Enterovirus Implications in Type 1 Diabetes

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Human enteroviruses (HEVs), particularly Coxsackie B viruses (CVBs), might trigger the onset of type 1 diabetes (T1D), either by direct infection of the insulin-producing beta-cells or by an indirect inflammatory response. The overall aim of this thesis was to study the tropism of HEVs in isolated human pancreatic cell clusters in vitro including virus effects on islet function, gene-expression and ultrastructure. Furthermore, the expression of the major CVB-receptor, CAR, was investigated in pancreatic tissue from T1D-related subjects and CVB-infected islets. Also, tissues and isolated islets from two adult organ-donors who died close to disease onset were studied. The results showed that beta-cells were destroyed through lytic infections with different strains of CVBs and that islets function did not depend on replication per se but on the degree of islet destruction. Virus particles were observed in beta-cells in association with insulin granules, however no virus replication or particles could be observed in the exocrine cell clusters, as opposed to in mice models. The virus-infected islets had a decreased expression of insulin mRNA and CAR mRNA/protein, possibly reflecting virus-killed beta-cells. Infected beta-cells contained a high number of insulin granules, which might indicate an impaired function. The in vivo studies showed presence of virus proteins in the islets of both donors who died close to onset of T1D and elevated expression of innate immunity genes, potentially indicating viral infection, but direct evidence is lacking. Both donors were immune-reactive for insulin but the isolated islets had an impaired or completely lacking glucose response. Ultrastructural analysis showed both damaged beta-cells and normal-looking beta-cells, indicating that the latter might still have the potential to function but were blocked. CAR-expression was significantly increased in T1D-related subjects which might indicate tissue damage and/or inflammation in these subjects. To conclude, these results showed that CVBs could infect human primary beta-cells, likely by binding to CAR and lead to functional abnormalities, indicating that they could cause T1D in vivo. Exocrine cells were not permissive to CVB, which raises the question if mice-models should be used to study human pancreatitis. Also, unique materials from two T1D organ-donors were described.

Keywords: Enterovirus, Coxsackie B virus, Type 1 diabetes, Pancreatitis

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List of Papers

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


II Hodik, M.*, Skog, O.*,Korsgren, O., Frisk, G. Enterovirus infection reduces beta-cell function and leads to decreased glucose stimulated insulin secretion in dissociated but not in intact pancreatic human islets. Submitted manuscript.
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Results and discussion

Paper I - CVB-5 can replicate in human primary endocrine islets but not in exocrine cell clusters in vitro

Paper II - Virus-induced islet destruction but not replication per se impairs beta-cell function

Paper III - Expression of the Coxsackie B and adenovirus receptor in islet auto-antibody positive donors, recent-onset T1D donors and longstanding T1D donors

Paper IV - On-going pathogenic processes in the pancreata of two organ donors at disease onset

Conclusions

General discussion

Future perspectives and concluding remarks

Acknowledgements

References
Abbreviations

ADP  Adenosine diphosphate
Arf1  ADP ribolsation factor 1
ATP  Adenosine triphosphate
CAR  Coxsackievirus and adenovirus receptor
cDNA  Complementary deoxyribonucleic acid
CHO  Chinese hamster ovary
COP II  Coat protein complex II
COP I  Coat protein complex I
CPE  Cytopathic effect
CVB  Coxsackie virus group B
DAF  Decay accelerating factor
DC  Dendritic cell
DIPP  Diabetic Prediction and prevention
dsRNA  Double-stranded RNA
eIF4G  Human eukaryotic translation initiation factor 4G
ELISA  Enzyme-linked immunosorbent assay
EM  Electron microscopy
ER  Endoplasmatic reticulum
GBF1  Golgi brefeldin A resistant guanine nucleotide exchange factor 1
HEV  Human enterovirus
HEVP1  Human enterovirus protein 1
HSP  Heat shock protein
GAD65  Glutamic acid decarboxylase 65
GLUT  Glucose transporter
GMK  Green monkey kidney cells
GSIS  Glucagon stimulated insulin secretion
IA-2  Islet cell antigen-2 (Tyrosine Phosphatase)
IFN  Interferon
Ig  Immunoglobulin
IHC  Immunohistochemistry
IL  Interleukin
IRES  Internal ribosomal entry site
IP-10  Interferon-γ-inducible protein 10
ISG  Insulin secretory granules
MafA  Mammalian homologue of Avian
MDA-5  Melanoma differentiation-associated protein 5
MCP-1  Monocyte chemotactic protein-1
mRNA   Messenger RNA
MHC    Major histocompatibility complex
MODY   Maturity diabetes onset of the young
NK cell Natural killer cell
NO     Nitric oxide
NOD    Non-obese diabetic
nPOD   Network for Pancreatic Organ donors with Diabetes
OAS    Oligoadenylate synthetase
PABP   Poly(A)-binding protein
PBMC   Peripheral blood mononuclear cell
PCBP2  Poly(rC) – binding protein 2
PDX1   Pancreatic and duodenal homebox 1
PKR    RNA activated protein kinase R
PIII4Kβ Phosphatidylinositol-4-kinase IIIβ
Poly(I:C) Polyinosinic:polycytidylic acid
PVR    Poliovirus receptor
PTB    Polypyrimidine tract-binding protein
RANTES Regulated upon activation, normal T cell expressed, and secreted
RIG-I Retinoic acid-inducible gene I
RNA    Ribonucleic acid
SEM    Standard error of mean
ssRNA  Single stranded RNA
TCID50 Tissue culture infectious dose 50
TEDDY The Environmental Determinants of Diabetes in Young
TLR    Toll-like receptor
T1D    Type 1 diabetes
UTR    Un-translated region
VP     Virus protein
VPg    Viral protein genome-linked
ZnT8   Zinc transporter 8
Insulin, produced by the beta-cells in the pancreatic islets of Langerhans, is a hormone important for providing cells throughout the body with energy. It binds to the insulin receptor which initiates a signalling cascade leading to increased expression of glucose transporter molecules on the plasma membrane. This in turn facilitates uptake of glucose in the blood, which will metabolize and generate energy-rich ATP molecules, required to carry out various functions in the cell. In type 1 diabetes (T1D), the insulin-producing beta-cells are destroyed and unless insulin is administered externally cells start starving which will lead to ketoacidosis and ultimately death. By the time of diagnosis the majority of the beta-cells has been destroyed and/or is dysfunctional. Even though scientists have suggested specific features that might collectively lead to T1D, including genetics, auto-immunity, infections, diet, gut-flora, toxins etc., the full picture is far from clear as yet. One major issue is the lack of pancreatic islet tissue close to disease onset. Also, taking biopsies from living T1D-patients is hazardous due to the risk of leakage of pancreatic juice. Furthermore, even if tissue is collected, there are several factors that make interpretation of data difficult, including medication and condition of T1D-patients prior to death, cause of death, post mortem changes, handling of tissues etc. The Network for pancreatic organ donors with type 1 diabetes (nPOD) is a recently started collaboration for scientists studying the aetiology of T1D, in which they are trying to address many of the issues that have restrained the progress of this field. Efforts include a common bio-bank of T1D-related material through which scientists can apply for information on donors and discussions on how certain key-concepts (e.g. “insulitis”) should be defined.
Background

The pancreas
The pancreas runs from the concavity of the duodenum up to the spleen. Although there are no defined anatomical boundaries, the organ can be roughly divided into three regions; the head, located close to the duodenum, the body and the tail. The bulk (~80% of total pancreatic mass) consists of exocrine acinar cells which produce digestive enzymes that are secreted into the duodenum via a duct running through the pancreas. Endocrine cell clusters (~1-2% of total pancreatic mass) of varying sizes, named the islets of Langerhans, can be found scattered in the exocrine parenchyma. These islet cells secrete hormones into the bloodstream and are important in the regulation of metabolism. The cell-types include beta-cells, alpha-cells, delta-cells and pancreatic poly-peptide-producing (PP) cells, which produce insulin, glucagon, somatostatin and polypeptides respectively [1]. Recently a fifth hormone type, ghrelin, was discovered [2]. The beta-cells are most numerous (~55%), followed by alpha-cells (~30%) and delta-cells (~10%). In contrast to murine islets, in which the beta cells normally are located in the core of the islet and are surrounded by alfa-, delta- and PP-cells, the spatial anatomy of the human hormone cells is more heterogeneous [3-4]. In addition, the islets are highly vasculated with fenestrated capillaries and are also rich in autonomic innervations [1]. The insulin, glucagon and somatostatin granules can easily be distinguished ultrastructurally in the electron microscope. The granules in beta-cells often have an electron-dense, crystalline core which is surrounded by a translucent halo and the medium diameter size varies between ~250-350 nm, depending on the type of tissue fixation [5-6]. Glucagon granules are of approximately the same size as insulin granules and have been described as resembling the eyes of a bull. Somatostatin granules are approximately 100 nm larger than insulin and glucagon granules and have variable electron densities [1].
Type 1 diabetes

History
Diabetes was first described 2,000 years ago by the Indian physician Shushruta, who described a sweet taste in urine from affected subjects. At around 250 B.C. we find the first recorded use of the word “diabetes” which is derived from the Greek word for “a siphon”, referring to the excessive urination, common in these patients, i.e. water is siphoned from the mouth out of the body. In 1861, Paul Langerhans discovered clusters of cells in the pancreas which were later named after him. In 1889, Joseph Mering and Oskar Minowski discovered that diabetes develops when the pancreas is removed, but it was not until 1921 that Fredrik Banting and Charles Best succeeded in showing that pancreatic extract can lower glucose levels in diabetic dogs and thus is crucial in the glucose homeostasis. The purity of the pancreatic extract was improved and one year later Banting injected a 14-year-old diabetic boy named Leonard Thompson with insulin from dog and managed to prolong his life. This awarded him and his college John Macleod with the Nobel Prize in Physiology or Medicine in 1923 [7-8]. Companies soon started deriving insulin from slaughtered animals (pigs and cows), to make drugs which helped millions of people. However, to avoid e.g. allergic reactions, recombinant insulin was developed in the early 1980s [9]. Before insulin was available, the survival time in T1D-patients ranged from a few months to a few years. Even though the survival rate is high today, fluctuations of glucose levels in the blood often lead to long-term secondary complications, especially in nerves, eyes, kidneys and blood vessels [10].

