Master's Thesis

Detecting Enterovirus Infection in Type 1 Diabetic Pancreas Tissue Using Immuno-Electron Microscopy

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Type 1 diabetes is a chronic autoimmune disease in which the glucose homeostasis is disrupted. The patient's pancreatic beta cells are destructed and therefore the insulin production is decreased. Genetic background has been shown to be associated with the development of Type 1 diabetes but also some environmental factors such as viruses seem to have a role. The prime viral suspects are human enteroviruses: the small RNA viruses that cause mild diseases such as common cold but also serious acute and chronic infections. This thesis was focused on detecting enteroviruses from Type 1 diabetic pancreas tissue and especially from insulin producing beta cells. In addition, the different cell types of pancreas were confirmed with the marker proteins. The pancreas tissue was examined for virus infection with Tokyuasu's immuno-electron microscopy technique. First, semi-thin sections were labeled for virus capsid and RNA by immunofluorescence. Then infected areas were chosen for thin sectioning, protein A gold labeling and electron microscope imaging. From the diabetic pancreas tissue, only a few beta cells were found. Instead, the amount of glucagon producing alfa cells was generous. The other pancreatic cell types were found poorly or not found at all in both diabetic and control pancreas tissues. Majority of the cells showed no indication of virus infection. Only a few beta cells from autoantibody positive and diabetic tissues gave low but statistically significant signs of infection suggesting that this technique may be used to detect low signal but is tedious method for finding small infected areas.

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Tyypin 1 diabetes on krooninen autoimmuunisairaus, jossa kehon glukoositasapaino on häiriintynyt. Tautia sairastavan potilaan haiman beta-solut ovat tuhoutuneet, minkä takia insuliinia ei muodostu tarpeeksi kehon sokeriaineenvaihdunnan ylläpitämiseen. Geenien lisäksi jotkin ympäristötekijät, kuten virukset, vaikuttavat mahdollisesti taudin puhkeamiseen. Pääepäiltynä ovat enterovirukset, pienet RNA-virukset, jotka aiheuttavat flunssaa, mutta myös vakavia akuutteja ja kroonisia tulehduksia. Tämän tutkimuksen tarkoituksena oli selvittää, voidaanko enterovirusinfektiota havaita tyypin 1 diabeteshaimakudoksessa ja erityisesti insuliinia tuottavissa beta-soluissa. Lisäksi päämääränä oli erotella haiman eri solutyypit markkeriproteiinien avulla. Haimakudoksen tarkastelu tapahtui Tokyuasun immuno-elektronimikroskopiatekniikan avulla. Ensin puoliohuista leikkeistä leimattiin fluoresenssitekniikalla viruskapsidi ja RNA. Tämän mukaan valittiin positiivisia näytteitä ohutleikkeisiin ja proteiini A-kultaleimauksiin, jotka kuvattiin elektronimikroskoopilla. Kartoittaessa eri solutyyppejä, huomattiin beta-solujen harvalukuinen esiintyminen diabeteshaimassa. Glukagonia tuottavia alfa-soluja sen sijaan löytyi reilusti ja muiden solutyyppien edustajia vain yksittäisiä, jos lainkaan. Vain muutama virusinfektiolle heikosti positiivinen beta-solu löytyi diabetes- ja autoantibody-positiivisesta näytteestä. Tämä tekniikka soveltuu siis heikkojen signaalien havaitsemiseen, mutta on hyvin työläs etsittäessä harvassa olevia positiivisia näytekohtia.

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ABBREVIATIONS

Aab	Autoantibody
CVB	Coxsackievirus B
EM	Electron microscope
HEV	Human enterovirus
nPOD	Network for Pancreatic Organ Donors with Diabetes
PAG	Protein A gold
PBS	Phosphate buffered saline
PP	Pancreatic polypeptide
T1D	Type 1 diabetes

1 INTRODUCTION

1.1 Enteroviruses

Human enteroviruses (HEV) are small RNA viruses in the family of Picornaviridae (Vuorinen *et al.* 1999). The classification has been changed a lot during the last decades. Earlier it was based on viral nucleic acid, host species and pathogenesis. However, after genome sequencing the classification has been defined based on the use of host cell receptors, the viral protein identities and genome organization and processing identities. Now the enterovirus genus is divided into 12 enterovirus species and three rhinovirus species where approximately 75 enterovirus serotypes and 100 rhinovirus serotypes have been classified (ICTV Virus Taxonomy 2017).

