific function in some of the cases in the isolated islets removed from the diabetogenic milieu should encourage further characterization of the underlying mechanism(s) of this functional impairment. Hopefully, this will allow initiation of clinical intervention trials specifically aimed to restore beta cell function alone or when combined with drugs targeting the injurious processes ongoing within the pancreas at onset of T1D.

Insulitis was present in all cases and fulfilled the new criteria for insulitis. Although great variability exists, many islets contained insulin and several resembled completely normal islets, showing a significant reservoir of insulin. The islet infiltrating T cells showed only background levels of cytokine/chemokine release and displayed marked differences at the messenger RNA level when compared with allogeneic T cells. Understanding the role of these infiltrating immune cells is the key to developing efficient intervention strategies designed to spare the significant remaining reservoir of beta cells present at onset of T1D.

DiViD is the first study of living newly diagnosed type 1 diabetic patients demonstrating the presence of enterovirus in pancreatic islets using multiple techniques across several independent laboratories including the detection of enterovirus specific sequences in the islet culture medium. The results do for certain not prove causality between enterovirus infection and T1D, but still they support the view that a low grade enteroviral infection is present in the islets of Langerhans at diagnosis of T1D. These findings should encourage studies in which anti-viral medication and/or vaccines against enteroviruses are tested in attempt to reduce disease progression or prevention of T1D.
10.1 Limitations in DiViD

Even though DiViD represents a unique cohort of T1D patients, and has already provided important knowledge regarding the pathogenesis of the disease, it has several important limitations.

The most obvious limitation is the small number of cases. As previously explained, this is due to the unexpected number of complications experienced, which made it unethical to continue recruiting patients to the study. Two out of six patients (33%) suffered from a postoperative leakage of amylase-rich pancreatic juice from the margin of the resection. Today, distal laparoscopic pancreatectomy is still recognized as the gold standard treatment for small tumours of the pancreatic body-tail, with several advantages over the traditional open approach in terms of patient recovery (130) and is considered safe and feasible. That of course has to be considered in light of the indication for the procedure. Whether the method is improved further since we stopped recruitment in December 2012 is uncertain, but unlikely. In three recent publications, the incidence of pancreatic fistula ranges varied considerably irrespective of the approach (131-133)]. The wide range, due to different definitions and classifications of fistula adopted by different centres, makes it difficult to determine with certainty if the risk of fistulas has decreased. This has to be documented in each surgery unit. Nevertheless, until the risk of complications is reduced with certainty, or there are clear possible benefits for the patients based on findings in the collected specimen, distal laparoscopic pancreatectomy should not be performed in T1D patients. The possible presence of different subtypes of T1D distinguishable by differences in the pancreatic pathologies (134), thereby leading to different intervention strategies for recovery, may be a potential benefit justifying the procedure in the future. Nevertheless, it should be emphasized that a marker for these pancreatic differences in peripheral blood would be preferable.
Another major challenge when interpreting the results from the DiViD-cohort is the lack of a non-diabetic control group undergoing the same surgical procedure and tissue sampling. When planning DiViD, different possibilities for control groups were discussed. As described, we decided to use otherwise healthy, but diseased organ donors. In retrospect, it might have been better to collect pancreatic tissue from patients undergoing the same distal laparoscopic pancreatectomy for various other reasons (mainly tumours, benign or malign). That would have been possible and ethical, as these patients must invariably undergo operations. The reasons we did not initially choose such patients as controls were uncertainties about how the tumour and ongoing pathological process in the pancreas could possibly affect the tissue, even though located more distally and outside the tumour itself. Also very few patients in the age group of DiViD (18-40 years) undergo such surgery. Nevertheless, this would have enabled us to process the collected specimens exactly the same way as we processed the DiViD-samples, which would have been an unquestionable advantage when interpreting our results. The optimal choice of controls would undoubtedly have been completely healthy volunteers. If and when planning a similar study to DiViD in the future, the surgical procedures ought to be safe enough to include such healthy volunteers as controls. That will ensure an optimal scientific quality in respect to the collected material. Equally important, this approach would have made blinded analyses much easier to carry out.

