Studies of Enterovirus Infection and Induction of Innate Immunity in Human Pancreatic Cells

MAHESH ANAGANDULA
Several epidemiological and clinical studies have indicated a possible role of Enterovirus (EV) infection in type 1 diabetes (T1D) development. However, the exact causal mechanism of these viruses in T1D development is not known. The aim of this thesis is to study various EVs that have been shown to differ in their immune phenotype, lytic ability, association with induction of islet autoantibodies, ability to replicate, cause islet disintegration and induce innate antiviral pathways in infected pancreatic cells in vitro. Furthermore, EV presence and pathogenic process in pancreatic tissue and isolated islets of T1D patients was also studied.

Studies in this thesis for first time show the detection of EV RNA and protein in recent onset live T1D patients supporting the EV hypothesis in T1D development. Further all EV serotypes studied were able to replicate in islets, causing variable amount of islet disintegration ranging from extensive islet disintegration to not affecting islet morphology at all. However, one of the EV serotype replicated in only two out of seven donors infected, highlighting the importance of individual variation between donors. Further, this serotype impaired the insulin response to glucose stimulation without causing any visible islet disintegration, highlighting that this serotype might impaired the insulin response by inducing a functional block. Infection of human islets with the EV serotypes that are differentially associated with the development of islet autoantibodies showed the islet cell disintegration that is comparable with their degree of islet autoantibody seroconversion. Suggesting that the extent of the epidemic-associated islet autoantibody induction may depend on the ability of the viral serotypes to damage islet cells. Furthermore, one of the EV strains showed unique ability to infect and replicate both in endo and exocrine cells of the pancreas. EV replication in both endo and exocrine cells affected the genes involved in innate and antiviral pathways and induction of certain genes with important antiviral activity significantly varied between different donors. Suggesting that the same EV infection could result in different outcome in different individuals. Finally, we compared the results obtained by lytic and non lytic EV strains in vitro with the findings reported in fulminant and slowly progressing autoimmune T1D and found some similarities. In conclusion the results presented in this thesis further support the role of EV in T1D development and provide more insights regarding viral and host variation. This will improve our understanding of the possible causative mechanism by EV in T1D development.

Keywords: Type 1 Diabetes, Enterovirus, Innate Immunity, Pancreas

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ISSN 1651-6206
urn:nbn:se:uu:diva-284370 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-284370)
List of Papers

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


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<td>2’-5'-oligoadenylate synthetase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackievirus and Adenovirus receptor</td>
</tr>
<tr>
<td>CBV</td>
<td>Coxsackie B virus</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CCL5</td>
<td>C-C motif chemokine ligand 5</td>
</tr>
<tr>
<td>CXCL10</td>
<td>C-X-C motif chemokine ligand 10</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay accelerating factor</td>
</tr>
<tr>
<td>DTZ</td>
<td>Dithizone</td>
</tr>
<tr>
<td>DiViD</td>
<td>Diabetes Virus Detection Study</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
</tr>
<tr>
<td>EV</td>
<td>Enterovirus</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GAD65</td>
<td>Glutamic acid decarboxylase 65 kDa</td>
</tr>
<tr>
<td>GADA</td>
<td>GAD65 antibody</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose stimulated insulin secretion</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>IRF</td>
<td>IFN regulatory factor</td>
</tr>
<tr>
<td>IFNAR</td>
<td>Type I Interferon Receptor</td>
</tr>
<tr>
<td>ISGF3</td>
<td>Interferon-stimulated gene factor 3</td>
</tr>
<tr>
<td>ICA</td>
<td>Islet cell autoantibodies</td>
</tr>
<tr>
<td>IA2</td>
<td>Tyrosine phosphatase-like insulinoma antigen 2</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IAA</td>
<td>Insulin autoantibody</td>
</tr>
<tr>
<td>IFIH1</td>
<td>Interferon induced with helicase C domain 1</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser capture microdissection</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial antiviral signaling protein</td>
</tr>
<tr>
<td>MDA-5</td>
<td>Melanoma differentiation-associated gene</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>Mx1</td>
<td>Myxovirus resistance protein 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MYD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kB</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>nPOD</td>
<td>Network for Pancreatic Organ donors with Diabetes</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerization domain (NOD)-like receptor</td>
</tr>
<tr>
<td>OCT</td>
<td>Octamer transcription factor</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PTPN2</td>
<td>protein tyrosine phosphatase, non-receptor type 2</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T cell expressed, and secreted</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid gene I</td>
</tr>
<tr>
<td>RLH</td>
<td>RIG-I-like helicase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription PCR</td>
</tr>
<tr>
<td>RNAseL</td>
<td>Ribonuclease-L</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>ss</td>
<td>Single-stranded</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>UTR</td>
<td>Un-translated region</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number of tandem repeats</td>
</tr>
<tr>
<td>VP</td>
<td>Virus protein</td>
</tr>
<tr>
<td>VPG</td>
<td>Viral protein genome-linked</td>
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</table>
Introduction

Enteroviruses
Virion structure
Enteroviruses (EV) are small non-enveloped viruses that belong to the Picornaviridae family. They consist of single stranded positive-sense RNA as their genome. The genome is encapsulated in an icosahedral protein capsid. The capsid is made up of 60 copies of the four capsid proteins (virus proteins 1-4). VP1, VP2 and VP3 make the outer surface of the capsid while VP4 is located inside the capsid. Four of these proteins together make a protomer, which is then repeated four times to make a pentamer. Twelve of these pentamers build the icosahedral capsid around the viral genome. The capsid protects the viral genome from external conditions and is also involved in the cell attachment. A narrow deep cleft-like structure (canyon) exists around each of the five fold axes on the capsid surface, which has been shown to be involved in the receptor-binding (1, 2).

Figure 1. Enterovirus capsid. The illustration is adapted from www.viralzone.expasy.org with permission.
EV genome

The EV genome is composed of a 7.5 kb long single stranded positive sense RNA. The RNA contains an open reading frame, flanked by un-translated regions (UTRs) at the 5’ and 3’ ends. These UTR regions form extensive secondary structures that play an important role in viral RNA replication and translation by acting as contacting points for the interaction of cellular and viral proteins. The 5’ UTR also contains an internal ribosomal entry site (IRES) that directs the cap-independent translation initiation. A small protein, called viral protein genome-linked (VPg), is attached at the 5’ end of the viral RNA and acts as a primer for the positive and negative strand synthesis during RNA replication. A poly A tail is attached at the 3’ end and acts as a template for the uridylation of the VPg protein. The coding region is divided into three parts called P1, P2 and P3 regions. P1 encodes the structural proteins VP1 to VP4. The P2 and P3 regions encode the non-structural proteins involved in various functions such as polyprotein processing, RNA replication, cleavage of host cell proteins and modifying the host cell to facilitate the virus lifecycle (2, 3). Functions of these proteins are summarized in Table 1 (1).

![Figure 2. EV genome structure](image)

Table 1. EV proteins and their functions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1-VP4</td>
<td>Structural proteins, make the virus capsid (3)</td>
</tr>
<tr>
<td>2A</td>
<td>By cleaving the host translation initiation factor it will shutdown the cap dependent translation (4)</td>
</tr>
<tr>
<td>2B</td>
<td>Disrupts the golgi complex and secretion pathways, alter the membrane permeability and Ca$^{2+}$ levels in host cells. (5, 6)</td>
</tr>
<tr>
<td>2C</td>
<td>Rearrangement of membranes of vesicles (7).</td>
</tr>
<tr>
<td>3A</td>
<td>Inhibition of cellular trafficking and secretion (8).</td>
</tr>
</tbody>
</table>
3B (VPg) Acts as an RNA primer for positive and negative strand synthesis (9, 10).

3C Cleaves the host cellular proteins important for in host gene expression. By cleaving the transcription factor TATA-box binding protein inhibits the cellular transcription (11). RNA-dependent RNA polymerization, VPg uridylation (9, 12).

3D\textsuperscript{pol} RNA-dependent RNA polymerization, VPg uridylation (9, 12)

**EV receptors**

EVs use a wide variety of cellular receptors to attach and infect the cell. Some of these receptors are listed in Table 2 (13, 14).

**Table 2. EV receptors**

<table>
<thead>
<tr>
<th>Species</th>
<th>Virus</th>
<th>Receptor</th>
<th>Receptor family</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV-B</td>
<td>Coxsackie B virus</td>
<td>Coxsackie and Adeno virus receptor, Decay accelerating factor (CD55)</td>
<td>Immunoglobulin superfamily</td>
</tr>
<tr>
<td></td>
<td>Coxsackie A virus 18, 21</td>
<td>Intercellular adhesion molecule 1 (ICAM1)</td>
<td>Complement control family</td>
</tr>
<tr>
<td></td>
<td>Echovirus1</td>
<td>Integrin α2β1</td>
<td>Immunoglobulin superfamily</td>
</tr>
<tr>
<td></td>
<td>Echovirus 6 and 7</td>
<td>Decay accelerating factor (CD55)</td>
<td>Integrin</td>
</tr>
<tr>
<td>EV-C</td>
<td>Poliovirus</td>
<td>Polio virus receptor (CD155)</td>
<td>Complement control family</td>
</tr>
<tr>
<td></td>
<td>Enterovirus-70</td>
<td>Sialic acid</td>
<td>Immunoglobulin superfamily</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carbohydrate</td>
</tr>
</tbody>
</table>
EV life cycle

Cell entry and uncoating

Virus attachment to the viral receptor initiates destabilization and conformational changes in the virus particle. These changes result in altered virions called A particles. These particles are devoid of the VP4 protein and contain the normally internalized hydrophobic N terminus of the VP1 on the outer surface (3). These particles have a lower sedimentation coefficient and increased affinity for membranes than the native virion. The A particle is thought to participate in the pore formation on cell membranes to release the genome (3). Different EVs have been shown to use different pathways and mechanisms to mediate the cell entry and the release of the viral genome (15, 16). This appears to be dependent mainly on the cell type, and the receptor used. For example, it was shown that CBV3 uses the caveolin-dependent, dynamin independent pathway in polarized CaCo2 cells and the dynamin lipid raft dependent and clathrin caveolin independent pathway in non-polarized Hela cells (15, 16).

