Title page

Title: Detection of a low-grade enteroviral infection in the islets of Langerhans of living patients newly diagnosed with type 1 diabetes
Short running title: Enterovirus in pancreas in patients with T1D

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Abstract

The Diabetes Virus Detection study (DiViD) is the first to examine fresh pancreatic tissue at the diagnosis of type 1 diabetes for the presence of viruses. Minimal pancreatic tail resection was performed 3-9 weeks after onset of type 1 diabetes in 6 adult patients (age 24-35 years). The presence of enteroviral capsid protein 1 (VP1) and the expression of class I HLA were investigated by immunohistochemistry. Enterovirus RNA was analyzed from isolated pancreatic islets and from fresh frozen whole pancreatic tissue using PCR and sequencing. Non-diabetic organ donors served as controls. VP1 was detected in the islets of all type 1 diabetes patients (2 of 9 controls). Hyperexpression of class I HLA molecules was found in the islets of all patients (1 of 9 controls). Enterovirus specific RNA sequences were detected in 4 of 6 cases (0 of 6 controls). The results were confirmed in different laboratories. Only 1.7 % of the islets contained VP1 positive cells and the amount of enterovirus RNA was low. The results provides evidence for the presence of enterovirus in pancreatic islets of type 1 diabetic patients, being consistent with the possibility that a low grade enteroviral infection in the pancreatic islets contribute to disease progression in humans.

Despite intensive research efforts over the past century, the precise causes of type 1 diabetes are still unknown although it is well established that the illness results from a complex interplay between genetic predisposition, the immune system and various environmental factors (1). One such influence is viral infection, first postulated in 1927 by Gundersen (2), who observed an increase in the incidence of type 1 diabetes following epidemics of mumps. Since then numerous studies have addressed the possible role of viruses as causative agents in type 1 diabetes and it has emerged that infection with enteroviruses provides association with disease development (3-6). Nevertheless the question of causality remains open and data which confirm the presence of enterovirus in the pancreases of type 1 diabetic patients would significantly strengthen the conclusions.

One of the principal factors limiting progress in the field has been the lack of availability of well-preserved tissue samples for study (7). The majority of published studies have made use of pancreatic tissues collected at autopsy from type 1 diabetic patients having varying duration of diabetes (8-10). Much of the material has been affected by post-mortem changes and preserved in the form of formalin-fixed, paraffin-embedded blocks, which means that many of the more modern techniques used to detect viruses cannot be applied with confidence. It would be preferable if well-preserved human organ samples could be accessed, since these should be more amenable to the detection of viral RNA sequences, viral antigen production and the cellular responses to viral infection (11).

The main objective of the Diabetes Virus Detection-study (DiViD) was to collect pancreatic tissue from living subjects very soon after the diagnosis of type 1 diabetes to investigate the presence of viruses. The sampling procedures and clinical data from the recruited patients have been reported (12). Here we provide evidence that the islets of Langerhans of these

patients display features consistent with a low-grade enteroviral infection. The study was approved by the Government's Regional Ethics Committee in Norway.

Material and methods

A total of six type 1 diabetic patients (three women, three men), age 24-35 years (median 28 years) were recruited to the study after giving written informed consent. Pancreatic biopsies, approximately 3 cm of the tail, were taken 3-9 weeks after diagnosis of type 1 diabetes (median five weeks). Details regarding the patients are shown in Table 1.

The biopsies were processed under sterile conditions and immediately divided into multiple smaller pieces snap-frozen in liquid nitrogen in the operating theatre (Table 1) and subsequently stored at -80 ^{o}C. Formalin fixed, paraffin embedded tissue blocks were prepared simultaneously. Other parts were allocated for the purification of living pancreatic islets in ViaSpan© medium. 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 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, BMI and auto-antobodies and HLA of the controls are shown in eTable 1.

Islet isolation

Pancreatic islets were isolated in Uppsala using methods previously developed for clinical islet isolation (13). 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).

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. For details regarding the methods, see supplementary material.

Results

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 RNA (Table 2). Four patients were enterovirus positive in the pancreas by two methods, one with three methods.

Detection of enterovirus genome

Enterovirus RNA was detected in the enriched islet preparation in three of the six type 1 diabetes 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 2). In addition, islet culture medium from one patient was detected as 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 2). 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 perfect match with enterovirus sequences (Table 3). Due to the low virus titer 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.

Detection of enterovirus protein

Pancreatic islets from all six type 1 diabetes 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.

Expression of class I HLA molecules

Hyperexpression of HLA class I molecules was observed homogeneous in the islets of all six type 1 diabetes patients but in only one of the nine controls ($p < 0.01$) (Table 2). Among the six patients, all insulin containing islets showed HLA-class I hyper expression irrespective of the presence of enterovirus VP1 protein. Figures 1 shows an insulin-containing islet with hyper expression of HLA class I and the presence of enterovirus VP1 protein.