However, the DiViD study is, so far, the largest cohort of well-preserved pancreatic tissue from individuals examined at onset of T1D, allowing molecular characterisation that cannot be performed elsewhere. Also, our data may supplement the relatively limited cohort of recent-onset T1D cases described previously.
11 Possible implications for our current understanding of T1D

Even though the DiViD-cohort consists of only 6 cases of T1D, the cohort has several important advantages that should not be underestimated. In fact, freshly frozen well-preserved tissue allowing an in-depth molecular characterization of the events so soon after T1D onset is extremely rare. Research to explain and understand T1D is, for obvious and already stated reasons, most often performed in either mouse models, specimens collected from cadavers or at its best from organ-donors. In a recent paper discussing the issues that pose a major stumbling point in T1D research, the authors noted that “perhaps past research into new treatments has relied too heavily on studies of animal models, in-vitro systems and, in the case of human research, scrutiny of peripheral blood rather than the human pancreas” (135). The DiViD-cohort consists of pancreatic material from live patients, soon after the onset of T1D, with no other ongoing illnesses or medications possibly affecting the local conditions in pancreas. Therefore, our results are extremely valuable.

Two questions to discuss are:

1. Is the common and established previously referred notion, expressed in over 50 000 scientific publications, that T1D is a T-cell-mediated autoimmune disease really true? In a provocative and much cited review, Skog et al debated that there is limited support for T1D being primarily an autoimmune disease (136). Based on the absence of (or at least sparse) insulitis in a majority of T1D cases, the slow progression of the disease and the very limited benefit of potent immunosuppressive drugs, the authors argued that T1D should be regarded as an innate inflammatory disease affecting the entire pancreas. How does the findings in DiViD support or weaken these two seemingly conflicting theories?
2. What is the triggering factor for the observed (auto) immune response observed in T1D?

11.1 Is T1D an autoimmune disease?
Autoimmune diseases arise from an abnormal immune response of the body against substances and tissues normally present in the body (autoimmunity). Although much is known about the pathogenesis behind an autoimmune disease, the aetiology is very often unknown. For a disease to be regarded as an autoimmune disease it needs to answer to Witebsky's postulates, first formulated by Ernst Witebsky and colleagues in 1957 (137) and modified in 1994 (138):

- Direct evidence from transfer of disease-causing antibody or disease-causing T lymphocyte white blood cells
- Indirect evidence based on reproduction of the autoimmune disease in experimental animals
- Circumstantial evidence from clinical clues
- Genetic evidence suggesting "clustering" with other autoimmune diseases

T1D has until recently been perceived to be a consequence of progressive loss of beta-cell mass caused by an immune attack from T-cells against the insulin-producing beta cells, and hence having an autoimmune pathogenesis. It should be emphasized this pathogenesis does not exclude an environmental trigger, implicating the aetiology of T1D being the previously described combination of genes, environmental factor(s) and abnormal immune response. This view of T1D as a T-cell-mediated autoimmune disease leading to beta-cell destruction has recently been challenged (139) for several reasons. Among the reasons for this are the observed involvement of the innate immune system (13), and the observed pathogenic role for
disease initiation in the NOD-mouse model for neutrophils, plasmacytoid dendritic cells and B-cells (140). In respect to this, it is noteworthy that we did observe an ongoing T-cell insulitis in all six cases. This is a novel finding in adults with T1D, also recently reported by Reddy et al. (108), pointing towards insulitis as a pathognomonic feature not only in children with T1D but also in older patients. However, it is important to notice that few signs of T-cell activation were present. This is questioning the exact pathological role of the infiltrating T-cells in T1D. Are these T-cells the driving force of the pathological process, and thereby actively destroying the beta-cells, or are they cleaning up after self-destructing processes going on in the beta-cells or at least inside the islets of Langerhans induced by an external factor? Already in 1986 this question was raised by Bottazzo (141), and 30 years later the jury is still out (142).