Definition and Classification
The definition of diabetes according to the World Health Organization (WHO) is a “metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both”. In 1985, the terms type 1 diabetes (T1D) and type 2 diabetes (T2D) were proposed by the WHO, the former refers to subjects who “lack insulin” and the latter to subjects whose “bodies cannot effectively use the insulin they produce”. T2D, which is not covered in this thesis, normally develops in older individuals and is often associated with obesity, lack of exercise and other environmental factors. It accounts for approximately 90% of all diabetic cases. In contrast to T1D, T2D can sometimes be reversible with the right diet and physical activity. In addition to the two main types of diabetes, other, less common, categories of diabetes exist. These include for example
different forms of maturity-onset diabetes of the young (MODY), which are
associated with genetic defects in beta-cell function and are characterized by
mild hyperglycaemia. Gestational diabetes can sometimes occur at the end of
pregnancy. In addition, other diseases in the pancreas, such as pancreatitis
and tumours, increased levels of certain hormones (cortisol), medication
(pentamidine, nicotinic acid, glucocorticoids, thyroid hormone, phenytoin)
and poisons (Vacor) may also cause diabetes [10].

Diagnosis
The first clinical symptoms of T1D usually include polyuria, weight loss,
hunger, thirst, headache and nausea, but the disease can sometimes be
asymptomatic [10]. It has been estimated that about 40-90% of the beta-cells
have been destroyed or are not functioning by the time affected individuals
seek medical attention [11-14]. The number of remaining insulin-positive
cells close to diagnosis appears to be higher in adults [12, 14]. To measure
glucose levels in the blood, the clinician can use different methods, e.g. fast-
ing glucose test, oral glucose tolerance test, random glucose test and HbA1c
with specific cut-off points [10, 15]. The HbA1c measures blood glucose
during the past 2-3 months and a person with a value above 6.5 % is consid-
ered diabetic, whereas measurements between 5.7-6.4% indicate high
risk/sub-diabetes [15]. To distinguish T1D and T2D, the clinicians usually
consider factors such as age, BMI and potential family history of diabet-
es. They can also measure islet auto-antibodies (see below) or endogenous insu-
lin release by so called C-peptide assays in the blood for more accurate dia-
gnosis. C-peptides are released in a 1:1 ratio to insulin and are absent or very
low in T1D patients [16-17]. After T1D diagnosis is made it is crucial that
the patient is treated with insulin.

Features

Islet auto-antibodies
Appearance of islet auto-antibodies in the blood has been suggested as
marker for islet damage. It is estimated that up to 90 % of newly diagnosed
T1D-patients have antibodies against one or multiple islet antigens. The islet
auto-antibodies which are screened for today in clinical trials include glu-
tamic acid decarboxylase 65 (GAD65) [18-19], tyrosine phosphatase or
insulinoma associated protein 2 (IA-2) [20-21], insulin/IAA [22-23] and
Zink transporter 8 (ZnT8) [24]. In addition, auto-antibodies against exocrine-
specific proteins have also been observed indicating that this part of the pan-
creas is also undergoing damage [25]. Presence of multiple islet auto-
antibodies is correlated with an increased risk of developing T1D – however,
occasionally it can take several years before hyperglycaemia is established
GAD is the most prevalent of these islet auto-antibodies and can be found in ~2% of the adult population [27]. There is no evidence yet that the islet auto-antibodies are involved in the actual beta-cell destruction in humans [28].

**Insulitis**

Insulitis is defined as peri- or intra-islet infiltration of immune-cells in the islets of Langerhans and considered to be a hallmark of recent-onset T1D [11, 29]. The immune cells consist mainly of CD8+/CD3+ T-cells and CD68+ macrophages, however CD20+ B-cells, CD4+/CD3+ T-helper cells [30] and NK cells [31] have also been observed. The infiltration is normally of a patchy character, i.e. it can be found in certain lobes, and is usually localized in insulin-containing islets [29-30]. In the two animal models of T1D, the non-obese diabetic (NOD) mouse and the BioBreed (BB) rat, a massive insulitis can be seen; however, in humans the infiltration is more mild [32]. Since the concept was first described over 100 years ago, there have been approximately 150 reported cases with insulitis; the two studies by Gepts and Foulis are considered to be the most large-scale ones. In a meta-analysis comprising 213 T1D patients the prevalence of insulitis was investigated. It was found that insulitis was more common in individuals ≤ 14 years of age than in adults (~73% vs. 29%), close to disease onset (≤ 1 month). This frequency declined with duration of the disease and after one year insulitis was found in only 2-4% of the patients [33]. In addition, insulitis has not been convincingly shown in islet auto-antibody positive (“pre-diabetic”) donors. In a large-scale study comprising 62 donors, only 2 donors with multiple antibodies showed insulitis (in 3-9% of islets), whereas none of the single auto-antibody-positive donors had insulitis [27].

One of the big concerns in the studies of insulitis is that there has been no general threshold for how many infiltrating immune cells are required for the condition to be defined as insulitis and not just “normal” variation. In February 2013 it was decided at a conference in Florida within the networks for Pancreatic Organ Donors with Diabetes (nPOD) collaboration that a new “golden definition of insulitis” will be used from now on: At least 15 CD45+ cells, either within or touching the islets, is defined as insulitis. The CD45 receptor is expressed on a variety of immune-cells, including precursor and mature B-cells and T-cells, macrophages, NK-cells and dendritic cells. Hopefully it will lead to a more homogeneous evaluation of the future materials studied.

**Atrophic pancreas**

By the time of T1D-diagnosis and in chronic patients atrophic islets with fibrotic stroma can sometimes be seen [29].
Epidemiology of T1D

The prevalence of T1D has been increasing by 2-5% worldwide during the last years; especially in children 0-4 years of age [34-38]. The incidence varies greatly between countries from 0.1/100,000 per year in China and Venezuela [39] to 64/100,000 per year in Finland [38]. The disease also has a seasonal variation suggesting an infectious etiology [40]. Approximately half of those diagnosed today are adults [41]. The incidence generally peaks at 4-6 and 10-14 years of age [42].

Diabetes subgroups with similarities to T1D

Latent autoimmune diabetes (LADA) shares some of the characteristics of T1D such as presence of islet auto-antibodies and decreased C-peptide. It is often diagnosed in adults over 30 years of age and is estimated to be misdiagnosed as T2D in approximately 10% of the cases due to the high age of the affected subjects and because they tend to respond well to dietary changes and medication during the first part of the disease [43-44].

In Japan, a subgroup of T1D has been observed in about 20% of the population. It is called fulminant diabetes, due to its rapid progress, which often leads to ketoacidosis and death. The patients with fulminant diabetes are usually adults, have virtually no C-peptide, lack islet auto-antibodies and have increased levels of exocrine enzymes in the blood [45].

Genetic risk factors

Many loci have been associated with the development of T1D and some have been shown to be protective. The most important of these genes can be found on the short arm of chromosome six, which encodes the highly variable cell-surface human leukocyte antigen II (HLA II) molecule. This molecule, which typically is expressed on B-cells and DCs, is important for antigen presentation of foreign peptides to CD4+ T-cells and plays a crucial role in graft rejection during organ transplantations. There are three major and two minor forms of this molecule: HLA-DP, HLA-DR, HLA-DQ and HLA-DM and HLA-DO which encode heterodimers of the HLA-II molecule consisting of one alpha chain (A) and one beta-chain (B). The loci that have been associated with T1D are HLA-DR3, HLA-DR4, HLA-DQ2 and HLA-DQ8 [46]. Approximately 95% of those who develop T1D carry at least one of the DR3 and DR4 alleles in comparison to ~40% of the general, white non-diabetic population in the US. Approximately 85% of those who are diagnosed with T1D do not have any near relatives with the disease. The risk of developing diabetes in siblings who have either DR3 or DR4 is approximately 3-4% and if they have both DR3 and DR4 this number increases to 16% [47]. A long-term study on monozygotic twins showed that approxi-
mately 67% of the twin-pairs developed diabetes and/or islet auto-
antibodies, although the time of the disease onset varied. Concordance was
higher if one twin developed diabetes at young age [48].

Although the genes encoding HLA apparently are the most important dis-
ease risk indicators, other genes have also been shown to be associated with
the disease. These include the insulin gene (INS) [49], the cytotoxic T-
lymphocyte associated protein 4 (CTLA4) [50], the protein tyrosine phospha-
tase, non-receptor type 22 (PTPN22) [51], the interleukin 2 receptor, alpha
(IL2RA) [52]. Two genes involved in the innate immunity response, OAS
[53] and interferon induced with helicase C domain 1/MDA5 (IFIH1) [54],
have also been shown to be linked to T1D. It has been suggested that it is the
complex interplay of, not only a few, but a large number of inherited genes
that probably leads to the disease development.