Enteroviruses are one of the most common human viral pathogens (Harris and Coyne 2015). They cause mild diseases but can also cause serious acute and chronic infections (Marjomäki *et al.* 2015). The primary transmission pathway of the HEVs is the fecal–oral route via the ingestion of contaminated water or food (Hober *et al.* 2013). Their first targets are polarized epithelial cells of the respiratory tracts where the infection can cause upper respiratory symptoms (Royston and Tapparel 2016). Due to the high tolerance to acidic environment HEVs can travel through the stomach all the way to the small intestine and invade the epithelial cells of the intestinal tract (Rhoades *et al.* 2012). If the infection spreads, HEVs can enter the bloodstream, travel through the body and transmit to pancreatic cells, cardiomyocytes, and neurons (Harris and Coyne 2015, Hyöty and Taylor 2002). Viral infections at these secondary sites can lead to serious diseases. At heart, HEVs can induce the cell death of cardiomyocytes or trigger an autoimmune response against cardiomyocytes, which can lead to development of

myocarditis and further to dilated cardiomyopathy (Harris and Coyne 2015). Infections at neurons can induce the apoptosis and cause damage at central nervous system (Rhoades *et al.* 2012). At pancreas HEVs can destroy the insulin producing beta cells or activate an autoimmune response against them leading to the development of Type I diabetes (T1D) (Harris and Coyne 2015).

1.2 Virus life cycle

The virus is armed with single-stranded positive RNA genome, encountered with icosahedral protein capsid (Figure 1) (Hober *et al.* 2013; Jiang *et al.* 2014). The capsid is composed of four structural proteins known as VP1, VP2, VP3 and VP4 (Marjomäki *et al.* 2015). With 60 copies of each, they display 2-, 3-, and 5-fold icosahedral symmetry axes. VP1, VP2 and VP3 form the outer surface of the capsid and the small VP4 molecules are located inside of the virion (Jiang *et al.* 2014).

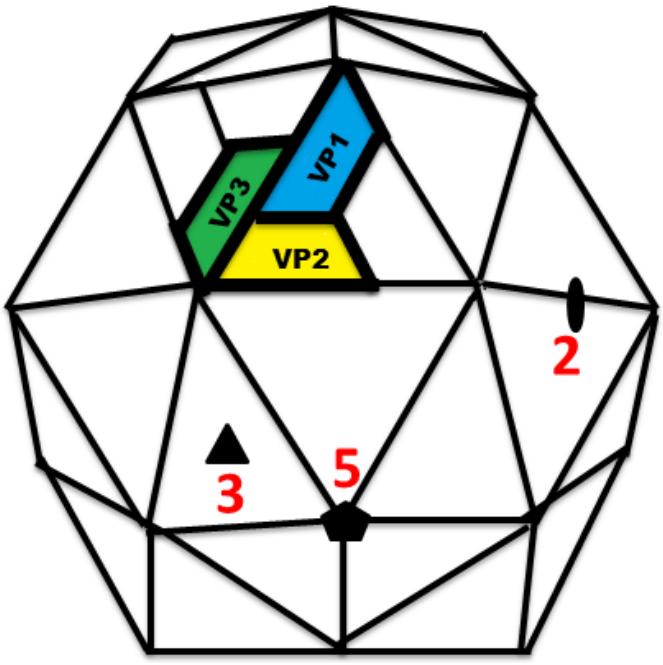

Figure 1. Human enterovirus capsid structure. The 2-, 3-, and 5-fold axes of symmetry are indicated with red numbers. The capsid proteins VP1 (blue), VP2 (yellow), and VP3 (green) form the icosahedral outer surface, whereas VP4 is located inside of the capsid. (Modified from Jiang et al. 2014).