Even though insulitis seem to be pathognomonic for T1D in both children and adults, still there are important differences in the character of the inflammation in different age groups. Two distinct types of insulitic lesions distinguishable by the degree of cellular infiltrate and presence of B cells in number, termed “hyper-immune” or “CD20Hi” and “pauci-immune” or “CD20Lo” have recently been reported (91). The CD20Hi group, defined as mean of > 3 B-cells/inflamed islet consists mainly of young patients below the age of 18, while the CD20Lo (mean of <3 B-cells/inflamed islet) profile occurs in older patients > 18 years. The authors conclude that different immunopathological processes (endotypes) may underlie T1D, carrying important implications for treatment and prevention strategies. When staining sections from the DiViD-cases for CD20, we found that they all categorize as “CD20Lo”, completely as expected due to their age at onset of disease (134).
The DiViD results show that at the onset of T1D, the islets display different stages of pathology, some apparently normal and others completely devoid of insulin-producing cells. The functional studies of insulin secretion from live islets show that insulin production might be improved, or even restored in vitro, and suggest that this may also be true in vivo if only the unknown drivers of pathology were halted. The immunohistochemical analyses confirmed this potential, identifying a large number of insulin-positive, although (perhaps) dysfunctional cells remain in subjects at onset of T1D. If disease progression could be halted, this beta cell volume would be of significant clinical importance, especially if their insulin secretion capacity in response to glucose could be further improved.

Even so, maybe the most interesting islets described in DiViD are the ones with an ongoing inflammation. Sampling pancreatic material very close to the diagnosis of T1D led to an opportunity to study the pathological processes while they were actually happening. This is of great advantage compared to pancreatic tissue from patients with long-standing T1D, or from patients diseased with serious major acute complications (diabetic ketoacidosis or hypoglycaemia, possible affecting the local conditions in the pancreas). As previously stated, insulitis was clearly present in all the DiViD-cases. The majority of the T-cells were located in the exocrine-endocrine interface, outside the actual islet parenchyma and outside the basal membrane of the islets. To understand the pathogenic role of these T-cells better will be important. To explore that we will perform gene expression analyses of different LCM-areas, with or without T-cells, within and outside the islets. This might explain the role of the T-cells in T1D better; are they merely passive bystanders or active contributors even when located in the peri-islet area, not in direct contact with the endocrine cells? Or are they caught just in a migration process of invading the islets, where they subsequently will destroy the remaining beta-cells?
11.2 Is T1D a viral disease?

Given the presence of insulitis in most patients with T1D, the most crucial questions remain the same: what triggers the observed insulitis and the beta cell destruction? We have shown the presence of enterovirus in both pancreas and other materials, leading to speculation if viruses could be the actual trigger of the immune system in genetically susceptible individuals. A model explaining how enteroviruses might accelerate or precipitate T1D in some individuals has recently been proposed by Morgan and Richardson (56). The authors argue that replication-deficient viruses are generated during an initial infection elsewhere in the body before reaching the beta-cells in the pancreas. These viruses rarely form functionally competent virions that can be released to infect neighbouring cells, but they lead to the formation of double-stranded RNA, which stimulate the production and release of interferons and subsequent upregulation of MHC class I. This hyperexpression enhances the antigen presenting efficiency, leading to the observed immune response involving beta-cell specific T-cells and B-cells of unknown specificity.

When exploring the potential causality between enteroviruses and T1D, it will wise to remember the Koch’s postulates regarding infectious diseases from 1884, originally stating:

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.

2. The microorganism must be isolated from a diseased organism and grown in pure culture.

3. The cultured microorganism should cause disease when introduced into a healthy organism.
4. The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

As modern nucleic acid-based microbial detection methods have made Koch’s original postulates less relevant, they have been revised as follows (143):

1. A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease. Microbial nucleic acids should be found preferentially in those organs or gross anatomic sites known to be diseased, and not in those organs that lack pathology.