Discussion

This study of fresh pancreatic tissue collected close to the time of diagnosis of type 1 diabetes suggests 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 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 (14) while it is reduced during persistent infection (15). This is because enteroviral persistence is characterized by naturally-occurring deletions at the 5′ terminus of the genome (16;17), which reduce the replication rate of the virus. It is recently shown that these terminally deleted viruses also can persist after inoculation in murine pancreas in the absence of cytopathic virus weeks past the acute infectious period (16). 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 type 1 diabetes. The biopsies were taken from the pancreatic tail, not discarding that the infection could be affecting other parts first slowly disseminating 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 type 1 diabetes patients were positive for viral RNA when analyzed 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.

The high sensitivity of PCR makes it susceptible for wrong positive results due to viruses which may contaminate the samples during the analysis. Several actions were taken to avoid such contaminations and to be able to detect them if they occur. First, the two virus laboratories got the same results even if they carried out all RNA extraction and RT-PCR steps independently and used different primers and PCR protocols. Second, the amplified enterovirus sequences differed from each other suggesting that they originate from different enterovirus strains thus excluding a common contaminating virus. Third, exocrine cells which were isolated from the same pancreases in the same time and place, as well as virus negative internal control samples included in each test run, were all PCR negative. In addition, all pancreas samples were PCR negative for all other tested viruses. Finally, control pancreases from non-diabetic individuals were PCR negative even if they were processed using exactly the same procedures and in the same laboratory as the samples from diabetic patients.

The antibody used to detect VP1 is known to recognize this protein from multiple different enteroviruses in formalin fixed samples (18). However, it has also been shown that, under some conditions, the antibody may label certain human proteins (19). We were careful to employ the antibody under conditions optimized to avoid such interactions without compromise of virus-specific binding (20). Thus, we are confident that the immunolabelling achieved in human pancreas sections is likely to represent the presence of viral protein.

In addition to virus specific markers, the expression of class I HLA molecules was upregulated in the islets of all type 1 diabetes patients. This fits with previous observations

showing that pancreatic islets of type 1 diabetes patients hyperexpress class I HLA molecules and interferon-alpha (21;22). This might indicate ongoing virus-induced interferon secretion in the islets. We have previously shown that enterovirus infection in human pancreatic islets leads to such HLA class I hyperexpression in vitro, partly mediated by secretion of type 1 interferon's (23).

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 were processed as the cases and served mainly as methodological controls in PCR analyses being negative for all tested viruses in PCR.

In conclusion, 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 islets. The results do not prove causality between enterovirus infection and type 1 diabetes but they support the view that a low grade enteroviral infection is present in the islets of Langerhans at diagnosis of type 1 diabetes. These findings should encourage studies in which anti-viral medication and/or vaccines against enteroviruses could be tested to reduce disease progression and prevent type 1 diabetes.

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LK participated in all parts of the study, and was responsible for clinical coordination and recruitment of patient, data collection, analysis and interpretation, and drafted the manuscript. BE and TB performed the surgery and participated in writing of the article. OK, GF, OK, OS, MA, MCE, NGM, SJR, PL, HH, SO, MO, JEL and JL contributed to data analysis and interpretation. JL, HH, GF and OK also contributed to the study design. KFH and DU contributed to study design, data interpretation and writing of the manuscript. KDJ was the principal investigator of the study, had the initial idea of the DiViD study, and participated in study design, funding, regulatory issues, international collaboration, data collection, analysis

and interpretation, and in writing of the manuscript. LK and KDJ are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1 Clinical data of cases with newly diagnosed type 1 diabetes.

Case	Age	Sex	BMI	Weeks from diagnosis until biopsy	HbA1c at biopsy % (mmol/mol)	Insulin (U/kg/day)	Anti GAD (<0.08 ai)	Anti-insulin (<0.08 ai)	anti-ZnT8 (<0.12 ai)	Anti IA2 (< 0.10 ai*)	HLA risk alleles†	Seconds until snap-freezing of pancreatic tissue
1	25	F	21.0	4	6.7 (50)	0.5	1.76	0.7	0.28	0.16	Yes	110
2	24	M	20.9	3	10.3 (89)	0.35	0.79	<0.01	0.44	>3	Yes	150
3	34	F	23.7	9	7.1 (54)	0.17	1.77	< 0.05	1,.45	> 3	Yes	150
4	31	M	25.6	5	7.4 (57)	0.4	0.77	0.1	< 0.01	2.54	Yes	160
5	24	F	28.6	5	7.4 (57)	0.36	0.46	0.1	0.06	>3	Yes	240
6	35	M	26.7	5	7.1 (54)	0.52	1.85	< 0.05	< 0.01	< 0.04	Yes	190

* Arbitrary units according to Diabetes Antibody Standardization Program (DASP) (24)
† Presence of HLA DR3-DQ2, HLA DR4-DQ8, or both

Table 2 Detection of enterovirus protein, enterovirus RNA and expression of class 1 HLA molecules in the pancreas of newly diagnosed type 1 diabetic patients.