Environmental risk factors

There are numerous observations that indicate that environmental factors are
involved in the development of T1D. These include for example- the rapidly
increasing incidence of T1D, which cannot be explained by genetic shift, an
increase in disease development in individuals moving from low-incidence
countries to high-incidence countries, discordance in monozygotic twins and
seasonal variations. Environmental factors that have been suggested to trig-
ger T1D include infections, lack of D-vitamin, dietary factors, gut micro-
environment, growth, stress and toxins [55-56]. Among the infective agents
mainly enteroviruses have been associated with T1D [57] and these will be
discussed in more detail in the next chapter.
Human Enteroviruses

Taxonomy

Picornaviridae is a family of small viruses consisting of a single-stranded positive senses RNA wrapped in a non-enveloped, icosahedral capsid. It consists of 17 genera, including the human enteroviruses (HEVs), which in turn are divided into seven groups of species: human enterovirus A, B, C, and D and Rhinoviruses A, B and C. The viruses which have mainly been associated with T1D, i.e. Coxsackie B viruses and Echoviruses, belong to the HEV-B group [58-59] (Fig. 1). Previously, these viruses were subdivided into serotypes through neutralization assays by adding type-specific serum. Nowadays genotyping is the most common way to classify new viruses. The serotypes/genotypes in turn, consist of many strains or quasi-species, which arise due to the high error rate of the viral replication system (approximately 1 error per 100-100,000 transcribed nucleotides) [60].

![Human enterovirus group B taxonomy](image)

**Figure 1.** Human enterovirus group B taxonomy
Virion genome and structure

The HEV genome is approximately 7,500 nucleotides long and is protected in an icosahedral capsid, composed of 180 subunits of the polypeptides VP1-VP4, symmetrically arranged in 12 pentamers and 20 hexamers. The genome has been divided into three regions, P1, P2 and P3, based on what type of proteins each region codes for. P1 codes for the structural proteins VP1-VP4, whereas the proteins in P2 and P3 are involved in membrane remodelling and replication respectively. The open reading frame (ORF) is flanked by stretches of untranslated regions (UTRs), consisting of stem-loops of RNA. The 3’ end of the UTR contains a poly-A tail. The 5’ end lacks the cap structure, which is essential for eukaryotic translation, and instead has a protein (VPg/3B) linked to it [61]. The virus particles line up in arrays and can easily be detected in the electron microscope (Fig. 2).

Figure 2. A) Poliovirus genome structure and the proteins encoded. The circles represent cleavage sites for virus proteases. A protease responsible for the cleavage of VP0 to VP4 and VP2 has not yet been identified. B) The genome is packed in an icosahedral capsid consisting of 180 subunits of the proteins VP1-VP4. C) Micrograph showing virus-particles in a beta-cell. The icosahedral structure was modified with permission from www.viralzone.expasy.org.
Enterovirus infectious cycle

Virus attachment and entry
The virions are too large to diffuse into cells. Instead they utilize various receptor molecules, often more than one, distributed across the cellular membranes. The virions belonging to the Picornavirus family use a variety of cellular proteins including several immunoglobulin-like proteins and integrins [62]. Crystallography and cryo-electron microscopy have provided us with detailed computer-simulated images of several receptor-virus interactions, including poliovirus (PV) and Coxsackie B3 [63-64].

Several capsid proteins, such as poliovirus and CVBs, have depressions on their surface at the five-fold axis to which the receptors bind. Polioviruses are believed to release their genomes by the so called “zipping pathway”, although this is debated. In this pathway, viruses enter the cell through endocytosis and the virus receptor interacts with the capsid and creates a pore in the endosome membrane, through which the virus RNA can escape into the cytoplasm [65-67]. The major receptors for Coxsackie B viruses are the Coxsackie and adenovirus receptor (CAR) [68] and the complement decay-accelerating factor (CD55 or DAF) [69-70], described in more detail below. Other, not defined, receptors have also been shown to be used by these viruses [71-72].

Figure 3. After internalization, the enterovirus genome is released in the cytoplasm.

The Coxsackie and adenovirus receptor (CAR)
CAR is the main receptor for all six serotypes of Coxsackie B viruses [73]. It belongs to the large IgG family and consists of two extracellular IgG-like domains, D1 and D2, one trans-membrane domain and an intracellular domain which has been associated with PDZ-binding proteins [68, 74-75]. At least five genomic splice variants of the CAR protein has been characterized in humans, two of which differ only in three amino acids at the C-terminal
(the TTV and SIV isoforms) and three shorter forms which lack a trans-
membrane domain [76-78]. The latter isoforms were shown to be secreted
when expressed from plasmids in HeLa cells [79]. Crystal structures of the
D1 domains were shown to form homo-dimers in a head-to-tail orientation at
pH 5.2 in vitro and it has been suggested that CAR-molecules on adjacent
cells bind to each other in this formation [80]. CAR-deficient mice cannot
develop heart muscle and die during early embryonic stages [81]. Further-
more, it has been shown in mice that CAR is highly expressed in various
tissues during embryogenesis, mainly in the brain, nervous system and mus-
cles, but expression declines during the neonate period and then becomes
restricted to only a few organs [82]. Presence of CAR mRNA has been ob-
erved through in situ hybridization in the mouse exocrine pancreas, but not
in the endocrine islets [83]. In adult humans, CAR protein has mainly been
found in the tight junctions of epithelial cells of various tissues, where it is
believed to have adhesion properties [84]. Coxsackie B viruses have been
shown to bind to the D1 region of CAR, whereas the cytoplasmic domain is
not necessary for infection [85]. Recently, it was proposed that CAR is ex-
pressed on beta-cells specifically in the pancreas (Dotta, F., nPOD abstract
2013).

Figure 4. The left image shows a schematic image of a CAR-molecule. The right
image shows a cryo-EM image of CAR-molecules (red=dimerized extracellular Ig G
loops, green=transmembrane and intracellular domains) attached to a CVB-3 parti-
cle (grey). (Cryo –EM image published with permission from [64])

Complement decay- accelerating factor (CD55 or DAF)
CAR is a component of tight junctions of epithelial cells and it was a mys-
tery how the virus could access this receptor to infect these cells. It has been
shown in cultured polarized Caco-2 cells that the virus can initially bind to a
receptor found at the apical surface, CD55, which then initiates a cascade of
signalling pathways leading to restructuring of actin and translocation of the virus particles to the tight junctions where they can bind to CAR [86].

Translation

The enteroviral genome is like a messenger RNA, in that it can be translated as soon as it enters into the cell. Transfection of cells with the virus genome alone leads to generation of functional virus particles. The ribosomes recognize and bind to a region in the 5’ UTR, the so-called internal ribosomal entry site (IRES) [87]. With the help of VPg/3B, which works as a primer, the virus genome can then be translated in an un-capped manner. Several host proteins, which have not been shown to facilitate cap-dependent translation, have been demonstrated to bind to the 5’ UTR, including PTB, PCBP2 and La [88-91]. These have been suggested to act as chaperons, stabilizing the viral mRNA structure. By cleaving proteins important in cap-dependent translation, such as eIF4G and PABP, with the viral proteases 2A, 3C<sup>pro</sup>, 2A<sup>pro</sup>, the virus can itself utilize the translation machinery [92-93].

![Diagram of viral translation]

*Figure 5. Viral translation. The enteroviruses have been shown to shut down the cap-dependent translation pathway.*

Viral RNA replication

Picornaviruses replicate their genome in the cytoplasm on intra-cellular membranes derived from the endoplasmatic reticulum (ER) and the Golgi apparatus. These membranes could provide protection of the viral genome
against cellular proteins that recognize foreign intermediates such as dsRNAs. It has also been suggested that concentration of the viral replicase complexes on membranes might facilitate replication by several orders of magnitude [94]. Shortly after infection, mainly single membranes are observed. In later stages of infection increased numbers of double-stranded membranes start to accumulate, suggesting activation of the autophagic pathway. It has been suggested that replication can occur on both types of membranes [95-96]. The morphology of these membrane structures were previously referred to as vesicle-like, but have recently been shown through ultrastructural tomography to have tubular-like shapes [95]. Several enterovirus proteins have been shown to induce these membrane structures; the viral protein 2C was shown to have the most dramatic effects, giving rise to membrane swirls and swelling of the ER [97]. In addition, it has been shown that enteroviruses utilize proteins from the secretory pathway for replication. When cells were treated with Brefeldin A, a fungus metabolite which binds to the important regulator of protein transport ADP ribosylation factor 1 (Arf1), no PV replication could occur. This suggested that enteroviruses require activated Arf1, which is unable to form when Brefeldin A binds to it [98]. This activation is accomplished by binding of a guanine nucleotide exchange factor (GEF), specifically GBF1, to Arf1, leading to a switch from the cytosolic Arf1-GDP to the membrane-bound Arf1-GTP [99-100]. During PV infection active Arf has been shown to re-localize from the Golgi apparatus and ER, where it is normally found, to replication membranes [98]. Furthermore, the virus proteins 3A and 3CD has been shown to translocate Arf1 to membranes in vitro [100]. GBF1 has been reported to be a beta-cell specific protein in the pancreas [101]. The effectors which are recruited to activated Arf1 include COP-I protein and clathrin, important for budding, and phosphatidylinositol-4-kinase IIIβ (PI4KIIIβ), which catalyses the production of phosphatidylinositol-4-phosphate (PI4P) lipids, shown to facilitate the binding of viral RNA polymerase (3Dpol) and thus viral synthesis [94].
Figure 6. The virus replication is believed to take place on intra-cellular membranes.