Translation

EV carries out the RNA replication and translation in the cytoplasm of the infected cells. Once the viral genome enters into the cell, VPg is cleaved by cellular unlinkase enzyme (2). The viral RNA is then translated into a single poly protein via a cap-independent mechanism by using the IRES. The newly synthesized poly protein is first cleaved into three precursor proteins P1, P2 and P3. The P1 protein is then further cleaved into structural proteins VP0, VP1 and VP3. Proteins P2 and P3 undergo subsequent cleavages to produce non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3CD, 3Dpol). All these non-structural proteins carry out various functions (summarized in Table 1) to facilitate the virus replication cycle (2, 3).

RNA Replication

Replication of EV RNA occurs on the membranous vesicles, which are formed by rearrangement of cellular membranes by the viral proteins 2B and 2C. It is hypothesized that these membranes act as scaffolds for RNA stabilization and also to facilitate a proper positioning and concentration of viral replication proteins to efficiently catalyze RNA synthesis (1).

Viral RNA replication starts with the synthesis of the negative strand by using the genomic RNA as template. This is carried out by 3D pol at the 3’ poly A tract of the genomic RNA by using uridylated VPg protein as primer. The newly synthesized negative strand RNA then serves as a template for the production of new positive strand RNA. A single negative strand RNA can produce several positive RNA strands. During RNA replication, ds RNA exist as an intermediate form. These newly formed positive RNA
strands can be packed into virions or can be translated to produce viral proteins (2, 3).

**Assembly and Release**

The assembly of new virions starts with the cleavage of P1 precursor protein into VP0, VP1, and VP3. These three proteins form a protomer and five protomers make the 5S pentamer. Twelve of these pentamers assemble to form the procapsid, which encapsulates the viral genome. The final step involves the cleavage of VP0 protein into VP2 and VP4 to produce the full-matured virion. The newly formed virions are released by cell lysis during a lytic infection. During non-lytic infection it is believed that the viral particles are released in vesicles (3, 17).

*Figure 3 EV infectious cycle*
EV persistence

EVs generally cause cytolytic infection due to their ability to affect different host cell functions. However, several studies have showed that EVs can also cause persistent infections. It has been shown that EVs can develop persistence both in vitro and in vivo in various organs such as heart, skeletal muscle and also in different types of cultured cells [18,19,20,21]. The mechanism of EV persistence is not totally understood however; both viral and cellular determinants, as well as the host immune response, are thought to play important roles in driving EV persistence. EVs depend on host factors for their infectious cycle; availability of these factors can affect the outcome of infection. It has been shown that the same EV strain can cause non-lytic or lytic infection in different cell cultures (18), indicating the importance of cellular environment in outcome of the infection. The status of the host cell cycle is also attributed to the development of EV persistence. Feuer et al reported maximum level of CBV protein expression and replication in proliferating cells (cells at G1/S phase) and very low amount of viral protein with minimum level of infectious virus in non dividing quiescent cells (cells at G0)(19). It was postulated that cellular factors required for EV replication might be optimal in proliferating cells resulting in productive infection, while absence of these factors in non-dividing quiescent cells might favor persistence.

Mutations in viral receptors have been observed in cells that harbor EV persistence (20). In addition to cellular factors, mutations in the EV genome have also been shown to contribute to EV persistence. Mutations in the capsid-coding region have been shown to result in EV persistence in neuroblastoma and Hep-2c cells (21, 22). These mutations were thought to affect the EV interaction with its receptor, affecting virus binding and capsid conformational changes further resulting in the EV persistence. After the acute lytic infection, EVs have been shown to develop deletions in the 5'UTR region (23, 24). These terminally deleted mutants are associated with lower replication rate and absence of cytopathic effect. These terminally deleted viruses have also been detected in in vivo studies in heart and pancreas of mice 30 days post infection (23, 25). In addition to these defective variants, EVs were also shown to persist through dsRNA complex in muscle cells (26). EV persistence has been associated with several chronic syndromes such as dilated cardiomyopathy, type 1 diabetes, post-polio syndrome and chronic fatigue syndrome (18, 27-30).

EV persistence in myocytes was shown to induce morphological and structural alteration in infected myocytes. In cardiomyocytes transfected with a CBV3 cDNA mutant, with genome replication resembling restricted persistent virus replication, cytopathic effects were induced (31). This suggests that a low-level of viral replication, without formation of infectious progeny virus, is sufficient to induce myocyte injury. Experiments with
transgenic mice with heart muscle-specific expression of the CBV3 mutant that synthesizes the viral plus- and minus-strand RNA without formation of infectious viral progeny was shown to induce myocardial interstitial fibrosis, hypertrophy, and degeneration of myocytes, resembling the clinical features of dilated cardiomyopathy in humans (32). These studies indicate that restricted persistent viral replication is sufficient to alter the myocyte function. EV persistence is also implicated in T1D development. EV persistence associated with chronic inflammation was reported in gut mucosa of T1D patients (29). Prolonged persistent replication of EV in human pancreatic islet cells has also been shown in vitro (33). Further, EV persistence in a pancreatic ductal cell line was shown to disturb the islet-like cell aggregates formation (34), suggesting that EV persistence in ductal cells could affect the trans differentiation of ductal cells into endocrine cells (34).

EV effect on the host cells

EVs exert profound effects on host cell biology to facilitate its replication cycle. One of the characteristics of EV is its ability to shutdown the cellular cap-dependent translation to maximize its IRES-driven protein synthesis. EVs accomplish this through the cleavage of eIF4G, one of the key components of the cap dependent translation initiation complex (35, 36). In addition to translation, EVs were also shown to affect the host transcription. EV 3C protease has been shown to cleave several factors and regulators such as TATA box-binding protein, octamer binding protein (OCT-1) and cyclic AMP-responsive element-binding protein that are essential for cellular DNA-dependent RNA polymerase activity (37). Another prominent feature of EV is rearrangement of intra-cellular membranes to form membranous vesicles associated with viral RNA synthesis (38, 39). EV proteases can cleave and down regulate the key factors involved in host innate and adaptive immune response (40). EVs were also shown to induce both apoptotic and necrotic pathways in infected cells (41, 42). Several morphological features such as rounding up of the cells and a highly distorted nucleus with condensed chromatin have been observed in EV-infected cells (43).

EV pathogenesis

EVs transmit through the fecal oral route. They initially replicate in the gastrointestinal tract, resulting in mild viremia, and the majority of infections are controlled at this stage without causing any symptoms. Further replication occurs in regional lymph nodes, resulting in dissemination of virus into blood through the lymphatic vessels and thoracic duct. Subsequent dissemination of virus from blood into various organs such as the CNS, heart and muscle can resulted in severe complications like aseptic meningitis, myocarditis, and paralytic poliomyelitis (37, 44). The factor that determines why the
EV infection is asymptomatic in some individuals and severely pathogenic in others is not understood. The host genetic constitution, immunological statuses along with viral factors are thought to influence the outcome of infection.

**Innate Immunity**

The innate immune system plays an important role as the first line of defense mechanism against microbial infections. Innate sensing of viral infection is usually activated through the recognition of different viral pathogen-associated molecular patterns (PAMPs) by a variety of pattern recognition receptors (PRRs). Sensing of viruses occurs mainly through three different families of receptors: Toll-like receptors (TLR), RIG-I like receptors (RLR) and Nucleotide-binding oligomerization domain (NOD) like receptors (NLR) (45). Sensing of viral PAMPs through TLR and RLR leads to the activation of different adaptor proteins such as IPS-1 (MDA5/RIG-I), MYD88 (all TLRs except TLR3) and TRIF (TLR3), which further leads to the activation of different kinases. Upon activation, these kinases phosphorylate the transcription factors such as NF-κB, IRF3 and IRF7. Phosphorylated transcription factors then translocate into the nucleus and bind to interferon stimulated response element (ISRE), resulting in the induction of type I IFN and pro-inflammatory chemokines (46).