The infectious entry into the host cell starts when the virus binds to the receptor on the plasma membrane (Marjomäki *et al.* 2015). The recognized surface molecules are integrins, decay accelerating factors (DAFs) and Coxsackie and adenovirus receptors (CARs). The receptors are widely available which explains why HEVs can cause such a variety of acute and chronic infections in various tissues. Typically, the receptor binds at canyon; a depression in the viral capsid which surrounds the fivefold axis of symmetry (Linden *et al.* 2015). The conserved amino acid residues of the canyon bind with the amino-terminal domains of the receptors causing clustering of the receptors (Lin *et al.* 2009). This triggers the signaling events and with the support of the host cell molecules the virus is taken into the cell (Marjomäki *et al.* 2015). However, enteroviruses are exploiting different internalization methods. Stuart *et al.* (2002) showed that lipid rafts and caveolae are used as an entry site for Echovirus 11. Yuan *et al.* (2018) revealed that Enterovirus 71 and Cocxackievirus-A16 are using clathrin and dynamin independent endocytic pathway for cellular entry. Marjomäki *et al.* (2015), on the other hand, stated that Enterovirus B species are using macropinocytic mechanisms to enter the host cell. However, it is known that the virus ends up into the host cytoplasm inside of an endosome after 15–30 minutes. The viruses start to uncoat and the endosomes develop into ESCRT-driven (endosomal sorting complexes required for transport) multivesicular bodies after 1 hour. Following the successful entry, the uncoating continues for at least 2 hours. The viral genome is then released into the cytoplasm ready for the replication (Marjomäki *et al.* 2015).

The HEV genome is constructed approximately from 7 000 - 8 500 nucleotides (Jiang *et al.* 2014). In the middle of the genome is one single open reading frame, which encodes the capsid structural polypeptides and the nonstructural replication proteins (Jiang *et al.* 2014). Before the reading frame is located a long 5′-untranslated region (5′ UTR), which contains an internal ribosome entry site

(IRES) with a length of 450 nucleotides (Su *et al.* 2018). IRES initiates the viral translation of internally by a cap-independent manner (Thompson and Sarnow 2003). The 5′ end of the viral genome has also a small covalently linked virus-encoded peptide called VPg (Hober *et al.* 2013). VPg is needed as a primer in RNA synthesis because RNA polymerase cannot initiate the replication on its own (Sun *et al.* 2012). At the 3′end of the genome is a polyadenylated poly(A) tail, which coordinates the actions of the 5′ and 3′ ends during the translation and replication (Kempf *et al.* 2013). In addition, essential RNA secondary structure, cis replication element (Cre), is located within the open reading frame (Jiang *et al.* 2014). It is involved in the function of VPg and therefore plays crucial role in the synthesis of positive and negative RNA strands (Cordey *et al.* 2008).

The host cell translation is shut down during infection so that the virus has full access to its own processes (Jiang *et al.* 2014). In the cytoplasm, the viral RNA genome is translated to single polyprotein, which is further cleaved into viral proteins by 2A and 3C proteases. The genome is also replicated but it cannot occur simultaneously with translation on the same RNA molecule (Zhang *et al.* 2015). Therefore, translation must be shut down in order to start the replication and vice versa. 3D RNA polymerase synthesizes a negative-strand RNA that, in turn, functions as a template for the synthesis of a positive-sense genome. Due to that, the replicative intermediate is partially double-stranded during the synthesis (Wimmer *et al*. 1987). The 3D RNA polymerase functions fast but in an error prone way creating continuously point mutations during viral replication (Ward *et al.* 1988, Jackson and Coyne 2018). For instance, the replication of eukaryote cells is controlled by several checkpoints and repair mechanisms in order to produce identical replicate of the genome. However, the errors in the virus genome are crucial for the survival of the virus population as generating more fit mutants they can to adapt to the changing environmental conditions.