Virus assembly and release

It is believed that the virus lyses the cells, leading to the release of newly formed virus particles. It has also been suggested that virus particles could be exocytosed in a non-lytic way by fusion of single or double membrane vesicles and plasma membranes [96].
Persistent infections

Enteroviruses are normally associated with lytic infections, causing tissue damage and inflammation, but persistent infections have also been implicated [102]. Human enteroviral RNA has been detected in hearts of human subjects with cardiomyopathy and myocarditis [103], but isolating lytic virus particles in cell cultures from these tissues has been difficult. The same phenomenon was shown in murine hearts and primary cell cultures, i.e. viral RNA could be detected long after isolation of infectious particles was possible. Analysis of the viral genome by PCR showed a 7-49 nt deletion in the 5’ end, which might explain the persistency of these viruses [104-105]. Interestingly, this type of truncated virus (CVB-2) was detected in the heart of a patient with dilated myocardite [106]. It has been hypothesized that persistent EV strains might lead to generation of defective plus-stranded RNAs and thus slower replication of non-lytic viruses.

Host response to RNA virus infection

The host has several mechanisms for protecting itself against virus infections. These include physical and chemical barriers like the skin and basement membranes. If the virus passes these barriers, first the innate and later the adaptive immune system are activated to prevent further spread. Much of the pathology seen in virus infections is due to the immune response.
Innate system

The cells that are normally among the first to reach a site of infection/cellular injury are the granulocytes (neutrophils, eosinophils, basophils, monocytes, NK cells and mast cells) and macrophages.

The innate system is initiated after only minutes or hours by certain transmembrane proteins that recognize foreign pathogen patterns, so-called toll-like receptors (TLR). There are several members of TLRs in humans and mice, and they are conserved in plants, animals and humans. Double-stranded RNA (dsRNA), formed as an intermediate during virus replication, is recognized by the so-called TLR3 receptor, which can be found in the endosomes [107]. In addition, there are helicase proteins in the cytoplasm, such as RIG-I, MDA-5, which can recognize dsRNA formed during RNA replication. Recognition of the dsRNA by either of these proteins triggers a signalling cascade which eventually leads to the production of inflammatory cytokines such as TNF-alpha (early warning), IL-6, IL-12 and type I interferons (IFN-alfa and IFN-beta) and chemokines (e.g. IP-10, MCP-1, RANTES and IL-8), which leads to recruitment of various immune-cells (dendritic cells, macrophages, T-cells etc.) to the infected area [108]. IFN-alpha and IFN-beta can be produced by basically all nucleated cells. IFN-gamma is produced after some time and is mainly made by activated T-cells and NK cells. The interferons are released from the cell and bind to their cell-surface receptors, which stimulate the expression of a number of cell proteins, the function of many remaining unknown. Several of the proteins have an antiviral effect, e.g. PKR which inhibits cellular translation, and OAS and RNAse L, which work together to cleave cytoplasmic mRNA [109]. IFNs, mainly IFN-gamma, and TNF-alpha also stimulate nitric oxide synthase synthesis causing production of nitric oxide (NO), which has also been shown to have antiviral effects. Furthermore, NO has been shown to lead to creation of free radicals, which are harmful to cellular proteins, and cause a major part in the pathology seen in virus infection [110]. The cytokines cause an inflammation response which includes increased blood flow, increased capillary permeability, influx of phagocytotic cells and tissue damage. The cytokines act locally, but spread systemically after some time, leading to drowsiness and fever [111].

Dendritic cells and the complement system

Dendritic cells are the cells which link the innate and adaptive immune -system. The immature forms of these cells patrol in the host tissue. They express receptors for cytokines and HLA-II antigens on their surface. When they encounter a dead or dying cell, dendritic cells respond by producing cytokines like IFNs, which might be enough to eliminate any potential virus spread. If this does not solve the problem, the dendritic cells can
phagocytose the dead cells and break them down into smaller peptides which are then incorporated into HLA-II molecules in the endosomes and transferred to the cell surface. After dendritic cell homing to the lymph nodes, the peptides can be presented to T-cells on the HLA-II molecules. If the peptides are found to be foreign, an activation of the adaptive immune-system might occur [112].

The complement cascade also works as a bridge between the innate and adaptive systems. These include a number of proteins that recognize foreign pathogens by pattern-recognition similar to that of toll-like receptors. They can kill infected cells through different mechanisms, for example, opsonization of pathogens, promoting phagocytosis as well as cell lysis [113].

Adaptive immune system

This is the final step to eliminate the invading pathogens and includes a humoral and a cell-mediated response. The cells important for each response are B-cells and T-cells respectively. These cells can be found in the lymph nodes. Dendritic cells enter the lymph nodes and present a foreign peptide to specialized T-cells, so-called T-helper cells or CD4+ T-cells. Depending on the cytokine mixture produced by the dendritic cells and the T-helper cells, the T-helper cells will then differentiate to specialized T_H1 or T_H2 cells. T_H1 cells promote differentiation of another subset of T-cells, the so-called CD8+ T-cells, into cytotoxic T cells, whereas T_H2 cells will differentiate B-cells into antibody-producing plasma cells. Cytotoxic T-cells migrate to the site of infection by chemotaxis and kill the virus-infected cells through lysis, either by transferring perforin or granzyme-containing granules or by apoptosis [114]. Secreted antibodies function as defence against virus infections by neutralization.

HEV pathology

Enteroviruses are a commonly circulating in the environment. They enter their host through the oral-faecal pathway, by ingestion of contaminated food, inhalation of virus containing aerosols or contact with contaminated sewers etc. The primary replication site is in the mucous-covered epithelial cells of the respiratory and gastrointestinal tract. If the virus manages to get across the basement membrane, the barrier between the epithelial cells and the underlying cells, it might enter the lymphatic capillaries and the blood. From the blood, the virus could disseminate to several organs in the body (i.e., systemic infection) and cause more serious diseases such as aseptic meningitis, poliomyelitis, and myocarditis [115]. The viruses have also been linked to pancreatic diseases such as pancreatitis [116-117] and T1D [118-119]. In neonates
or immune-deficient individuals the infection could be fatal, however the majorities of enterovirus infections (~80%) are asymptomatic or give mild symptoms [120-121]. Also, during poliovirus epidemics, only ~1% of the infected population developed symptoms whereas the remaining 99% remained asymptomatic [122]. It has been shown through isolation from faeces that the virus is normally cleared between 3-4 weeks post infection but can sometimes be shed for 2-3 months. In addition, viral RNA has been detected in the blood for longer periods using PCR [104-105].

**HEV implications in pancreatitis**

Pancreatitis is an inflammation in the exocrine part of the pancreas, and is usually divided into an acute and a chronic form. The acute form can be mild or severe, and pathologic findings include acinar cell necrosis, oedema, and inflammation. In the chronic form of the disease, acinoductular metaplasia and fibrosis can also be seen. The most important risk factors include gallstones and alcohol consumption; however, it is believed that 22% of the factors behind the disease are idiopathic. CVBs have been shown to infect mouse pancreatic acinar cells *in vivo* leaving the islets apparently uninfected [83, 123-124], and the pathological findings resemble those in pancreatitis patients. The link between CVBs and pancreatitis in humans has been found in a small number of case studies [125] and serological studies [116, 126-128] but the tropism of CVB to human exocrine tissue was unknown until now.

**HEV implications in T1D**

The first association of T1D and enterovirus was made over 40 years ago. In one study, it was reported that T1D followed a seasonal pattern which correlated well with enterovirus infections and an increase of neutralizing antibodies against CVBs was observed in recent-onset T1D subjects as compared with controls [129]. In the 70s, CVB-4 was isolated from the pancreas of a young boy who died of ketoacidosis within a week after diagnosis [130]. Since then, there have been numerous studies linking HEVs to diabetes, including epidemiological, case studies and experimental observations [57], of which some are presented in “Cross-sectional and longitudinal studies”.

How could HEV reach the pancreas and destroy the beta-cells?

Several routes of infections have been suggested by which the virus could reach the pancreas, including 1) blood, 2) duct and 3) infection of PBMC

**Blood:** Virus RNA has been observed in higher frequency in T1D donors compared with uninfected controls, which suggests that this could be a likely route for infection to reach the pancreas [131]. The viruses might infect the beta-cells directly or other cells present in the pancreas, e.g. exocrine cells,
ductal cells (see below) or endothelial cells, and cause an immune-response which could be toxic for the beta-cells and/or trigger auto-immune-cells. Exocrine cells could not be infected with CVB-5 (paper I). It does not preclude that there might be permissive individuals in which replication could take place or infectious strains. CVBs have also been shown to infect islet-derived endothelial cells in vitro [132].

**Duct:** It has been proposed that a virus infection in gut mucosa could lead to virus particles slipping in through the duct and infecting the epithelial cells. Some groups have observed prolonged virus infection in the gut mucosa of T1D donors which might increase this risk [133]. Viral RNA has also been detected in the ductal cells in T1D donors [118]. Furthermore, CVBs have also been shown to infect primary ductal cells in vitro which was suggested to lead to impairment of ductal-derived islet regeneration [134].

**PBMC:** CVBs cannot infect PBMC in vitro [135], however it has been shown that PBMC, as well as monocytes and DCs can take up virus-antibody complexes with a Fcγ-receptor, and induce IFN-alpha production [136]. These cells could thus act as "Trojan horses" and transfer virus to the pancreas and/or cause an inflammatory response.