**Interferon system**

The interferons are a family of cytokines involved in eliciting different antiviral effects. These are divided into three groups: type I consisting of IFN α and β, type II consisting of IFN γ, and type III including IFN λ. Type I interferons (IFN α / β) are induced by viral infection and play a major role in antiviral defense. Upon induction, type I IFNs bind to the common heterodimeric receptor composed of IFN α receptor 1 and 2 (IFNAR1 and IFNAR2), which leads to dimerization of these subunits and subsequent phosphorylation of the associated Janus kinases JAK1 and TYK2. Activated kinase then phosphorylates the STATs which leads to the binding of the STAT complex to IRF9 to form IFN-stimulated gene factor 3 (ISGF3). After formation, ISGF3 translocates into the nucleus and binds to interferon-stimulated response element (ISRE) in the promoters of interferon-stimulated genes (ISGs), resulting in the transcription of more than 300 ISGs with different antiviral activities leading to establishment of an antiviral state (47-49).
EV interaction with innate immunity

An intact innate immune response is crucial for control and clearance of EV infection; mice lacking innate immune components have been shown to be more susceptible to EV infection than wild type mice (50, 51). Several viral sensors are shown to be involved in sensing the EV infection (51-54). Among toll like receptors, TLR 3, 7 and 8 have been implicated in sensing EV infections (52, 53). TLR 7 and 8, known to sense ss-RNA have been shown to detect CBV infection in human cardiac cells (52, 53). A synergic effect of TLR7/8 sensing of CBV was shown to induce an inflammatory response and thought to be involved in myocardial tissue damage(53).

Although protective, the role of these receptors in controlling the EV infection is not known. In addition, TLR4 has also been proved to be involved in sensing EV infection in a pancreatic cell line (55). In in vivo studies using mouse models it was demonstrated that TLR3 plays an important role in limiting the EV infection (56). Infection with EV showed increased virus titer and mortality in TLR 3 knockout mice (TLR3KO) compared to wt mice (56, 57). Furthermore, it was also shown that TLR3 signaling on macrophages is critical in controlling the EV in mice (57). In addition to TLRs, the cytoplasmic RNA helicase MDA5 has also been shown to play a critical role in sensing and controlling EV infection (51). The viral sensor MDA5 has been shown to detect EV infection through double stranded RNA, which forms as an intermediate during EV replication (54). Mice deficient in MDA5 and its adaptor molecule MAVS showed early mortality and reduced type I IFN induction compared to wt mice upon EV infection (51). In vitro infection with CBV also showed that the expression of genes encoding MDA5 and RIG-I was increased in isolated pancreatic human islets in vitro (58, 59).

The type I IFN response plays a critical role in limiting and inhibiting infection. It has been shown that mice lacking type I IFN receptor (IFNR) showed higher and early mortality than wt mice upon EV infection (50). In in vitro experiments using cultured cells it was shown that pre-treating these cells with type I and II IFNs can inhibit EV replication (60). IFNs exert their antiviral activity through the up-regulation of ISGs. Among these several ISGs such as PKR and OAS have been shown to exert their antiviral activity against EV (61). EVs have developed several strategies to counteract the innate immune response. EVs have been shown to interfere with several aspects of innate immune pathways, from sensing of infection to IFN induction and to evade the antiviral activity. Degradation of MDA5 and RIG-I proteins was demonstrated in cells after infection with poliovirus (62, 63).

It has been shown that EV-3C protease can cleave the adaptor proteins MAVS and TRIF to block the IFN signaling (64). It has also been shown that EV72-3C protease can cleave the transcription factor IRF7 to attenuate the IFN signaling. In addition, it was also shown that EV71 can disrupt the
interferon signaling by targeting IFNAR1. EVs are also known to shutdown the host cap-dependent translation and disrupt the intra-cellular trafficking, which also will interfere with the innate immune pathways (65).

Figure 4. EV innate sensing pathways
The Pancreas

The pancreas is a long flat oval shaped gland, which lies deep in the abdomen. It contains both endocrine and exocrine functions. The exocrine fraction constitutes up to 80% of the pancreas and these exocrine cells synthesize and release the digestive enzymes into duodenum to digest the carbohydrates, lipids and proteins (66). The endocrine fractions contain highly vascularised small island-like structures called islets of Langerhans and constitute about 1-2% of the pancreas. Edouard Laguesse, a histologist who deduced islets endocrine function, coined the term Islets of Langerhans in memory of Paul Langerhans who was the first to describe them as island of cells (66). Islets contain five different types of endocrine cells. These are insulin-secreting beta cells, glucagon-secreting alpha cells, somatostatin-secreting delta cells, pancreatic polypeptide-secreting PP cells, and ghrelin-secreting epsilon cells. Of these cells, beta cells comprise the majority of islet mass (up to 55%) followed by alpha cells (33%) and other endocrine cells (66, 67). However, the relative proportion of these endocrine cells has been shown to vary between different islets (68). In addition to these endocrine cells, islets also contain several non-endocrine cells such as endothelial cells, fibroblasts, macrophages and dendritic cells (66). In humans, these endocrine cells are heterogeneously organized without any obvious pattern, in contrast to mice which have a beta cell core followed by a non-beta cell rim (66, 67).

Type 1 Diabetes (T1D)

Type 1 diabetes (T1D) is a chronic metabolic disorder resulting from the selective destruction of insulin-producing β-cells in the pancreas (69). Insulin is a hormone that regulates the blood sugar levels. Lack of sufficient insulin in T1D patients due to beta cell loss results in high blood-glucose levels, which will eventually lead to serious health complications such as ketoacidosis, kidney failure and blindness (70-72). The etiology of T1D is multi factorial and thought to develop as a result of a combination between genes and environmental factors.

Autoantibodies

One of the hallmarks of T1D is the appearance of auto-antibodies against the antigens Glutamic acid decarboxylase (GAD), Zinc transporter protein 8 (ZnT8), Tyrosine phosphate (IA-2) and Insulin. Studies have shown that 70-80% of newly diagnosed patients with T1D have at least one of these antibodies (73). The risk of progression to clinical onset of T1D in autoantibody positive individuals appears to be associated with multiple number and high-
er titer of antibodies (73, 74) Even though auto-antibodies are useful in prediction and classification of the T1D the exact role of these antibodies in disease pathogenesis is not understood. Apart from insulin, the T1D-related auto-antigens are not beta cell-specific, and it is possible that auto-antibodies appear as a response to beta cell damage leading to exposure of antigens that were previously hidden intracellularly.

**Glutamic acid decarboxylase 65 (GAD)**

GAD65 is an enzyme that catalyzes the α-decarboxylation of L-glutamic acid to synthesize neurotransmitter γ-aminobutyric acid (GABA) (75). GAD65 is expressed in pancreatic islet β cells, ovaries, testis and in GABA neurons (76). In pancreatic islet β cells, it is stored in synaptic vesicles (76), but the role of this enzyme in islet β cells is not known (77, 78). It has been shown that autoantibodies against GAD65 are present in 70-80% of T1D patients (79).

**Tyrosine phosphate (IA-2)**

IA-2 is a transmembrane protein that belongs to the protein tyrosine phosphatase family (80). In pancreatic islet β cells it is located on the insulin secretory granule membrane (81). It is estimated that about 55-75% of newly diagnosed T1D patients are positive for IA-2 auto antibodies (82). The prevalence of these antibodies varies with the age and HLA type. Highest frequency was reported in younger children and in patients with DR4 HLA type (79, 83).

**Zinc Transporter 8 (ZnT8)**

ZnT8 is a dimeric membrane protein. In islet β cells it is located in insulin secretory granule and acts as a zinc transporter (84, 85). ZnT8 auto antibodies have been detected in 60-70% of T1D patients (86). Its frequency is inversely related to the age of onset of T1D. Highest frequency was reported in children below 10 years (86).

**Insulin**

Insulin is a polypeptide hormone produced by the β cells of the islets of Langerhans. It is essential for the intracellular transport of glucose into tissues. Insulin is composed of two polypeptide chains, A and B, which are connected by a disulfide bridge. Insulin is synthesized as its precursor molecule pre-proinsulin on the ribosomes of the rough endoplasmic reticulum. Cleavage of signal polypeptide from pre-proinsulin forms the proinsulin. It is transported to Golgi where it is converted to an insulin hexamer molecule by excision of C-peptide(87). Antibodies against insulin have been found in 70-80% of newly diagnosed T1D patients (79).
Genetic factors
Several genetic factors such as HLA, Insulin, PTPN2, CTLA-4, IL-2RA, and IF1H1 (MDA5) are associated with predisposition to T1D (88-90).

**Human Leukocyte Antigen (HLA)**
HLA is one of the major genetic loci associated with T1D development. In humans, HLA is located on the chromosome 6p21. It is estimated that this region alone contributes to around 40-50% of the overall genetic risk to develop T1D (91). HLA is divided into class I, II and III regions. HLA class I molecules are encoded by genes A, B and C and these molecules are expressed on most of the nucleated cells. DP, DQ and DR genes encode HLA class II molecules and expression of these molecules was found on most of the antigen presenting cells. The main function of these molecules is presentation of processed antigen to immune cells (89, 92). Different combinations of HLA class II genes are associated with susceptibility vs. protection towards development of T1D.

**Insulin**
Susceptibility to T1D in the insulin locus was mapped to variable tandem repeats (VNTR) in the insulin promoter region. Tandem repeats occur in three variable sizes such as shorter (class, I, 26-63 repeats), intermediate (class II, 80) and longer repeats (Class III 141-209) (93). Functional studies showed that class I VNTR repeats are associated with lower insulin expression in thymus, which is thought to result in the less efficient negative selection of insulin specific T lymphocytes. In contrast class III VNTR alleles are associated with higher insulin expression in thymus thereby more efficient in the deletion of insulin specific T cells and in turn induction of immune tolerance (94). It has been reported that presence of class I/I homozygous genotype is associated with 2-5 fold risk of developing T1D (89).