	Enterovirus VP1 protein expression		Enterovirus genome				Class I HLA expression
Methodology	**IHC**		**Enterovirus specific RT-PCR**			**RNA**	**IHC**
Tissues	Pancreatic islets		Supernatant from cultured purified pancreatic islets		Snap-frozen pancreas 30 mg	Snap-frozen pancreas 30 mg	Pancreatic islets
Laboratory	Tampere	Exeter	Uppsala	Tampere	Tampere and Uppsala	Oslo	Exeter
Case							
1	Positive	Positive	Negative	Negative	Negative	Negative	Hyper expression
2	Positive	Positive	Positive *	Positive †	Negative	Negative	Hyper expression
3	Positive	Positive	Negative	Negative	Negative	Negative	Hyper expression
4	Positive	Negative	Positive *	Positive †	Negative	Negative	Hyper expression
5	Positive	Positive	Positive ‡	Positive ‡	Negative	Negative	Hyper expression
6	Positive	Positive	Negative§	Positive ‡	Positive	Negative	Hyper expression

* 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

Table 3. Sequence alignment of PCR-products, Uppsala. Variable nucleotide sites between different cases are highlighted with yellow.

```
Case2  GTACCTTTGTGCGCCTGTTTTATTACCCCCTCCCCTAACTGTAACTTAGAAGTAATACACACCGATCAACAGTAAGTGTGGCACGCCAGCCATGCTCCGATCAAGCACTTCTGTTCCCCC
Case4  ----------------------------------------------------------------------------------------------------------------------------
Case5  ----------------------------------------------------------------------------------------------------------------------------
Case6  ----------------------------------------------------------------------------------------------------------------------------

Case2  GGACCGAGTATCAATAGACTGCCCACGCGGTTGAAGGAGAAAGTGTCCGTTATCCGGCCAACTACTTCGAGAAGCCCAGTAACACCATGGAAGTTGCAGAGTGTTTCGCTCAGCACAACC
Case4  ----------------------------------------------------------------------------------------------------------------------------
Case5  ----------------------------------------------------------------------------------------------------------------------------
Case6  ----------------------------------------------------------------------------------------------------------------------------

Case2  CCAGTGTAGATCAGGTCGATGAGTCACTGCATACCCCACGGGTGACCGTGGCAGTGGCTGCGTTGGCGGCCTGCCTATGGGGAAGCCCATAGGACGCTCTAATACAGACATGGTGTGAAG
Case4  ----------------------------------------------------------------------------------------------------------------------------
Case5  ----------------------------------------------------------------------------------------------------------------------------
Case6  ----------------------------------------------------------------------------------------------------------------------------

Case2  AGTCTATTGAGCTAGTTGGTAGTCCTCCGGCCCCTGAATGCGGCTAATCCTAACTGTGGAGCATACGCCCTCAAGCCAGGGGGCAGTGTGTCGTAACGGGCAACTCTGCAGCGGAACCGA
Case4  ----------------------------CCCTGAATGCGGCTAATCCTAACTGTGGAGCACATGCCCACAAACCAGTGGGTGGTGTGTCGTAACGGGTAANTNNNNNGNNGAACCGA
Case5  ----------------------------------------------------------------------------------------------------------------------------
Case6  ----------------------------CCCTGANTGCGGCTAATCCTAACTGCGGAGCANATACCCACAAACCAGTGGGCAGTCTGTCGTAATGGGTAACTCTGCAGCGGAACCGA

Case2  CTAC------------------------------------------------------------------------------------------------------------------------
Case4  CTACTTTGGGTGTCCGTGTTTCTATTTATTCCTATACNGGC-----------------------------------------------------------------------------------
Case5  ----------------------------------------------------------AANAGTAAAAGAAACACGGACACCCAAAGTAGTCGGTTCCGCTGCNNAGTTACCCATTACG
Case6  CTACTTTGGGTGTCCGTGTTTCT-TTTACTCTTTTATTGTCACCATAAGCAGCCAATAAAAGAGTAAAAGAAACACGGACACCCAAAGTAGTCGGTTCCGCTGCAGAGTTACCCATTACG

Case2  ----------------------------------------------
Case4  ----------------------------------------------
Case5  ACAGACTGCCCACTGGCTTGTGGGTNNCTGCTCCGCAGTTAGGATTA
Case6  ACAGACTGCCCAC-----------------------------------
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Figure 1 Pancreatic islet from one of type 1 diabetic patient , stained for insulin (brown) and glucagon (red) (A), HLA class 1 molecules (B) and enterovirus protein VP1 (C). (Cells positive for enterovirus protein are marked by arrows)

A. B. C. VP1-positive cells.