2. Fewer, or no, copies of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease.

3. With resolution of disease, the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, the opposite should occur.

4. When sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship.

5. The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms.

6. Tissue-sequence correlates should be sought at the cellular level: efforts should be made to demonstrate specific in situ hybridization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located.

7. These sequence-based forms of evidence for microbial causation should be reproducible.
If one considers these postulates in relation to T1D, postulate 1 and 2 are fulfilled by the results from DiViD. Postulate 4 still needs to be explored and eventually confirmed, but it is difficult to collect high quality pancreatic tissue from pre-diabetic individuals, and almost impossible to collect from individuals later developing T1D but still not even autoantibody positive. Regarding postulate 5, enteroviruses, although cytolytic, can establish persistent infections in vitro and in vivo (144). This persistence has been shown to contribute significantly to chronic myocarditis and dilated cardiomyopathy (121), both through direct effects of viral replication and as induction of inflammation in the heart. The theory is that a persistent infection by cytolytic viruses such as enterovirus as a result of a virus-host coevolution which combines a resistance developed by the cell and an adaptation to the virulence of the viral strain (144). These viral and cellular factors have recently been reviewed (145), and are summarized in figure 16. Postulate 6 is indirectly fulfilled by the presence of vp1 mainly in insulinproducing, insulitic islets. Attempts have been made on the DiViD samples to show the presence of viral RNA inside the islets by ISH, but these results are still pending. Finally postulate 7 is fulfilled as the results from DiViD are confirmed in several independent laboratories.

Summarized, the modified Koch’s postulates provide necessary reminders that we still have not proved causality between enterovirus infection and T1D. It is mainly postulate 3 that is missing confirmation. I may also be argued that the modernized Koch’s not cover the situation observed in persistent, chronic infections, where small amounts of a non-replicating, not-cytopathic virus is the suspected cause of a low-virulent infection. However, the basis for claiming that enteroviruses may, in some way or another, contribute to the development of T1D is strengthened by our findings. The viruses may enter the pancreas before the
development of auto-antibodies, but also at the same time or after the auto-antibodies develop. The present viruses may even be innocent bystanders in vulnerable individuals. Nevertheless, based on the very low amounts of viruses observed, it seems that how the pancreas and the immune system respond to the virus are more important than the presence of enterovirus. Different HLA-serotypes and tropisms in the virus-receptors are some of the differences that may explain the different response.

![Figure 16 Possible mechanisms involved in the persistence of Coxsackievirus B](image)

A cytolotic virus such as Coxsackievirus B (CVB) can under certain circumstances establish a persistent infection in susceptible cells. Changes in cell and virus characteristics leading to a decreased or suppressed viral replication can be observed when the infection is persistent. Reproduced with kind permission, Didier Hober (145).
11.3 The DiViD intervention trial

As mentioned above; If the results in DiViD are judged by Koch’s postulates, it is obvious that the most important missing point is to show that the copy number of pathogen associated nucleic acid sequences becomes undetectable or decreases as the disease is resolved (postulate 3). That has led us to plan a randomized controlled double-blind intervention study, where the combination of ribavirin and pleconaril or placebo will be given to newly diagnosed type 1 diabetic children aged 6-18 years. Pleconaril inhibits enteroviruses by preventing these viruses from uncoating and replicating once inside the cells; it also prevents some picornaviruses from attaching to host cells by integrating into a specific hydrophobic site within the viral capsid. In vitro studies show that pleconaril inhibits replication of >90% of the enterovirus strains with clinical significance (146). This mode of action might be a limitation in T1D, as the enteroviruses identified in DiViD not seem to be fast replicating, at least not in vitro. Therefore ribavirin will be added as intervention. Ribavirin is a nucleoside analogue with broad-spectrum antiviral activity against a variety of viruses, including picornaviruses (147). Primary end points will be mean residual insulin secretion measured by Boost® stimulated C-peptide two hour area under the curve profile two years after inclusion, while secondary endpoints will be the number of patients with stimulated C-peptide >0,2 \( \mu \text{mol/l} \), insulin dose and HbA1c. Follow-up is to be 36 months, and power analyses have shown that 192 children must be included. The study will be launched in 2016, and children will be recruited from Sweden, Denmark, Finland and Norway. Hopefully, this study will show improved conservation of the ability to produce insulin in the treatment group.
12 Future investigations and research on the DiViD material