**Interferon induced with helicase domain 1 (IF1H1)**
In humans IF1H1, present on chromosome 2, encodes a cytoplasmic helicase (95). It is involved in recognition of dsRNA generated during replication of RNA viruses (96). Single nucleotide polymorphisms in this gene are associated with the susceptibility vs. protection towards T1D development. It has been shown that individuals with susceptible genotypes had higher IF1H1 gene expression levels. In contrast, protective IF1H1 variants showed reduced function when expressed in cell lines (97, 98).

**Epidemiology**
The incidence of T1D has been increasing worldwide and according to published data; there was a pooled increase of 3.0% per year in 37 populations
all over the world. The incidence rate is highly varied among different countries (99). Highest incidence rate was reported from Finland, with nearly 60 per 100,000 in 2006, and lowest in Zunyi (China) and Carcass (Venezuela), with 0.1/100,000 per year (99). Several epidemiological reports also reported a variation of incidence according to season, where the highest number of cases were diagnosed in the autumn and winter and lowest in the spring (100).

Environmental factors
Even though T1D has a strong genetic component, the rapid increase of T1D incidence all over the world, the low concordance rates to develop T1D between monozygotic twins (50%) and dizygotic twins (10%) (101, 102) and the increase of incidence of T1D in low risk HLA groups (103) cannot be explained by genetics and support the role of environmental factors in T1D development. Several environmental factors such as viruses (enterovirus, rotavirus, mumps virus, cytomegalovirus, and rubella virus), gut microbiota, dietary factors (Cow milk, wheat protein), toxins and vitamin D deficiency are implicated in the role of T1D development (92).

EV Implication in T1D development
Several epidemiological and experimental studies emphasized the association of EV infections in T1D development. In 1969, Gamble et al. showed that serum from T1D patients had higher CBV neutralizing antibody titers than in control subjects. Further they also reported a coincidence of seasonal variation of T1D cases with EV infections (100, 104). In 1974, Yoon et al. isolated CBV4 from the pancreas of a 10-year-old child who died of diabetic ketoacidosis. In addition they also showed that infection with this strain lead to hyperglycemia, β-cell necrosis and inflammation of islets of Langerhans in mice (105). Several cross sectional studies have also reported that EV RNA is more frequently found in blood and serum of T1D patients than controls (29). Studies also showed that the antiviral cytokine IFN α up-regulated in T1D patients (106).

In prospective studies from Finland, where they followed non diabetic siblings of T1D children until they developed clinical onset or auto antibodies, revealed that EV infections are more frequent in children who became diabetic than others (107). This was further confirmed by another study in Finland where they followed risk HLA children and reported that EV infections are more frequent in children who turned autoantibody positive than in others (108). Further other studies observed that EV protein 1 (VP1) was found in multiple islets of 44 out of 72 (61%) T1D cases versus 3 islets out of 50 (6%) in controls (109). Studies from Japan demonstrated the presence of EV RNA and also up-regulation of several viral sensors in pancreata of
patients who died from fulminant type 1 diabetes (110). It has also been shown that several EV serotypes can infect and replicate in islets in vitro (111). Infection with different serotypes and strains of a serotype of EVs also caused up-regulation of several chemokines such as CXCL10, CCL5 in vitro (58). It has also been shown that serum of T1D patients contains higher level of CXCL10 compared to controls (112). In conclusion, all these data from epidemiological, animal and pancreas studies support a casual relationship of EV infections in T1D development. However more studies are needed to show the casual role of EV in T1D development.

Possible Mechanisms of EV in T1D development

The exact mechanism of viruses in T1D development is poorly understood. However several mechanisms are hypothesized.

Direct damage
Direct infection with EV can destroy the insulin-producing β-cells by virus-induced cytolysis. It was shown that several EV serotypes and clinical isolates can infect, replicate and destroy the human islets in vitro (58, 111). Alternatively, persistent infection with virus can impair the β-cell function in several possible ways. For example continuous replication of virus can damage the β cell function and eventually release of insulin.

Bystander Mechanism
According to this mechanism, infection of β-cells leads to inflammation induced tissue damage, which in turns results in the release of sequestered islet antigens. Presentation of these antigens may activate and recruit auto reactive T lymphocytes that evoke β-cell damage (113).

Molecular Mimicry
It was observed that there was partial sequence homology between islet-cell auto antigens (GAD-65, IA-2/IAR, and HSP-60) and EV proteins (2c, VP1and VP0) (114). This leads to speculation that similarity between these proteins may lead to generation of antiviral Cytotoxic T lymphocytes that cross react with islet cell proteins and in turn causes β-cell death. However most of the studies disproved this hypothesis.
Aims

The overall aim of this thesis is to study different EV strains on their ability to replicate, cause cytopathic effect/islet disintegration and induce innate antiviral pathways in explanted human islet of Langerhans and in exocrine cells clusters. In addition, the presence of EV RNA, protein and pathological changes in pancreatic tissue of T1D donors was investigated.

Paper-I

The aim of this study was to investigate the presence of EV genome, EV protein and the expression of MHC1 in islets and biopsies collected from live recent onset T1D patients.

Paper-II

To study the effect of infection with two different strains of CBV-1 on the induction of cytopathic effect/islet disintegration and genes involved in the viral sensing pathways leading to the synthesis of chemokines and cytokines in explanted purified, human islets.

Paper-III

In this study we have used three different Echovirus serotypes; these were associated with development of diabetes-related autoantibodies during meningitis epidemics in Cuba. The effect of these serotypes on beta cell function and their ability to cause islet cell disintegration and induction of innate immunity genes in human islets of Langerhans were studied.

Paper-IV

In this study, three echoviruses that were found to have islet tropism in Paper-III were used, along with strains of Echovirus 6, endemic in Cuba, with the aim to analyze their tropism and ability to replicate and cause induction of antiviral genes in human exocrine and endocrine cells.
Paper-V

The aim was to characterize islets with a large number of endocrine cells displaying signs of hydropic degeneration in pancreatic tissue collected from a T1D donor. Also the study aimed at examining the feasibility of using islet tissue microdissected by laser capture technology to quantitatively analyze gene expression.

Paper-VI

The aim was to study the effect of lytic and non-lytic CBV strains to mimic fulminant or more slowly progressing type 1 diabetes. By comparing our findings on in vitro-infected islets with previously reported clinical findings, we aimed investigating whether some of the clinical manifestations of these two subtypes of T1D could be explained by islet infection with lytic and non-lytic EVs.
Materials and Methods

In this section some of the materials and methods, and rationale for using those, are described. More detailed and thorough information about the methods is described in the material and methods section of the respective papers.

Virus
The strains CBV-1-7-10796 and CBV-1-11-10802 were isolated in Argentina during 1983 and 1998 respectively, by Centers for Disease Control and Prevention (CDC), Atlanta, USA. These two strains were selected because they have showed difference in their ability to induce innate immune responses in peripheral blood mononuclear cells in vitro (115). The CBV-1-7-10796 strain was shown to be weakly immunogenic and the CBV-1-11-10802 strain strongly immunogenic (116). CBV-1-3 was isolated in the USA. The CBV-4 strain, VD2921, was isolated from a patient suffering from aseptic meningitis, plaque purified, and the plaques previously shown to cause a non-lytic infection in explanted human islets (111) were used. The Echovirus serotypes 4, 16 and 30 were isolated from stool samples of children during Cuban meningitis epidemics in the years 1986, 2000, and 2001, respectively (117). These three serotypes were associated with development of islet autoantibodies during these meningitis epidemics (118-120). Echovirus-6, endemic in Cuba, was isolated from the stools of sporadic cases of viral meningitis in Cuba during the years 1991 (E6/91), 1992 (E6/92), 1993 (E6/93), 1994 (E6/94), 1996 (E6/96), 2011(E6/11) and 2012 (E6/12).
Table 3. Serotypes of EVs and strains of serotypes included in the thesis

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Strain</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBV1</td>
<td>CBV-1-11-10802</td>
<td>Paper II and paper VI</td>
</tr>
<tr>
<td></td>
<td>CBV-1-7-10796</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBV-1-3</td>
<td>Paper VI</td>
</tr>
<tr>
<td>CBV4</td>
<td>VD2921</td>
<td>Paper VI</td>
</tr>
<tr>
<td>Echovirus 4</td>
<td>Echovirus 4</td>
<td>Paper III and Paper IV</td>
</tr>
<tr>
<td>Echovirus 6</td>
<td>Echovirus 6</td>
<td>Paper IV</td>
</tr>
<tr>
<td>Echovirus 16</td>
<td>Echovirus16</td>
<td>Paper III and Paper IV</td>
</tr>
<tr>
<td>Echovirus 30</td>
<td>Echovirus30</td>
<td>Paper III and Paper IV</td>
</tr>
</tbody>
</table>

Human explanted islets and exocrine cells (Paper II, III, IV and VI)

Most studies have used animal models or immortal cell lines to study EV-induced innate immunity pathways (121, 122). Although these models are useful in analyzing certain aspects, they may not reflect the effect of such infections in primary human cells. Especially when studying EV induced innate immunity in the context of T1D pathogenesis, these models may not mirror the complex environment of islets of Langerhans. Islets contain several endocrine and non-endocrine cells, all expressing different markers, receptors and immune or antiviral genes (123). In addition, recent studies have indicated that the expression of certain immune and antiviral genes differs between different islet endocrine cell types (124). In this context, it is
important to use primary human pancreatic islets of Langerhans to study the EV-induced innate immunity in order to gain more insights into the role of EV in T1D pathogenesis.