**Cross-sectional and longitudinal studies**

There are a number of cross-sectional studies that have shown association between HEVs and T1D in humans. These include immunohistochemical studies demonstrating presence of human enterovirus capsid protein (HEVP1) in higher frequency in recent-onset T1D donors than in controls [119], HEVP1 positivity in a child with islet auto-antibodies [137], in situ hybridization studies showing presence of viral RNA in higher frequency in the islets and ducts of patients close to T1D-onset and children with systemic HEV infections [138]. There have also been studies showing increased frequency of viral RNA in serum or blood detected through RT-PCR soon after T1D diagnosis [139] and close to seroconversion of islet auto-antibodies [140], whereas other studies have failed at detecting virus at these time-points [140-141]. Presence of virus is also supported by serological studies showing increased CVB IgM [142] and some studies of neutralizing CVB antibodies [143] in T1D patients. To test whether HEV can induce autoimmunity or increase the rate of progression to T1D from autoimmunity, larger longitudinal studies have been conducted, measuring EV RNA in serum and rectal swabs collected at regular time-points, in young children at risk of developing T1D. These studies, including e.g. the TEDDY, DIPP, DiMe, BABYDIAB, MIDIA and DAISY, have yielded heterogeneous results. The study designs, frequencies of sample collections and small numbers of subjects have been suggested as influencing the divergent results obtained [131].
A common observation in several of these studies was that the presence of virus in blood rather than in the faeces was associated with the development of T1D [131]. A potentially interesting observation in the TEDDY study, as well as in an Italian study, is that individuals who develop T1D have fever less frequently [141, 144]. What the significance of this might be remains to be elucidated. Another interesting study recently showed that seroconversion to islet auto-antibodies was observed in a number of individuals during ECHO epidemics by comparing acute sera with convalescence sera [145].

**Potential mechanisms of virus-induced T1D**

Several mechanisms have been suggested which could lead to virus-induced beta-cell destruction and/or dysfunction. Some of the main mechanisms are presented below.

1. **Direct infection:** Direct infection of beta-cells and the following immune-response could lead to destruction or impaired function of these cells. The release of previously hidden islet antigens could trigger an autoimmune response and lead to further destruction by the adaptive immune system. *In vitro* and *in vivo* studies have shown that enteroviruses are permissive to human islets, e.g. [118, 146]. Furthermore, we have previously shown that islets infected with enterovirus secrete IP-10 and MCP-1, which could have chemotactic properties, homing auto-reactive T-cells to the site of infection [147]. Previous studies showed positive immunolabelling of MHC-I and IFN-alpha in the islets in the majority of patients with T1D [148-149]. In addition, IFN-alpha was demonstrated in persistently CVB-infected beta-cells [150], collectively indicating that a virus infection might be involved in the disease pathogenesis.

2. **Bystander mechanism:** The virus might infect non-beta-cells present in the pancreas, which could lead to local inflammation. Cytokines and chemokines released from these inflammation sites might be toxic for beta-cells and lead to destruction through an indirect autoimmune response [151].

3. **Molecular mimicry:** It has been suggested that viral proteins share similar epitopes with islet antigens and thus could induce a cross-reactive immune response. One of the best studied cases is the homology between GAD65 and the virus antigen 2C [152] but its role *in vivo* is still unclear.

4. **Impairment of central tolerance:** It has been suggested from *in vitro* studies that enterovirus could infect thymic epithelial cells and potentially lead to impaired maturation of T-cells with autoimmune features in neonates [153].
5. **Hygiene hypothesis:** The increased hygiene standard and usage of vaccinations and antibiotics in the 20th century has been linked to the increase of several autoimmune diseases, including T1D, and allergies. The basis of this hypothesis is that immune systems are no longer exposed to foreign agents as often as previously which might lead to predisposition of autoimmune T-cells [154-155].

*Figure 8* Schematic model of T1D development. Modified with permission from [156].
Aims

The overall aim of this thesis was to investigate the tropism of CVBs to human primary pancreatic cells and effects of infection on gene-expression, function and ultrastructural morphology in isolated human islets *in vitro*. We also studied the pancreatic tissue from two donors who died of ketoacidosis close to onset, as well as pancreatic tissue from T1D-related subjects, including pre-diabetics, recent and longstanding diabetics. The more specific aims are listed below.

- To study the tropism of two CVB-5 strains to human primary pancreatic islets and exocrine cell clusters *in vitro*. (Paper I)

- To investigate the ultrastructural changes in CVB-5-infected human islets *in vitro*. (Papers I and II)

- To analyse the glucose-stimulated insulin response in CVB-infected and synthetic dsRNA (poly:IC)-inoculated human islets *in vitro*. (Paper II)

- To study the expression of beta- and alfa-cell-associated genes and the CAR gene and protein in CVB-infected human islets *in vitro*. (Papers II and III)

- To study the CAR-gene and protein expression in T1D-related subjects and non-diabetic controls *in vivo*. (Paper III)

- To study two donors who died close to T1D-onset with regard to pancreatic tissue morphology and ultrastructure, induction of innate immunity, islet function, insulin content and immune-cell infiltration in pancreatic tissue, presence of virus particles, viral proteins- and/or viral nucleic acid and mode of cell death. (Paper IV)
• To study innate immunity and pancreatic immune-cell infiltration and insulin content in organ donors several years post T1D onset and in islet auto-antibody positive donors. (Paper IV)
Materials and Methods

For more detailed information regarding materials and methods, please read the original papers and manuscripts.

Human primary isolated islets and exocrine cell clusters (Papers I-IV)

Human islets of Langerhans are isolated routinely at the Rudbeck Laboratory in Uppsala, Sweden from pancreata of organ donors within the Nordic Network. The isolation is performed using a protocol approved by the Local Ethics Committee and described by Goto [157]. The islets are initially kept in culture bags with 200 mL CMRL-1066 supplemented with 10 mM HEPES, 2 mM L-glutamine, 50 µL Gentamycin, 0.25 µg/mL Fungizone, 20 µg/mL Ciprofloxacin, 10 mM nicotinamide and 10 % heat-inactivated human serum. After hand-picking in a light microscope, islets were transferred to 6-well plates, treated to keep the cells free-floating, and cultured in 5.5 mM RPMI supplemented with FBS and 2 mM L-glut. Exocrine cell-clusters were collected after gradient separation and cultured in the same medium.

Primary cells derived from donors are heterogeneous, well-differentiated and have different genetic properties compared with cell lines. Thus, they represent the in vivo situations better than the immortalized cell-lines, although factors prior to organ procurement, during organ handling and islet isolation and maintenance are likely to add deviations.

Human pancreatic tissues (Papers III-IV)

During islet isolation it is routine to take a piece of tissue from the head of the pancreas. This tissue specimen is fixed in 4 % PFA, embedded in paraffin and stored in the Uppsala Biobank (UB). Additional biopsies from different parts of the pancreas are collected from T1D and T2D donors. Each pancreas is shipped to the laboratory in an organ-preserving UW solution, which limits the post-mortem changes often seen in studies of diabetes. Human tissue from T1D and islet auto-antibody donors was also obtained from the Network of Pancreatic Organ Donors with Diabetes (nPOD). In addition, we
had a unique opportunity to study the pancreata of living patients with recent-onset T1D enrolled in the DiViD study. In paper III, pancreatic tissue from control donors, islet auto-antibody positive donors and T1D and T2D donors and patients were studied.

In Paper IV, we obtained and studied pancreatic tissue from two donors (donor 1 and donor 2) who died close to onset of T1D. Tissue was taken from the head and the body, whereas the tail of the pancreas was used for islet isolation. The T1D donors, both males, suddenly developed hyperglycaemia and ketoacidosis and died in cerebral oedema only days after receiving their diagnoses. Donor 1 complained of thirst, diuresis, sore throat and he also had a skin infection on his lower back three weeks prior to diagnosis. In addition, we studied pancreata from donors who were positive for GAD65 and IA-2/GAD65 as well as donors with longstanding T1D.

Monolayers of cells used for virus replication, isolation and propagation (Papers I-IV)

Monolayers of Green monkey kidney (GMK) cells were used for TCID\textsubscript{50} titrations and virus propagation. They were cultured in EMEM supplemented with 10 % FBS (Papers I-IV). For attempts of virus isolation, monolayers of HeLa cells, Rhabdomyosarcoma (RD), and human primary islet single cells/clusters and exocrine cell clusters were used in addition (Paper IV).

Virus strains

Among the enterovirus species, mainly CVBs have been associated with the development of T1D, which is the reason why we chose to focus on these viruses in our studies. The following virus strains were used: CVB5/Adr (Papers I-II), CVB5/V89-4557 (Papers I-II), CVB4/VD2921 (paper II), CVB4/E2-Yoon (paper II) CVB1/3 (paper III), CVB1/7 (paper III), CVB1/11 (paper III) and Echo-virus6/2C (paper III).

CVB5/Adr was isolated from the faeces of a young boy who was diagnosed with T1D [158]. CVB5/V89-4557 and CVB4/VD2921 were originally isolated from patients with aseptic meningitis and were plaque-purified in GMK cells [146]. V89-4557 has been shown to replicate in Chinese Hamster Ovary (CHO) cells, which lack the two major CVB receptors CAR and DAF [72], reflecting a wider tropism of this strain. VD2921 generally replicates non-lytically in isolated human islets and might be a good candidate for a persistent virus infection in vivo [146]. In the 70s, the CVB4/E2-Yoon strain was isolated from the pancreas of a young boy close to T1D-onset [130]. The CVB1 strains were obtained from The Center of Disease Control and
Prevention (CDC). CVB1/7 and CVB1/11 were isolated in Argentina in 1983 and 1998 respectively whereas CVB1/3 was isolated in the USA. They have all been shown to replicate lytically in isolated islets with variable effects on the insulin gene expression (Anagandula, M., manuscript).