The amount of material collected in DiViD implies that we still have samples left for further analyses. As discussed earlier, the quality of the material is excellent. More importantly, the interest among the diabetes research community to investigate pancreatic tissue from newly diagnosed live patients with T1D has been quite overwhelming. To ensure best utilization of the collected material, we have, through the Persistent virus infection in diabetes network (PEVNET) and nPOD, been openly inviting other researchers to collaborate. That has led to multiple material transfer agreements, and so far material from DiViD has been sent to more than 10 different research groups in Denmark, Italy, the Netherlands, Israel, the US and New Zealand. It is too extensive to refer to all plans, but some will be mentioned briefly in the following.

12.1 Uppsala, Sweden

In Uppsala, Oskar Skog and Olle Korsgren will continue the work characterizing the T-cells and the inflamed islets. By laser capture microdissection of islets shown to be infiltrated by T-cells in consecutive sections, and also by separately microdissecting defined areas of the islets (islet core and the surrounding immune cells), the gene expression will be compared to the expression in islets with no inflammation. This will hopefully yield new information on questions raised in paper III, and further exploring the role of the T-cells in T1D. Results from these experiments will be published shortly.

Also in Uppsala, pancreatic innervation in the DiViD-samples will be compared with long-standing type 1 diabetic subjects and healthy controls. By using immunofluorescence and morphometry, the sensory, sympathetic and parasympathetic nerve fibres will be located and quantified. Preliminary results suggest that the innervation pattern of the pancreas and islets
of type 1 diabetic T1D subjects is very similar to that of non-diabetic controls, but that the sensory nerve fibres occurred more often in the pancreata from T1D. The results will be published, but its clinical and pathological importance for T1D aetiology may be limited. One speculation might be that the observed higher density of sensory nerve fibres in recent onset T1D could contribute to the previously described dysfunction in insulin-secretion (Paper II).

12.2 Memphis, US
Gene expression in laser captured islets from the DiViD cases and controls has already been investigated by Ivan Gerling, University of Tennessee, Memphis, USA. The results have been partially published as an abstract at ADA 75th Scientific Sessions in 2015, and show a significant enrichment of gene ontologies and pathways involved in viral transcription, viral genome expression, cellular stress and mitochondrial dysfunction. Further analyses on the gene expression in these islets will be published shortly.

12.3 Exeter, UK
The inflammation at onset of T1D, with analyses of subsets of leucocytes, will be described in more depths in collaboration with Professor Noel Morgen and his co-workers in Exeter, UK. This work will, in addition to the CD20Hi and Lo analysis, also involve the staining of sections for other histochemical markers, such as CD4, CD8, CD20, CD11c and CD68. The possible presence of beta-cell proliferation (148) will be explored by staining for a cellular marker of proliferation (Ki67).

12.4 Varese, Italy
In collaboration with Antonio Toniolo in Varese, Italy, a search for enteroviruses using virus amplification in cell culture followed by gene amplification and sequencing is being
conducted. The results are still pending, but preliminary data suggest positivity in all 6 cases. These findings must be quality assured and compared with our previous results before they are to be published.