In this thesis, explanted human pancreatic islets and exocrine cell clusters from brain dead organ donors were used. The islets of Langerhans and exocrine cells were isolated from pancreata obtained from brain dead organ donors using a protocol approved by the local ethics committee. Islets are principally isolated for the purpose of clinical transplantation, however, when the islet volume is too low for transplantation they are used for research purpose with consent from organ donors or close relatives. The purity of the islets after isolation varies, ranging from 10% to 90%. In order to avoid exocrine contamination in the islet preparations, or endocrine contamination in the exocrine cell preparations, we have, in all studies, further purified islets and exocrine cells by handpicking with a micropipette under a light microscope. Islet purity was determined by using dithizone (DTZ) (Sigma–Aldrich Sweden AB, Stockholm, Sweden) staining. DTZ is a zinc-chelating agent known to selectively stain pancreatic beta cells because of their high zinc content (125). Islets after isolation and after additional purification can be seen in Figure 5.

![Figure 5. Islets stained with dithizone appeared red in color A) before purification B) after purification](image)

**Pancreatic tissue (Paper I and V)**

Studies of human pancreata from individuals with T1D have been very limited due to lack of availability of suitable tissue samples. However, the building of biobanks of organ donor pancreata such as nPOD, and laparoscopic pancreatic tail resections in the DiViD study, have made such tissue available for studies of T1D. Analysis of pancreatic tissue from T1D pa-
tients, close to onset, is important as it will enable us to study the endocrine and non endocrine cells in their endogenous environment. In paper V we studied the pancreatic tissue collected from an organ donor with T1D and from one non-T1D organ donor. The donor with T1D was 40-year-old healthy male who died due to complications associated with onset of T1D. The control donor was a 26-year-old healthy male without any pancreatic disease. Tissue samples were collected from the head and body of the pancreas and fixed in 4% PFA. In paper I we had a rare opportunity to study pancreatic tissue that was obtained from six living subjects with recent onset of T1D development. More details regarding the donors and methods used for collecting the tissue are described in elsewhere (126).

EV Detection Methods (Paper I)

Virus Isolation

EV isolation using cell cultures is regarded as the golden standard for detecting EV infections. However, due to its time consuming and labor-intensive nature, it is often replaced by easier and quicker molecular methods. The main advantage of virus isolation is that it allows the study of the viral isolate (virus phenotype) in more detail to understand its molecular and pathogenic characteristics. However, isolation proved to be less sensitive compared with molecular methods (127, 128). In addition, isolation might be not possible when the viral titers are very low or during slowly replicating non-cytopathic persistent infections. In paper I, we have used the cell lines HeLa, Green monkey kidney (GMK), EndoC-βH1 and Rhabdomyosarcoma (RD) cells, cultured as monolayer, for virus isolation. These cell lines were inoculated with the culture medium collected from cultured islets and exocrine cell clusters at different time points post islet isolation. The inoculated cells were screened for the presence of cytopathic effect under light microscope, all isolates were passaged once on the same cell line.

Immunohistochemistry (IHC)

IHC is the most widely used method to detect protein in tissue samples. It is also the most commonly used method to detect EV proteins in tissue from patients. In addition to detecting the viral proteins, this method enables us to study the localization of virus in different cells types in tissue by double staining for specific marker for different cell types. We have employed the monoclonal antibody clone 5D8/1(Dako) directed against EV-P1 in our studies. This antibody recognizes a peptide sequence located between 42 to 50 residues of the VP1 capsid protein, which is highly conserved among several
EV species (129) and this antibody was shown to detect different EV serotypes in infected cell cultures (130, 131).

Polymerase chain reaction (PCR)

PCR has been proven to be the most sensitive method among all EV detection methods (132). Studies have shown that it is possible to detect less than 20 viral particles by using 5’UTR semi-nested PCR (133).

However, the sensitivity of the PCR was shown to vary depending on the serotype (134). In addition PCR sensitivity might also vary depending on the sample type (amount of tissue inhibitors). In our study (Paper I), we have used a semi nested PCR targeting the 5’UTR of the EV genome and the primers used are listed in table 3. The advantage of targeting this region of the virus genome is that it is highly conserved among many EV serotypes and therefore primers can be designed to detect most EV types. However, since the region is conserved among EV, this region cannot be used to genotype the virus. The regions that were detected by PCR and IHC were depicted in Figure 6.

Table 4. Primers used for EV detection by using 5’ UTR semi nested PCR

<table>
<thead>
<tr>
<th>Location in 5UTR</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st PCR</td>
<td></td>
</tr>
<tr>
<td>EVfw (sense)</td>
<td>GCCCCTGAATGCGGCTAAT</td>
</tr>
<tr>
<td>450-468 nt</td>
<td></td>
</tr>
<tr>
<td>ECBV5 (antisense)</td>
<td>GATGGCCAATCCAATAGCT</td>
</tr>
<tr>
<td>640 nt</td>
<td></td>
</tr>
<tr>
<td>2nd PCR</td>
<td></td>
</tr>
<tr>
<td>EVfw (sense)</td>
<td>GCCCCTGAATGCGGCTAAT</td>
</tr>
<tr>
<td>450-468 nt</td>
<td></td>
</tr>
<tr>
<td>EV rev (antisense)</td>
<td>ATTGTCACCATAAGCAGCCA</td>
</tr>
<tr>
<td>596 nt</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6. Schematic representation of EV genome A) indicating the locations detected by PCR and VP1 antibody B) representation of the positions of the primers used in this study.
Results and discussion

Paper I - Detection of a low-grade enteroviral infection in the islets of Langerhans of living patients newly diagnosed with type 1 diabetes

EVs have long been associated with T1D development. Several studies have supported this hypothesis by detecting the EV protein, genome and neutralizing antibodies, in different samples such as stool, blood, and serum of T1D patients (104, 135, 136). However, studies in the pancreas are limited due to lack of well-preserved tissue. Detection of the virus in the pancreatic islets would be particularly important since it would provide a mechanistic explanation of EVs role in the T1D pathogenic process. However, due to the anatomic location of the pancreas, it has been difficult to obtain pancreatic samples. In this study we had a unique opportunity to study the EV presence in pancreatic tissue collected from living T1D subjects through pancreatic tail resection.

This study showed that three out of six patients were positive for EV-RNA in the culture medium of isolated islet cells with PCR in both labs in Uppsala and Tampere. Culture medium from islets isolated from one patient was only positive by PCR run in Tampere whereas culture medium from exocrine cells from the same patient was positive in Uppsala. All PCR products were sequenced in the 5’UTR of the EV genome and obtained partial nucleotide sequences were compared to reference EV strains available in GenBank. Database results revealed a perfect match with EV sequences. EV protein (EV-P1) positive cells were detected in the islets of all six T1D patients whereas this protein was detected only in two out of nine controls. Failure to isolate virus, absence of cytopathic effect in isolated endocrine and exocrine from biopsies and detection of low level of viral RNA and protein suggest that these patients might harbor a persistent infection. It has recently been shown that EV persists in the absence of cytopathic virus in mice pancreas through the evolution from cytolytic to non cytolytic EV variants due to genomic deletions in the 5’ UTR region (137). When the whole tissue (flash-frozen or stored in RNAlater) was analyzed, EV RNA was detected in only one of the six patients, and this patient was also found positive in culture medium. Even though the rest of the patients were found negative in pancreatic whole tissue, we cannot exclude that these patients might have had EV infection in other parts of the tissue. The presence of EV in tissue has been shown to be focal during infection, and using more biopsies also showed to increase the positivity in myocarditis pa-
This might also be the case in these patients that EV infection might be limited to some areas, and that analyzing more tissue might have allowed the finding of more patients positive for EV in the pancreas. However, due to limited availability of tissue, we were not able to use more than one part of a biopsy. In two patients that were found positive for EV by detecting EV-P1 cells by IHC analysis, no signal was obtained by PCR. One possible explanation for the lack of EV genome detection in spite of detecting EV protein might be due to PCR inhibitors in tissue that might have decreased the PCR sensitivity. Another explanation could be that the positivity obtained with IHC was from another part of the pancreatic resection and due to the focal presence of these viruses in tissue it is possible that this might have caused the difference in positivity between the methods.

In addition to presence of EV this study also shows the hyper expression of MHC1 in all six patients. This is in line with the previous finding showing up regulation of MHC1 in T1D patients (140, 141). However what causes the MHC1 up regulation is not known. It has been hypothesized that IFN alpha released from the beta cells could be responsible for this up regulation. In support of this, IFN has been detected in T1D patients (140). EV persistence in islets was shown to induce IFN alpha in in vitro infected islets (142). It seems likely that EV presence could induce IFN alpha which subsequently up regulate MHC, perhaps resulting in autoimmune beta cell death.

In conclusion, this study that for first time shows the detection of EV RNA and protein in live T1D patients, demonstrates that EV can spread to the pancreas; however, further studies are needed to prove a casual relationship between EV and T1D development.