In addition we used an Echovirus-6 strain/2C (kindly provided by Luis Sarmiento). This strain was isolated in Cuba 2011 from a patient with aseptic meningitis.

**Tissue culture infectious dose \(_{50}\) (TCID\(_{50}\)) titration (Papers I-IV)**

After virus inoculation, cell culture medium was withdrawn at different time-points and stored at -20 °C. For virus TCID\(_{50}\) titration, GMK cells were cultured in 96-well plates as monolayers in EMEM supplemented with 10 % FBS until reaching approximately 80 % confluence. The samples of collected cell culture medium were thawed, diluted in 10-fold steps in EMEM + 2 % FBS (i.e. 900 µL EMEM + 100 µL sample medium) and each dilution (à 200 µL) was then added in duplicates to the GMK cells. Cytopathic effects were monitored in the light microscope until no further changes were seen.

**Enteroviral detection methods (Papers I-IV)**

To link a virus to a certain disease, one must first be able to confirm the presence of virus in the host. The crucial viral detection methods used in the papers are described below.

**Electron microscopy (EM)**

Tobacco mosaic virus was the first virus ever to be visualized and this was done in 1939 by Ruska and colleges using the electron microscope. Since then, a number of viruses have been detected and characterized by this powerful method. The clear benefit of the electron microscope is that a visual study is usually very “open minded”, and one can detect new/unsuspected pathogens.

The tissue specimens and cultured islets were processed for morphology following a classical protocol, i.e. fixation in glutaraldehyde and osmium tetraoxide followed by embedding in epoxy resin. To preserve the antigenicity, materials aimed for immune-gold labelling were processed at +4° C, short dehydration in 50-95 % ethanol during a successive lowering of temperature to -20° C, embedding in the methacrylate-acrylate resin Lowicryl K4M at -20° C and polymerization in UV light at -20° C [159]. In the immuno-gold procedure, the ultrathin sections were first incubated with the primary antibodies, and subsequently with the secondary gold-conjugated antibodies [159]. Common to both EM techniques was the method to improve the contrast in sections, through contrasting with uranyl acetate and lead citrate be-
fore examination in either a Technai Bio Twin or a Hitachi H1700 electron microscope.

High resolution images of whole islets were generated by stitching together thousands of smaller images (paper II). This method creates a tool similar to “Google earth” and allows you to scroll around in the micrograph on your computer and study small ultrastructural details at high magnifications. High-resolution images were kindly provided to us by Ben Giepman. Quantification of ultrastructural organelles was performed manually in Photoshop and ImageJ/Fiji32.

Immunohistochemistry (IHC) (Papers I, IV)
The antibody against HEVP1 (Dako) is one of the most widely used detection markers for HEVs and was used to detect virus in in vitro infected -islets and exocrine cell clusters and also in vivo, to screen for virus in the pancreatic tissue of two recent onset T1D donors. Doubts have been raised about the specificity of this antibody in human tissue; it has been suggested to cross-link to e.g. mitochondrial proteins [160], IA-2 [161] and HSP60 [162]. Careful optimization of the dilution of this antibody does not normally show any immunoreactivity in non-diabetic control donors. The potential cross-reactivity against cellular proteins makes validation and confirmation with other viral antibodies and/or viral sensing antibodies very important (e.g. dsRNA, Coxackie B Blend or virus-induced proteins such as PKR and MDA5) as well as confirmation with other methods.

Real-time-PCR (RT-PCR) (Papers II-IV)
Real-time-PCR is the most common method used to study the presence of virus RNA. In these studies, RNA was isolated from infected and uninfected human islets, pancreatic tissue and micro-dissected frozen human pancreatic tissue. This method has a high sensitivity in cell cultures but it declines in tissue, probably due to presence of RNA inhibitory factors. Other limitations of this method include e.g. choice of primers.

Virus isolation in cell cultures (Paper IV)
Pancreatic tissue homogenates and culture medium of isolated pancreatic cell clusters were seeded on various monolayers of cell lines, in order to analyse cytopathic effects and isolate any existing viruses. Although many viruses can be isolated using this method, there are strains which are non-permissive for these cell types and thus might not be detected.
Results and discussion

Paper I - CVB-5 can replicate in human primary endocrine islets but not in exocrine cell clusters in vitro

When CVB is admitted intraperitonally in mice models, the virus usually finds its way to the pancreas, infects the exocrine cells and causes necrosis and inflammation, i.e. pancreatitis [83, 123-124, 163]. The islets in these studies have been surprisingly intact. If the virus is given orally on the other hand, the pancreas is protected from damage, although viral proteins are detected in both the exocrine and endocrine parts [124].

Even though some association of CVB infections to human pancreatitis has been shown, mainly in serological studies [116, 126-127] and a few case studies [125], little is known about their role in this disease. In this paper we wanted to address the question if human primary pancreatic exocrine and endocrine cell clusters are permissive to CVBs in vitro. Replication was not seen in 62 % (5/8) and 67 % (7/10) of the donors inoculated with Adr and V89 respectively. In those donors in which a slight increase in virus titre (0.5-2 log TCID$_{50}$/200µL) was observed, the titre culminated at day 3-5 post-inoculation and then declined or remained unchanged. IHC staining for virus protein revealed ~1% positive cells after surveying infected exocrine clusters from five donors. In contrast, the endocrine cell clusters revealed a clear titre increase. In addition, virus was also detected in islets from several donors using IHC and EM. The virus particles were restricted to beta-cells. Figure 9A shows positive HEVP1 staining in two cells (black arrows) in the exocrine cell cluster fraction infected with the total concentration 5.5 TCID$_{50}$ CVB-5/Adr day 1 p.i. Figure 9B corresponds to the HEVP1 staining in the endocrine fraction from the same donor infected under the same conditions. Since primary cultures always contain a heterogeneous population of cell types, we hypothesize that the increased virus titre and few number of positive cells seen in some donors and the small percentage of positive cells could be due to replication in one of these subpopulations, e.g. endocrine cells or ductal cells. Replication in ductal cells has been reported by the CVB-4/E2 strain in vitro [134] and viral RNA has also been observed in T1D-patients in vivo [138].

We cannot exclude that there are permissive hosts in which replication of these viruses could occur; however, in this study, no virus could be detected through IHC or EM in the exocrine cells.
That CVBs have tropism to human beta-cells has been shown before [118, 146, 158, 164] and was confirmed in this study. A novel finding in this paper was that virus particles were found in close association with insulin granules, suggesting that the viruses could utilize these membranes as replication scaffolds. HEVs have been shown to replicate on membranes derived from the Golgi apparatus and the ER [165], which supports this idea. Interestingly, the insulin granules have been reported to be the main sites of cholesterol accumulation in cultured beta-cells, in contrast to fibroblast cells in which the cholesterol accumulates in the trans-Golgi-network and recycling endosomes [166]. Both CAR and DAF have been associated with lipid rafts and many enteroviruses have been shown to utilize lipid raft micro-domains or cholesterol to gain access to susceptible cells [167-169]. Furthermore, cholesterol-enriched membrane platforms have also been suggested to be important for RNA virus replication [170]. This suggests that the cholesterol-enriched insulin granules could be of great importance for viral synthesis and should therefore be studied further. Ultrastructure analysis also showed a dramatic alteration of the cytoplasmic area including condensed nuclei and increased vesiculation.

The study did not reveal why exocrine cells are non-permissive. One possible explanation might be the lack of virus receptors which has been suggested in some studies [137] (Dotta, F., abstract nPOD 2013). However, in paper III we could see positive CAR immunolabelling in the acinar cells in ~40 % of the pancreata studied, but mainly in T1D-related donors, suggesting that there could be other reasons for the failure to replicate. An alternative reason why the virus fails to replicate in exocrine cells may be presence of proteolytic enzymes or lack of key proteins required to complete the virus cycle. One key protein, GBF1, which is important for CVB-replication, has been found selectively in beta-cells in the pancreas [101] and might partly explain the differences in tropism between the two cell types. A third alternative could be an effective anti-viral state.

In summary this paper showed that the virus cannot replicate in human primary exocrine cell clusters in vitro, which is the opposite to what has been shown in mice studies. Also, the paper supports the possibility that beta-cell destruction seen in T1D patients could be due to a direct lytic infection. The interesting observation that virus particles are associated with insulin granule membranes suggests that these could be used as replication scaffolds.
Figure 9 HEVP1 immunolabelling in exocrine (A) and endocrine (B) cell clusters from the same donor inoculated with high titre CVB-5/Adr day 1 p.i.

Paper II – Virus-induced islet destruction but not replication per se impairs beta-cell function.

T1D organ donors and T1D patients usually have a significant number of insulin-positive islets at disease onset. However, isolated islets from these donors occasionally cannot respond to glucose stimulation, which indicates a functional block (Paper IV, [31]). The primary aim of this paper was to test if HEVs could cause a functional block in human pancreatic islets infected in
vitro and during what stages of infection this blocking might occur. We also wanted to study the effect of virus infection on islet gene-expression and virus-induced ultrastructural changes.