12.5 San Diego, US
Teresa Rodriguez Calvo and Matthias von Herrath at La Jolla Institute, San Diego, California will try to measure autoreactive and enterovirus specific T-cells using tetramers and will also assess expression of inflammatory cytokines at both the mRNA level (RNAscope technology) as well as the protein level (direct protein staining) on consecutive sections. So far the biggest obstacle to this has been to develop stable and reliable tetramers that bind to T-cells located in the pancreatic tissue. Due to these challenges, it is difficult to know if and when results from this work will be ready for publication.

12.6 Auckland, New Zealand
Shiva Reddy in Auckland, New Zealand has recently published interesting data regarding the insulitis seen in organ donors with long-standing T1D (108). In samples from DiViD, he will do immunohistochemical analyses in an attempt to identify molecular markers of oxidative and nitrosative stress in beta cells during the early stages of human T1D. Preliminary results will be presented at the annual nPOD-meeting in February 2016, and then hopefully later published.

12.7 Brussels, Belgium
By investigating the gene expression in live islets (paper II), Professor Decio Eizirik in Brussels will evaluate alternatively spliced isoforms in islets isolated from T1D and respective controls for discovery of novel autoantigens and mechanisms of disease. The plan
is to analyse the RNA-seq data to identify all splice variants in human islets from the DiViD cases compared to controls and to compare these observations with the alternative splicing signatures present in human islets exposed in vitro to inflammatory or metabolic stress. The results are still pending.

12.8 Jerusalem, Israel

Formalin fixated sections have also been sent to Professor Yuval Dor at the Hebrew University-Hadassah Medical School in Israel. His group is exploring the DNA damage response and beta cell dedifferentiation in human T1D. DNA damage will be identified with antibodies against gammaH2AX, 53BP1 and phospho-ATM, all being components of the response to DNA-breaks. Results are expected in a few months.

12.9 Copenhagen, Denmark

The presence of sulfatide and the RNA expression of enzymes involved in sulfatide metabolism in islets from DiViD-cases will be compared to the presence and expression in healthy individuals by Karsten Buchard, The Bartholin Institute in Copenhagen. Sulfatide is a glycosphingolipid present in the brain, neural tissue, and pancreatic beta-cells. In the latter, sulfatide is a chaperone for insulin. It facilitates folding of proinsulin, preserves insulin crystals and promotes exocytosis of insulin granules. After secretion sulfatide opens ATP-sensitive K+ channels thereby providing rest to the individual beta-cell. The results will be published shortly.
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10. Lupi, R, Marselli, L, Dionisi, S, Del, GS, Boggi, U, Del, CM, Lencioni, C, Bugliani, M, Mosca, F, Di, MU, Del, PS, Dotta, F, Marchetti, P: Improved insulin secretory function and reduced chemotactic properties after tissue
culture of islets from type 1 diabetic patients. *Diabetes Metab Res Rev* 20:246-251, 2004


30. Serrano-Rios, M, Goday, A, Martinez, LT: Migrant populations and the incidence of type 1 diabetes mellitus: an overview of the literature with a


61. Fernandez-Cruz, L, Martinez, I, Gilabert, R, Cesar-Borges, G, Astudillo, E, Navarro, S: Laparoscopic distal pancreatectomy combined with


64. Fernandez-Cruz, L: Distal pancreatic resection: technical differences between open and laparoscopic approaches. HPB (Oxford) 8:49-56, 2006


111


Ref Type: Conference Proceeding


104. Oram, RA, Jones, AG, Besser, RE, Knight, BA, Shields, BM, Brown, RJ, Hattersley, AT, McDonald, TJ: The majority of patients with long-duration type 1 diabetes are insulin microsecretors and have functioning beta cells. *Diabetologia* 57:187-191, 2014


beta-cell-specific CD8 T cell phenotype in type 1 diabetes reflects chronic autoantigen exposure. *Diabetes* 2014


114


128. Foulis, A, Farquharson, M, Hardman, R: Aberrant expression of class II major histocompatibility complex molecules by B cells and hyperexpression of class I major histocompatibility complex molecules by


17 Papers I-IV