Table 5. Detection of EV RNA, Protein and MHC-1 expression in T1D patients

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/Sex</th>
<th>Diagnosis to biopsy</th>
<th>EV-RT-PCR (Uppsala &amp; Tampere)</th>
<th>EV-P1 (IHC) (Exeter &amp; Tampere)</th>
<th>Expression of HLA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Culture Medium</td>
<td>Biopsy</td>
<td>**</td>
</tr>
<tr>
<td>1</td>
<td>25/F</td>
<td>4</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>24/M</td>
<td>3</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>34/F</td>
<td>9</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>31/M</td>
<td>5</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive ++</td>
</tr>
<tr>
<td>5</td>
<td>24/F</td>
<td>5</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>35/M</td>
<td>5</td>
<td>Negative**</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

** Positive in exocrine fraction in Uppsala and in endocrine fraction in Tampere
++ Positive in Tampere negative in Exeter
Paper II - Infection of human islets of Langerhans with two strains of coxsackie B virus serotype 1: Assessment of virus replication, degree of cell death and induction of genes involved in the innate immunity pathway

In this study, we used two CBV-1 strains that are previously shown to differ in their induction of innate immunity in peripheral blood mononuclear cells (143) and investigated if these two strains also differ in their ability to cause cytopathic effect and innate antiviral gene induction in primary isolated human pancreatic islets. We showed that one of the CBV-1 strains (CBV-1-11) caused more cytopathic effect (islet disintegration) than the other CBV-1 strain (CBV-1-7). This can be explained by the fact that even a single point mutation in the EV genome has been shown to affect the virulence of the virus (144).

CBVs have mainly been shown to be sensed by the innate immune sensors TLR3 and MDA5. However, the relative importance of these factors seems to differ between different cell types (145). In this study, we showed that TLR3 expression is minimal at the mRNA level and almost absent at the protein level in islet cells; The absence of TLR3 in the endocrine cells might indicate that MDA5 plays the primary role in sensing the CBV in islets of Langerhans. In addition, we showed that infection with both of these CBV-1 strains caused the up regulation of genes encoding CXCL10 (IP10) and CCL5 (RANTES). These chemokines are implicated in the immunopathology of several inflammatory and autoimmune diseases (146) and have been shown to be elevated in serum of T1D patients (147). In addition, CXCL10 has been shown to be expressed in pancreata of fulminant T1D cases (148). In NOD and transgenic RIP-lymphocytic choriomeningitis virus (LCMV) models of autoimmune diabetes, selective and extensive expression of CXCL10 was shown to attract the activated T lymphocytes (149). In rat insulin promotor (RIP)-GP mice model, infection with lymphocytic choriomeningitis virus was shown to induce CXCL10 leading to expansion of auto-aggressive T cells and their migration into the islets leading to impairment of beta cell function (150, 151). It has also been shown that treating human islets with CXCL10 decreases the insulin synthesis (152). It seems likely that the induction of CXCL10 in vivo upon EV infection of islets would attract T-lymphocytes to the islets. This might lead to insulitis and possibly to immune-mediated damage.

Furthermore, both of these strains specifically reduced the expression of the gene encoding insulin while the glucagon gene expression was not affected. This, together with the detection of dsRNA in insulin positive but not in glucagon positive cells, indicates that these CBV-1 strains specifically replicated in beta cells. However, it is not known why only beta cells, rather than alpha cells, are productively infected but recent studies with purified rat
alpha and beta cells showed that alpha cell express higher basal and induction levels of antiviral genes than beta cells resulting in an antiviral state in the latter, thus virus replication will be affected (124). Infection with both of these strains resulted in the induction of genes involved in innate antiviral pathways and the gene induction levels did not differ between the strains. This might be surprising as one could expect that infection with more lytic strains would lead to less gene expression due to rapid cell death. However, islets consist of several types of endocrine cells so it seems possible that some of the genes induced might be a secondary effect of the infection and origin from other endocrine cells in the islets.

One of the important findings in this study was that expression level of some of the innate immunity genes (IFN-β, MDA5, IP-10), that are important in induction of an antiviral state, varied between different donors. IFN-β was shown to be important in controlling CBV infection in mice (153). IFN-β treatment of dilated cardiomyopathy (DCM) patients with EV persistence was also shown to eliminate EV genome from these patients (154). It is tempting to speculate that, if this situation replicates in vivo, the individual with lower expression of these genes might not be able to clear the infection efficiently and this might result in prolonged virus replication/virus persistence and beta cell death. MDA5 is a cytoplasmic sensor of dsRNA, a replication intermediate formed during replication of RNA viruses (155), including EV (156). Activation of MDA5 induces antiviral pathways to limit the viral infection (157). Studies using MDA5-deficient mice have showed an increased mortality after EV infection (158), demonstrating the important role of MDA5 in controlling the EV infection. Variation of this gene between donors might influence the outcome of the infection. It is possible that lower expression of MDA5 and other antiviral genes in vivo results in impaired EV sensing, leading to systemic spread of the virus. CXCL10 is important for the recruitment of auto-aggressive T cells to pancreatic islets (151). Inhibition of CXCL10 has been shown to decrease diabetes incidence in mice (149). Further, the pancreatic CXCL10 mRNA expression level was shown to positively correlate with the insulitis frequency in a NOD mice model (159). Transgenic mice expressing CXCL10 in β cells show spontaneous infiltration of lymphocytes as well as impairment of β cell function (160). The variation of this gene expression upon EV infection, leading to differential secretion of protein in vivo, might influence the infiltration of T cells and further determine the extent of insulitis and beta cell damage.

In conclusion, differential expression of these antiviral genes between different donors suggest that the same infection could have a different outcome in two different individuals, which might also explain why systemic EV infections initiate changes leading to the development of type 1 diabetes in certain individuals but not in others.
Autoantibodies against islet cell antigens can be detected in the serum of T1D patients even years before clinical onset of T1D and they are used as predictive markers for developing T1D (161). However, factors triggering the induction of islet autoantibodies are not known. Recent studies from Cuba reported sero-conversion to islet cell autoantibodies during three echovirus (echovirus16, echovirus 30, echovirus 4) epidemics, suggesting that EV infections may be one of the factors that initiate the development of islet autoimmunity (119, 162). Interestingly, the prevalence of these islet cell autoantibodies also varied between different epidemics. Prevalence of ICA was moderate during echovirus 4 epidemic and high during echovirus 16 and 30 epidemics. In addition to ICA, autoantibodies against insulin (IAA) and glutamic decarboxylase (GADA) were also detected during echovirus 16 and echovirus 30 epidemics (118-120).

In this study, explanted human pancreatic islets from seven organ donors were infected with echovirus serotypes 4, 16 and 30. The viral replication, islet disintegration/cytopathic effect, beta cell function, and induction of innate immunity genes were studied. Echovirus 16 and 30, which were associated with higher prevalence of autoantibodies, caused cytopathic effect/islet disintegration in all donors and there was no apparent difference between these serotypes. Islets infected with any of these serotypes showed signs of cytopathic effect already day one post infection and this was more pronounced day three-post infection, whereas in islets infected with echovirus 4, which is associated with moderate prevalence of autoantibody induction, no cytopathic effect/islet disintegration in islets from any of the donors were seen. Both echovirus 16 and 30 replicated in the islets infected from all seven donors with clear titer increase from day zero to day three post infection. Further, detection of EV-P1 and dsRNA in insulin-positive cells suggested the selective replication of these strains in beta cells. Echovirus 4 was replicated only in islets from two of the seven donors, with a titer increase between day three and five post infection. Despite that, no signs of cytopathic effect/islet disintegration were detected.

Viral replication ability has been shown to depend on both genetics of the virus and host determinants. The human islets used in the present study were obtained from different organ donors with different genetic background, and therefore, variability in the level of viral replication between different donors can be expected. However, selective replication of echovirus 4 in two donors and complete absence of replication in the other donors suggest that the is-
lets from these two donors express the receptors or other factors that are required for the echovirus 4 replication.

The non-cytolytic replication of echovirus 4 seems to be islet or beta cell specific since further experiments with beta cell lines also showed the similar results to what was obtained in isolated islets (163). However, infection of non-beta cell lines such as GMK and human rhabdomyosarcoma (RD) cells showed clear cytopathic effect, suggesting that the absence of cytopathic effect is beta cell specific. This beta cell specific non-cytolytic replication has also been reported for other EV strains (33). Studies have indicated that both viral genomic changes and availability of cellular factors could influence the outcome of the EV cytolytic ability. It has been shown that CBV undergoes lytic replication in immortalized cells lines while preferentially establishing non lytic slowly replicating persistent infection in primary cells, hypothesizing that differential expression levels of specific cellular factors in primary cells might favor the non-cytopathic persistent infection over the lytic (164). It might be possible that lack of host factors in insulin producing cells might favor the productive non-cytopathic echovirus infection in beta cells.

Infection with any of the three echoviruses affected the glucose-stimulated insulin secretion significantly compared to mock infected control islets. However, the effect was higher with echovirus 16 and echovirus 30 compared to echovirus 4. Effect of echovirus 30 and 16 infection can be expected due to islet cell disintegration. However the effect of echovirus 4 on glucose induced insulin secretion despite not inducing any islet disintegration suggests that this virus might cause a functional block. This also suggests that even non-cytolytic productive infection can impair the insulin secretion. Interestingly, impairment of insulin secretion without reduction in insulin content was reported in T1D patients (165). Further, this study also shows that infection with echovirus 16 and echovirus 30 resulted in the induction of innate antiviral genes. However, infection with echovirus 4 did not cause any induction of the genes evaluated.