It has previously been shown that CVB-infected islets might cause functional impairment due to lysis [164], however it was unknown whether replication itself will affect the function.

This study showed that the rapidness of virus-induced cytopathic effect differed between different strains but also between different donors. The insulin secretion was not impaired by replication per se. Instead, the more damaged the islets appeared, the less they responded to glucose stimulation. This could suggest that infection with a slowly replicating/non-lytic strain might not cause a functional block. However, in an in vivo situation, infection with a non-lytic/persistent strain might still lead to glucose sensing abnormalities and/or beta-cell destruction by immune-cell responses in the pancreas.

Ultrastuctural analysis of a high-resolution image-section of a CVB-5-infected islet showing moderate disintegration revealed virus particles in 11 beta-cells in the periphery of the islet. About ~80 % of the total number of beta-cells had signs of infection including condensed nuclei, increased frequency of vesicles, disassembled Golgi apparatuses, and occasionally capsids without any contents. The remaining ~20 % were apparently uninfected and could thus potentially still function and compensate to some extent for the virus-killed cells. There were also a few non-insulin containing cells that displayed vesicles (on average 1-2) in the cytoplasm, which suggests that virus could enter these cells but might not be able to replicate.

The beta-cell tropism was also confirmed by RT-PCR, in which beta-cell-associated genes coding for insulin, PDX1 and MafA were decreased in islets infected with CVB4/E2, CVB4/VD2921 and CVB5/V89-4557. V89-4557 also reduced alpha-cell associated genes coding for glucagon and MafB, suggesting that this strain might replicate in alpha-cells as well.

Interestingly, there was a higher insulin granule density in the 11 beta-cells with virus particles compared with apparently uninfected beta-cells from the same islet and compared with beta-cells in the periphery of an uninfected islet from the same donor. In addition, insulin staining was also observed in CVB5/V89-4557- and CVB4/E2-infected islets, with complete loss of glucose-stimulated insulin secretion 6 days post-infection. This could be due to insulin granules remaining in dying beta-cells. Ultrastructural analysis revealed cells of varying necrotic stages in the periphery of the islets with sometimes barely recognizable cytoplasm; however, there were still a few insulin granules left in these, suggesting that insulin molecules might not be rapidly degraded. The higher content of insulin granules in clearly infected cells with intact plasma membranes and reduced insulin gene-expression might also indicate a functional block in these cells. Enteroviruses have been shown to block cap-dependent cellular translation by cleaving eIF4A, which
likely leads to a number of abnormal functions in the host cell. In addition, viral proteins have been shown to hijack proteins from the secretory pathway to their replication sites, including e.g. Arf, GBF1, P4IIIβK [95-96, 171], which might explain the dissolved Golgi apparatus and thus partly why the insulin stores have not been emptied.

This paper also showed that Poly(I:C), a synthetic double-stranded RNA, potentiated insulin secretion upon glucose challenge. This is the opposite of what has been shown in murine models, in which this compound decreased function and also induced apoptosis [172-173]. One could speculate that this compound might interfere with the insulin secretion pathway.

In summary, this paper showed that the islet function was not impaired by viral replication per se, but on how damaged the islets appeared. Severely damaged islets likely reflect a high number of virus-killed cells whereas in intact islets, there are probably enough non-infected cells to compensate for any loss of function.

Moreover, the high content of insulin in virus-infected cells might reflect a block in insulin secretion.

Paper III - Expression of the Coxsackie B and adenovirus receptor in islet auto-antibody positive donors, recent-onset T1D donors and longstanding T1D donors

In this study, the expression of the main CVB receptor, CAR, was investigated in adult islet auto-antibody donors/"pre-diabetic" donors (n=14) and recent-onset T1D donors (n=7), longstanding T1D donors (n=6) and non-diabetic donors (n=24). The effect of HEV-infected human isolated islets with regards to expression of the CAR gene and protein was also studied.

The results in this study revealed that CAR protein expression could be found in 57 % (8/14) of the islet auto-antibody donors, 43 % (3/7) of the recent-onset T1D donors, 67 % (4/6) of the longstanding T1D donors and only 25 % (6/24) of the non-diabetic control donors. The expression could be observed both in the islets and in the exocrine acinar cells. In islets, the expression of CAR co-localized with endocrine cells. Among 60 % (31/51) of the donors, no CAR-staining could be detected in the pancreas. There was an odd ratio of 3.877 of CAR-staining in T1D-related subjects (15/27) compared with non-diabetic subjects (6/24). These results suggest that there might be an ongoing inflammation and/or tissue damage in the pancreata of these individuals, which might induce the expression of CAR and thus increase their vulnerability to CVB infections. Previous studies have shown that tissue healing increased the expression of this protein [174].
There was a higher frequency of CAR-staining in the ductal epithelial cells in tissue from the nPOD-biobank perhaps due to different handling of organ between the biobanks.

Previous studies of the CAR protein expression in the pancreas, using different CAR antibodies, have yielded very divergent results including expression only in ducts [84], only in beta-cells (Dotta, F. abstract nPOD 2013), only in islets [137] and both in islets and exocrine cells [175]. CAR is predicted to be expressed as at least five different isoforms; three of these lack the transmembrane domain and are suggested to be secretable proteins which cannot serve as virus receptors [77]. It will be necessary to further study the different CAR-isoforms and their distribution in the pancreas to fully understand the virus tropism of this organ.

This study also showed that CAR mRNA appears to be expressed in both exocrine and endocrine cells in pancreatic tissue, however due to the small donor size (n=2) nothing can be said about differences in expression levels between tissues and between donors. It has previously been shown that islet-derived endothelial cells also express CAR mRNA, which might contribute to the total CAR expression in islets [132], and perhaps in exocrine cells; however no co-localization of endothelial cells and CAR could be observed in this study. The gene-expression in human pancreata differs from the mouse pancreata, where in situ hybridization detected CAR mRNA only in the exocrine cells and not in the islets [83]. This might explain the difference in tropism between the two species.

The expression of the CAR gene declined in isolated human islets infected with four different CVB-1 strains but not with an Echo-6 strain, which has been shown to use other receptors [70]. For one CVB-1 strain, this was also confirmed on the protein level. Infection of islets with the CVB-1 strains revealed decreased expression of the insulin gene (Anagandula M. et al., manuscript) and previous studies have found virus -particles only in beta-cells, suggesting that these cells are the only islet cells in which productive virus infection can take place. Thus, the decrease of CAR might be a direct consequence of beta-cells being killed by viruses. Alternatively, the decrease of CAR -protein in islets might limit the number of viruses that could infect the same cells, so-called super-infection exclusion. This phenomenon has been observed in e.g. hepatitis C viruses [176] and could be favorable for slow-growing viruses.

The observed increase of CAR in T1D-related individuals speaks against a viral involvement in T1D since CVBs decrease the expression of CAR. It is possible that due to tissue damage, e.g. infection, the surrounding cells will start to express CAR as e.g. a part of healing process which is what we observe. Similar findings have been noted in individuals with dilated cardiomyopathy [177].
Paper IV - On-going pathogenic processes in the pancreata of two organ donors at disease onset

One of the problems in T1D research is the lack of pancreatic tissue from donors close to disease onset. Due to insulin-treatment of patients nowadays, scientists usually study events that occur in the pancreas months or even years after diagnosis. In this study, we had the rare possibility to study the pancreatic tissue and isolated islets from two adult donors who died of ketoacidosis within days after diagnosis. In addition, we studied a small cohort of adult donors with islet antibodies against GAD65 or GAD65 and IA-2 ("pre-diabetics") and donors with longstanding T1D.

The recent-onset T1D donors ("donor 1" and "donor 2") had the HLA types DR4/DR7 and DR4/DR4 respectively but were negative for GAD65, IA-2 and ZnT8. The light-microscopic morphology showed dramatically swollen-looking cells and enlarged endothelial cells/bleedings in several islets from donor 1. The pancreas from this donor also had a moderately high fat content. Swollen cells were first documented at the beginning of the last century by Weichselbaum, and has been referred to as "hydropic degeneration" [178]. These types of cells have also been observed in rabbits treated with cortisone [179] and in the islets of bank voles infected with Ljungan virus [180] and are believed to be a consequence of cellular oedema. Some hypotheses suggest that the swollen cells represent functioning cells which reached exhaustion due to work overload [181].

The cellular damage in donor 1 was also reflected in the ultrastructural analysis which showed several "empty"-looking cells with relatively intact-looking nuclei and increased number of cytoplasmic vesicles and cell debris. In donor 2 a high number of beta-cells had a normal appearance, although some cells were damaged and swollen-looking as well. In addition, glycogen deposits, fused granules negatively affected mitochondria, fragmented ER and fibrosis in e.g. the peri-vascular compartments were noted in both donors. The fused granules might be signs of crinophagy, i.e. an autophagic process to get rid of damaged secretory granules.

The islets of both recent-onset donors were immuno-positive to insulin, however dynamic perifusion analysis revealed that the glucose- stimulated insulin secretion was severely impaired in donor 1 and completely lacking in donor 2. Ultrastructural analysis revealed that beside damaged beta-cells there were apparently normal-looking beta-cells, particularly in donor 2, which in combination with the function analysis could indicate a block of secretion. Another possibility could be that the cells are injured beyond repair, but that insulin molecules have not been degraded yet.