In conclusion, cytolytic properties of these serotypes might explain the difference in the prevalence of islet autoantibody development during different Echovirus epidemics in Cuba.
Table 6. Effect of Echovirus infection on virus replication and islet disintegration in explanted human islets

<table>
<thead>
<tr>
<th>ECHO virus serotype</th>
<th>Prevalence of ICA during epidemics</th>
<th>Viral replication in infected isolated islets (n=7)</th>
<th>Cytopathic effect in infected isolated islets (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echovirus 4</td>
<td>36.1%</td>
<td>Replicated in 2/7 donors</td>
<td>Did not cause cytopathic effect/islet disintegration in islets from any donor</td>
</tr>
<tr>
<td>Echovirus 16</td>
<td>92.1%</td>
<td>Replicated in all donors</td>
<td>Caused cytopathic effect/islet disintegration in all donors</td>
</tr>
<tr>
<td>Echovirus 30</td>
<td>87.5%</td>
<td>Replicated in all donors</td>
<td>Caused cytopathic effect/islet disintegration in all donors</td>
</tr>
</tbody>
</table>

Paper IV - Field strains of Echovirus 6 infect human endocrine and exocrine pancreatic cells and induce pro-inflammatory innate immune responses

Considerable evidence implies the involvement of both endocrine and exocrine pancreas in T1D patients. In support of this, various studies reported signs of inflammation, fibrosis and atrophy in the exocrine pancreas of T1D patients (166) (167). However, the causative mechanism behind the pathological changes in endocrine and exocrine pancreas of T1D patients is not known. EVs are implicated as one of the major environmental factors that trigger or accelerate the T1D. However most of the studies have investigated the effect of EV strains in the endocrine islets of Langerhans and very little is known about their tropism and effect on pancreatic exocrine cells (59, 168, 169). To address this, we have infected human pancreatic isolated exocrine cells with different strains of echovirus 6 and further studied their tropism and if infection resulted in induction of innate inflammatory molecules in these cells.

We have showed that only one of the echovirus serotypes (echovirus 6) could replicate in both exocrine and endocrine cells. In contrast, the other echovirus serotypes (4, 16 and 30) that were shown to infect endocrine cells in paper III did not replicate in exocrine cells. All seven field-isolates of echovirus 6 caused clear cytopathic effect/islet disintegration and they replicated both in endocrine and exocrine cells with no significant difference in titer increase between endocrine and exocrine cells. Previous studies with CBV strains showed minor viral titer increase when exocrine cell cultures were infected, and this was shown to be due to embedded endocrine cells.
However, in our study, a clear titer increase of echovirus 6 and conversely viral titer decrease of other islet tropic echovirus serotypes indicated that the viral titer increase was solely due to echovirus 6 replication in exocrine cells and not results from embedded endocrine cells. Further, electron microscopic detection of virus particles in the cytoplasm and in enclosed vacuoles in echovirus 6 infected exocrine cells proves the echovirus 6 replication in exocrine cells. In contrast, infection with the other three echovirus serotypes did not result in any viral titer increase and cytopathic effect/islet disintegration in exocrine cells. This lack of tropism for acinar cells has also been reported for CBV strains (170).

This result shows the unique ability of echovirus 6 to infect and replicate in both exocrine and endocrine pancreatic cells. Virus tropism and replication ability depends on several factors such as the presence of the appropriate virus receptor, availability of cellular factors essential for virus replication and viral genomic regions that were able to interact with receptor and cellular factors. Several studies have indicated that even a single or few amino acid changes in the viral genome can influence the receptor usage and tissue tropism. A single amino acid change in the VP1 region of a CBV6 strain was shown to change the viral phenotype to use DAF as a receptor (171). Furthermore, experiments with chimeric viruses, constructed by exchanging 5’UTR of different EVs, have been shown to alter the tissue tropism in mice (172), demonstrating that genomic regions in the 5’ UTR could influence the tissue tropism and replication. It might be possible that echovirus 6 has other genetic determinants than other EV strains that facilitate use of the exocrine cellular factors, which enables its replication in exocrine cells.

This study further demonstrates the induction of genes involved in viral sensing (MDA5), antiviral defense (OAS1 and IFNB) and chemokines (CXCL10 and CCL5) in both echovirus 6 infected exocrine and endocrine cells, suggesting that, like endocrine cells, exocrine cells are also equipped with a machinery to detect and respond to echovirus 6 infection. Further, the expression of chemokines suggests that infection of exocrine pancreatic cells also might result in attraction of immune cells. The mere induction of genes does not show that the corresponding protein is synthesized and genes coding for antiviral proteins will either not be processed due to block of CAP dependent translation, or some of the cellular proteins involved in the antiviral pathway will be cleaved by some of the virus proteins (173-175). Efficient virus replication is not possible in a cell with an antiviral state, thus this cannot be the case in any of our studies of explanted islets.
Paper V - Gene expression analysis of human islets in a subject at onset of type 1 diabetes

Swollen islet cells have been documented already at the beginning of the last century by Weichselbaum, and it has been referred to as hydropic degeneration (176, 177). This phenomenon was also further reported by Gepts. This kind of islets were also observed in the pancreas of depancretized diabetic dogs (178), alloxan-induced diabetic rabbits (179), in bank voles infected with Ljugan virus (180) and in dogs and cats treated with anterior pituitary extract (181). The hydropic cells are hypothesized to develop due to accumulation of cellular edema. However, the characterizations of these cells are not yet reported. In this study we had a unique opportunity to study the pancreatic tissue and isolated islets obtained from cadaver pancreases of a T1D donor and compare it to a non-diabetic control. Formalin-fixed and paraffin-embedded pancreatic biopsies and isolated isles were obtained from one T1D and one healthy donor. Islet-enriched tissue samples from these pancreatic sections were extracted by using Laser Capture Micro dissection (LCM). IHC analysis showed several hydropic cells in almost all examined islets in sections from the T1D donor whereas it rarely appeared in the control donor. We carried out this study with two major aims, first, to characterize the islets from T1D patients and, second, to investigate the feasibility of using RNA extracted from microdissected islets from frozen pancreatic tissue for gene expression analysis.

To characterize the islets from T1D patients, genes involved in ER stress, beta cell function, cell death and inflammation were analyzed by qPCR in both microdissected and isolated islets from T1D and control donors. Increased mRNA levels of genes encoding the chemokine MCP1 and MHC class I were found in both LCM samples and isolated islets of T1D compared to control samples. Hyper expression of MHC1 was reported in Paper-I and also in other studies. This result further support those observations (140). Genes encoding ER stress transcription factors ATF4 and CHOP were found to be increased in islets from T1D donor whereas mRNA levels of these genes were undetectable in islets from control donor. Increased levels of CHOP protein was also previously reported in islets of T1D patients (182). Our study further confirms this and also suggests partial ER stress response in this donor. However, further more extensive analysis is needed to confirm this observation. There were no obvious differences in genes involved in inflammation or apoptosis between islets from T1D and control donors. In addition, staining for cleaved caspase-8 also showed no evidence of apoptosis in control or T1D islets. These data show that there is no obvious sign of inflammation and apoptosis in hydropic degenerated swollen islets from a T1D subject compared to islets from a control donor, indicating that these cells are responding normally to high glucose and finally exhausted by beta cell depletion.
It is well known that the pathological features in T1D patients are unevenly distributed over the different lobes of pancreas (141). Using the whole tissue for molecular analysis makes the interpretation difficult and even undermines some of the important changes. The advantage of using LCM is that it allows us to extract the selective population or regions of the tissue. However intact RNA extraction from microdisected pancreatic tissue is particularly challenging due to high abundance of RNases in pancreas. Even though using intact RNA is optimal for gene expression analysis sometimes it might be desirable to use even the degrade samples since the tissue availability from the T1D pancreas is very rare. This study shows the possibility of performing qPCR analysis on the RNA extracted from microdisected pancreatic tissue in spite of degradation.

Paper VI- Enterovirus-induced changes in explanted human islets of Langerhans resemble findings in islets of fulminant and conventional type 1 diabetes

Fulminant Type 1 diabetes is a unique subtype of T1D mostly described in the Japanese population and characterized by extensive islet cell lysis (183). In contrast, this dramatic islet cell death has not been observed in the pancreas of conventional T1D patients. EV infections are associated with the etiology of both fulminant and conventional T1D (109, 184). However the causative mechanism of either is not known but it seems likely that the fulminant variant could be caused by an acute lytic virus infection. After observing the similarities and differences between the findings in fulminant and conventional T1D with our results obtained with lytic CBV-1 strain in PAPER-III and the low grade persistent kind of infection in PAPER- I. We hypothesized that EVs capability to cause more lytic vs non-lytic infection and the nature of virus induced host immune response may have implications on the pathogenesis of these subtypes of T1D. To mimic the fulminant T1D situation we have used three CBV-1 strains that has been shown to cause lytic infection in human explanted islets and to mimic the slowly progressing conventional type 1 diabetes (TD1) we have used a strain of CBV4 which has been shown to cause non lytic persistent infection in explanted islets. All CBV strains replicated in the islets but only the lytic CBV strains caused cytopathic effect/islet disintegration whereas the non-lytic CBV4 strain did not cause any disintegration of the islets. Some of the innate and antiviral proteins that were detected in fulminant and conventional T1D were induced by both lytic and non-lytic CBV.

Genes encoding OAS2 and MX1 were induced by all CBV strains. These proteins were shown to induce an antiviral state in infected cells (185). OAS convert ATP into 2’, 5’-linked oligomers of adenosine and these oligomers
activate the latent form of RNaseL leading to degradation of both viral and cellular RNA to inhibit the viral replication (186, 187). OAS pathway has been shown to be protective against CBVs during infection of isolated murine pancreatic islets (188). PKR is a protein kinase that is induced by interferon and activated by dsRNA. Upon activation, it phosphorylates translation initiation factor eIF-2α, leading to inhibition of protein translation to inhibit the virus replication. The gene encoding PKR was induced by both CBV-1-11 and by the CBV-4 strains. PKR was also detected in the EV-P1 positive beta cells of T1D patients. The gene encoding IRF7 was induced only by the lytic CBV-1-11 strain. IRF7 is an important transcriptional factor that belongs to the interferon regulatory factors. IRF7 resides in the cytosol and, following activation, it translocates to the nucleus, where with other co-activators it initiates IFN mRNA synthesis (46). Hyper expression of IRF7 was detected in fulminant T1D patients. Some of the results obtained with lytic and non lytic strains compared with the reported findings in fulminant and slowly progressing T1D are summarized in Table 8.

Table 7. Clinical characteristics of fulminant and conventional T1D (189).

<table>
<thead>
<tr>
<th></th>
<th>Fulminant Type 1 Diabetes</th>
<th>Conventional Type 1 Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Onset</strong></td>
<td>Abrupt onset with flu like symptoms</td>
<td>Slow onset</td>
</tr>
<tr>
<td><strong>Elevated pancreatic enzyme in serum</strong></td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Autoantibodies</strong></td>
<td>Mostly absent</td>
<td>Present</td>
</tr>
<tr>
<td><strong>Hemoglobin A1c levels</strong></td>
<td>Slightly elevated</td>
<td>Markedly elevated</td>
</tr>
</tbody>
</table>
Table 8. Comparison between the T1D and FT1D

<table>
<thead>
<tr>
<th>Results from the present study</th>
<th>Finding in islets infected with lytic CBV strains</th>
<th>Finding in Fulminant T1D cases</th>
<th>Finding in islets infected with non-lytic CBV strain</th>
<th>Finding in Conventional T1D cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islet cell lysis</td>
<td>Detected (190)</td>
<td>Detected (191)</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>Expression of PKR</td>
<td>mRNA expression levels were up regulated</td>
<td>Unknown</td>
<td>mRNA expression levels were up regulated</td>
<td>The protein was detected in EV-P1 positive beta cell (109)</td>
</tr>
<tr>
<td>Expression of TLR4 and NOD2</td>
<td>Not affected</td>
<td>TLR4 was only detected in monocytes (184)</td>
<td>Not affected</td>
<td>Unknown</td>
</tr>
<tr>
<td>Expression of IRF7</td>
<td>mRNA expression levels were up regulated</td>
<td>Hyper expression was detected (110)</td>
<td>IRF7 not affected</td>
<td>Unknown</td>
</tr>
<tr>
<td>Results from the previous studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detection of the EV-P1 protein</td>
<td>Detected (192)</td>
<td>Detected (184)</td>
<td>Detected (33)</td>
<td>Detected (109)</td>
</tr>
<tr>
<td>Hyper expression of MDA5, RIG-I</td>
<td>mRNA expression levels were up regulated</td>
<td>Hyper expression of proteins were detected (110)</td>
<td>mRNA expression levels were up regulated</td>
<td>Hyper expression was not detected</td>
</tr>
<tr>
<td>Expression of IRF7, IFN (α/β)</td>
<td>mRNA expression levels were up regulated</td>
<td>Hyper expression was detected (184)</td>
<td>IRF7 not affected, IFN not analyzed</td>
<td>Expression of type I IFN was detected (193)</td>
</tr>
<tr>
<td>Expression of CXCL10</td>
<td>mRNA expression levels were up regulated</td>
<td>Expression was detected (184)</td>
<td>CXCL10 secreted from infected islets (194)</td>
<td>Expression was detected (195)</td>
</tr>
</tbody>
</table>
Conclusions

Paper-I

- All six T1D patients were positive for EV with at least one of the methods independently in two labs.
- EV RNA was detected in 4/6 patients and EV-P1 positive cells were detected in all six patients.
- All six T1D patients were found negative for other viruses (rhinovirus, norovirus, rotavirus, and paraechovirus) analyzed.
- MHC-I hyper expression was observed in all six T1D patients.

Paper-II

- The CBV-1-11 strain caused more cytopathic effect/islet disintegration than the CBV-1-7 strain in explanted human pancreatic islets.
- Infection of islets with any of the CBV-1 strains resulted in the induction of genes involved in innate and antiviral pathways.
- Expression of the genes encoding IFN-β (P < 0.007), CXCL10 (P < 0.037) and MDA5 (P < 0.042) varied between different donors.

Paper-III

- Infection of human explanted islets with three echovirus serotypes that were differentially associated with induction of islet cell autoantibodies showed a correlation between their islet autoantibody induction and their ability to cause islet cell disintegration in explanted islets.
- Echovirus 30 and 16, that were associated with higher prevalence of autoantibody-induction, replicated and caused cytopathic effect/islet disintegration in explanted islets from all donors.
- Echovirus 4 that was associated with lower autoantibody induction replicated only in islets from 2/7 donors and did not cause cytopathic effect/islet disintegration.
• Infection with all three serotypes of echovirus impaired the glucose stimulated insulin secretion, also echovirus 4 despite not causing any islet disintegration.

Paper-IV
• All seven-field isolates of echovirus 6 replicated and caused cytopathic effect in both human endocrine and exocrine cells.
• Electron microscopy of echovirus 6 infected endocrine and exocrine cells showed the presence of virus particles in both.
• The epidemic echovirus 4, 16 and 30, associated with induction of islet autoantibodies, were not able to infect and replicate in human exocrine cells.
• Infection with echovirus 6 induced a massive degradation of cytoplasm and formation of empty vacuoles in exocrine cells.
• Infection with echovirus 6 affected the glucose-induced insulin secretion and also led to accumulation of echovirus 6 particles in close proximity to insulin granules.

Paper-V
• There were no significant signs of apoptosis, inflammation and ER stress in islets of a T1D donor despite cells showing swollen hydropic morphology.
• Gene expression analysis is feasible on RNA extracted from tissue microdissected from frozen pancreatic tissue that had been stored for several years.

Paper-VI
• All CBV strains replicated in explanted islets but only the lytic CBV-1 strain caused cytopathic effect/islet disintegration whereas the non-cytoplytic CBV-4 did not show any islet disintegration.
• Infection with all CBV strains caused the induction of the genes encoding MX1 and OAS2.
• Only one of the lytic CBV-1 strains caused induction of IRF7 gene expression.
Acknowledgements

I would like to convey my gratitude and sincere appreciation to everyone who has helped and supported me during the past years

First I would like to express my sincere gratitude to my supervisor Gun Frisk for believing in me and accepting me as a PhD student in your group. Thanks for always taking the time to listen and discuss everything from science to life. For your patience in correcting my writing countless times and especially for supporting me when I needed the most.

My Co-supervisor Oskar Skog I can’t thank enough for your continuous support and help right from my Master’s project to PhD thesis, and also, for all the discussions and valuable inputs and proofreading my thesis.

I am also deeply thankful to My Co-supervisor Magnus Essand for your helping nature, kindness and your willingness to come forward to help me in the most difficult times.

I would like to thank Catharina Sevensson for your concern and valuable suggestions. I truly admire both your scientific, administrating skills and caring nature.

I would like to thank Karin Forsberg Nilsson and Claes Wadelius for all your effort to make sure that everything goes smooth for my PhD defense.

Monika Hodik and Therese Reosenling, I was fortunate to have both of you in lab during my PhD, Thanks for all the discussions, lunches, help and kindness. I really wished that both of you would have stayed some more time. I wish I had been more in touch with you than I was…

Heikki Hyötty thanks for willing to help during the final stages of my PhD. Sofia Nordling thanks for your kind words.

Luis Sarmiento, friend and coauthor, for the nice collaboration and for being cheerful always, I really enjoyed working with you.
Anna Wiberg, thanks for your patience listening to my stories!! And also for answering all my stupid questions. Marcus Lundberg thanks for your company in the lab and the numerous discussions we had...Your suggestions were also very helpful.

I would like to thank all my colleagues Enida Kuric (Thanks for your tips how to survive post PhD), Marie Karlsson (helping with Endo Cells and for you kind nature), Per-Anton Stenwall, Marcus Bergström, Maja Ericsson, Peter, Johan Olerud, Magnus Ståhle, Andrew Friberg, Anna-Maria Ullbors, Karin Fonnaland, Sofie Ingvast, Thank you very much for all your company during these years.

Sana Asif thanks for all the discussion ranging from Bollywood to cricket and life in general, Ram Thanks for all your help, discussions and fun times!

Many thanks to all my collaborators Sami Oikarinen, Noel G. Morgan, Sarah J. Richardson Corrado M. Cilio, Lars Korngold, Knut Dahl-Jørgensen.

I would like to thank all my friends here in Uppsala and in India

Last but not least I would like to thank my family for always supporting and believing in me
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)