The islet auto-antibody donors responded normally to glucose stimulation and displayed positive insulin-staining, whereas no insulin staining was found in the longstanding T1D donors.
Positive immunolabelling for the enterovirus capsid protein was shown in several islets of both recent-onset T1D donors and one islet auto-antibody donor but in none of the controls. In donor 1 there was, in addition, a positive immunolabelling for a blend of antibodies against CVBs. Also, the isolated islets from T1D donor 1 and two islet auto-antibody donors expressed elevated levels of the innate immunity gene RIG-I and one islet auto-antibody positive donor also expressed elevated levels of MDA5, which could indicate signs of viral infection. No viruses or viral fingerprints could be detected using any other methods, including RT-PCR, virus isolation methods or immunogold labelling and ultrastructural analysis, which makes it difficult to confirm or dismiss the presence of virus. If virus particles or RNA were present, this could have been missed due to several methodological factors which are discussed in the Materials and Methods chapter.

No insulitis was detected in any of the recent-onset T1D donors or in the islet auto-antibody positive donors. Instead, immune -cells could be detected evenly distributed over the pancreas with an increased number of immune -cells in donor 2. The immune-cells included CD8+/CD3+ T-cells and macrophages (CD68+ cells), whereas CD4+ T-cells and B-cells (CD20+ cells) were absent. There was an elevated expression of MCP-1 (donor 1) in isolated islets, which was also confirmed by gene -analysis of laser-captured islets in a published article including this donor [181]. Also, elevated levels of RANTES and IP-10 were detected in both recent-onset T1D donors. In islet auto-antibody positive donors 7 % (1/14) expressed elevated levels of IL-6 and 14 % (2/14) expressed elevated levels of IL-8.

Furthermore, there were no signs of apoptosis in either recent onset T1D donors. These results and the IHC results collectively imply that T-cells might not have a primary role in the beta-cell destruction of these two donors; however, it cannot be excluded that T-cells might have been present in the islets at an earlier time. Instead, the cellular oedema, degradation of cytoplasmic proteins, fused granules and increased vesiculation, rather indicate a cytonecrotic and/or autophagic mode of cell-death.
Conclusions

Paper I

- Two CVB-5 strains, Adr and V89-4557, replicated in primary human endocrine islets but not in exocrine cell clusters in vitro.
- Virus particles of the CVB-5 strain Adr could be found selectively in beta-cells in close association with insulin granules.

Paper II

- The glucose-stimulated insulin secretion in CVB-infected islets was not impaired by replication per se but by how disintegrated the islets appeared.
- A high number of insulin granules could be observed in beta-cells with virus particles, suggesting a functional block.
- Infected cells showed altered cytoplasm including condensed nucleus, increased vesiculation, disassembled Golgi apparatus and occasionally empty-looking capsids.
- Human primary endocrine islets inoculated with the CVB-5 strain V89-4557 showed decreased expression of alpha- and beta-cell associated genes, whereas the two CVB-4 strains, VD-2921 and E2-Yoon, showed decreased expression only in beta-cell associated genes.
- Addition of synthetic dsRNA, poly(I:C), to human islets potentiated the glucose-stimulated insulin secretion.
Paper III

- There was a significantly higher frequency of positive CAR-staining in the islets of T1D-related subjects as compared with non-diabetic controls.
- CAR protein was expressed in both human exocrine and endocrine tissue.
- Human islets showed reduced expression of the CAR mRNA when inoculated with three CVB-1 strains, CVB1-3, CVB1-7 and CVB1-11, but not with an Echovirus-6 strain, Echovirus-6-2C.

Paper IV

- Both donor 1 and donor 2, who died shortly after T1D diagnosis, stained positive for insulin. However, glucose-stimulated insulin secretion in isolated islets was impaired in donor 1 and lacking in donor 2, suggesting a functional block.
- Donor 1, in particular, revealed severely damaged endocrine islets including swollen-looking cells and dilated endothelial cells/bleedings.
- Ultrastructure analysis revealed empty-looking cells with debris, vacuolization, glycogen deposits, fused granules and dilated ER in the cytoplasm, but apparently intact nuclei. Fibrosis could also be observed in, for example, the peri-vascular area.
- No signs of apoptosis were seen in either donor.
- Immune-cells, mainly CD8+ cells and macrophages, could be observed evenly scattered in the pancreata of both donors, with increased numbers in donor 2.
- Signs of an activated innate immune response in islets were observed in both donors as well as up-regulation of some chemokines.
- Positive HEVP1-staining could be found in the islets of both donors and one islet auto-antibody positive donor. The positive cells co-localized to some extent with Coxsackie B Blend antibodies in donor 1. Viruses could not be confirmed with PCR, ultrastructural analysis or immunogold labelling against HEVP1.
General discussion

Understanding the tropism of HEV and the effects of virus-infection in the pancreas might increase understanding of the mechanisms underlying beta-cell destruction in T1D. Some studies, both in vitro and in vivo, have shown that human islets, ductal cells and islet-derived endothelial cells are permissive to these viruses and that the infection could result in islet-damage and recruitment of immune-cells [118, 132, 146, 158, 164, 182]. HEVs have repeatedly been shown to have tropism to human beta-cells in vitro ([118, 146, 164], papers I-II) and in vivo [118-119, 182]. Due to the increased frequencies of HEV- RNA in the blood of T1D donors compared with controls [131], it is likely that such systemic infection could reach the pancreas and directly infect and trigger beta-cell destruction. An infection, in the blood, correlates well with the patchy insulitis in pancreatic lobes seen in some T1D donors. Many scientists have searched for “diabetogenic” strains that triggers and accelerate the disease, however it is not likely that such strains exist, since all the viruses we have studied have tropism to beta-cells. Also, there has been a large variation in isolated HEV genomes in patients associated with T1D [31, 145, 158, 183-184], suggesting that there are several HEV-B serotypes that might lead to beta-cell damage in susceptible individuals.

In this thesis the tropism of CVB to human primary exocrine cell cluster was examined for the first time in vitro. The two CVB-5 strains used replicated in human islets but not in exocrine cell clusters. This is the opposite as to mice models and implies a difference in tropism between the two species. Thus, using mouse animal models to study human CVB-induced pancreatitis or diabetes should be done with caution. Depletion of cholesterol affects the replication of +stranded RNA viruses negatively. The close relationship of virus particles to cholesterol-rich insulin granules [166] might indicate that these membranes could be used as RNA replication -sites. It would hypothetically save the virus energy and time to use these membranes, which are numerous in the beta-cells, instead of re-arranging membranes from the ER and Golgi apparatus. The suggested block in insulin granule release, which was observed in paper II, might lead to accumulation of insulin granules in the cytoplasm and thus facilitate virus replication even more. Understanding the mechanisms behind replication of CVB in beta-cells and how the viruses are packed into the characteristic crystal rafts, might have implications for the treatment of virus-induced disease.
Why are endocrine cells and not exocrine cells permissive to CVB? One answer may lie in the presence of virus receptors. The expression of the main CVB receptor, CAR, was investigated in vivo and was found in both the exocrine and endocrine part of the pancreas among ~40% of the subjects analysed. The number was significantly higher in T1D-related individuals. The expression of CAR is influenced by tissue damage and inflammation and this might indicate an ongoing tissue damage and/or inflammation in this group of individuals which might increase their vulnerability to virus infections. The generally low frequency of CAR expression in islets in vivo is a bit perplexing since isolated islets generally are permissive to CVBs in vitro. One possible explanation might be that the isolation -procedure might damage the islets, resulting in expression of CAR, which also was shown by IHC-staining of CAR in isolated islets. Alternatively, unknown, receptors might potentially be used for virus-entry in the endocrine cells. The lack of permissiveness in exocrine cells still awaits answers.

In paper IV we had the rare opportunity to study pancreata from two donors close to T1D-onset. Several signs of virus - infection were detected including IHC-staining for virus proteins in several islets, dramatic ultrastructural alterations and activation of innate immunity genes. Despite this, no viral RNA could be detected. This could be due to a viral “hit-and-run” mechanism, which might only leave fingerprints behind to study. Or, the cellular injuries might be due to some other, still unknown, factors.

In summary the in vitro results suggest that HEVs could cause T1D by infection of beta-cells, but not exocrine cells. The role of viruses in vivo awaits further studies.
Future perspectives and concluding remarks

It is important to find the environmental factor(s) which trigger T1D in order to develop strategies to prevent the outbreak and progress of the disease in time. The search for enterovirus as the inducers or accelerators for T1D has been on-going for 50 years. During these years, a vast number of studies have been done, some supporting and some rejecting the significance of viruses in the disease development. Several factors have hampered these studies, including limited pancreatic material from T1D donors at or just prior to disease onset, poor quality material, varying study designs and laboratory techniques. Some of these problems are currently being addressed, including collection of T1D materials in large biobanks, which scientists all over the world can apply to perform experiments on, and standardizations of various techniques between different laboratories. This is probably a good foundation to build future studies on.

Many scientists have hoped to find “diabetogenic” HEV-strains, such as polio against which vaccination could be offered; however, studies have rather shown association of several different HEVs to T1D [130, 145, 158, 185]. Which HEV serotypes should be vaccinated against and who should be vaccinated? It has been suggested that a randomized vaccination study should be carried out in high-risk groups against a number of serotypes, to see if a decrease in frequency of disease outbreak could be achieved [131]. Alternative approaches could be development of antiviral-drugs or drugs that could modulate the immune-response; however, these strategies are still hampered by the lack of defined target(s). Hopefully better screening methods to detect “pre-diabetes” and defined environmental triggering factors will be developed soon so that the disease can be treated before clinical symptoms starts to appear.
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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.