With the help of viral and host cell factors, the replicated genomes are packed inside the produced capsid proteins and then new virus particles are ready to be transported from the cell (Zhang *et al.* 2015). The main exit mechanism of HEVs is cell death but other routes are also possible (Harris and Coyne 2015). Autophagy, a natural recycling mechanism of the cells, can be utilized by viruses during the exit (Robinson *et al.* 2014). The infection can be also persistent with low levels of cell lysis and continuous viral shedding (Harris and Coyne 2015). The virus is decreasing the lysis rate by restricting the viral RNA translation and replication (Cunningham *et al*. 1990). The new virus particles that are formed, are shed inside of extracellular vesicles which can help them to avoid the immune surveillance (Robinson *et al.* 2014). This way the virus can weaken the inflammation and the activation of the immune cells simultaneously releasing new particles.

At the same time the cell death is the exit mechanism of the virus but also the pathogen elimination mechanism of the infected cell (Harris and Coyne 2015). The cell aims to eliminate the virus before it completes the replication cycle and the virus tries to inhibit the early cell death in order to survive. The cell death, however, can be highly detrimental to the individual when concerning important cells and organs such as neuronal cells. As mentioned above the cell deaths are causing the most serious enteroviral infection induced diseases. Especially recently, the research have been focusing on the association between T1D and enteroviral cell deaths and persistent infections in the pancreas.

1.3 Diabetes

Pancreas is a complex organ constructed from exocrine and endocrine cells (Figure 2) (Leung and Ip 2006; Li *et al.* 2018). Exocrine cells (acinar and ductal) contain zymogen granules, which produce digestive enzymes, and endocrine cells (alfa, beta, gamma, delta and epsilon) in the pancreatic islets contain hormones

secreting granules that regulate the body's glucose homeostasis. If the body does not recognize the insulin producing beta cells in the pancreatic islets, consequences can be the development of T1D. The disease is defined as a chronic autoimmune disease caused by severe decrease of insulin secretion (American Diabetes Association 2014). Beta cells are identified as harmful and autoantibodies against beta cell proteins circulating through patients' blood are marking them for immune mediated destruction performed by autoreactive T-cells (Knip and Simell 2012; Wenzlau and Hutton 2013). The patient's glucose metabolism is in imbalance since the insulin is needed in glucose absorption from the blood (Wilcox 2005). The high concentration of glucose in the blood leads to chronic hyperglycemia, which is associated with long-term damage, dysfunction, and failure of different organs, such the eyes, heart, and blood vessels (American Diabetes Association 2014).

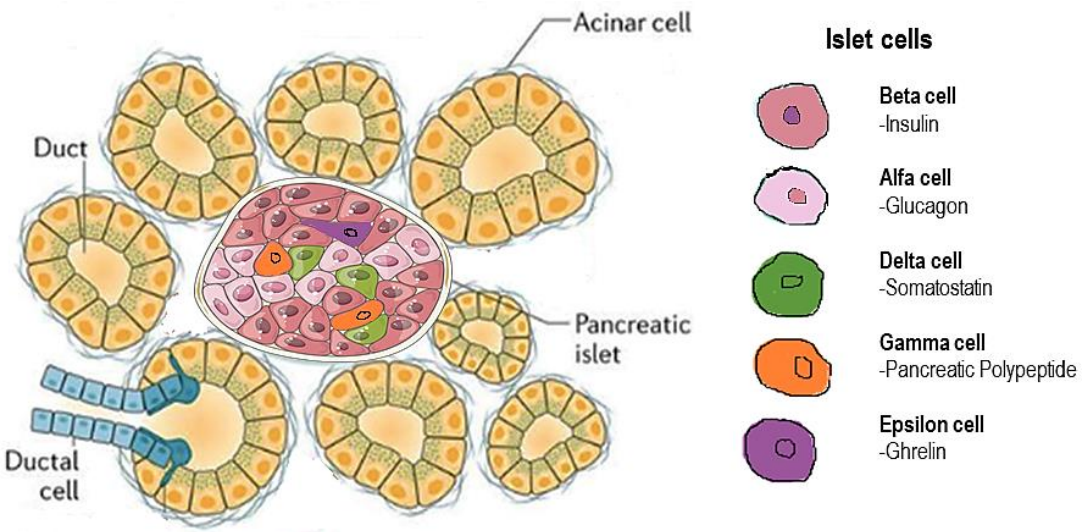

Figure 2. Presentation of the pancreatic cells. Exocrine tissue is composed of digestive enzyme secreting acinar cells and ductal cells. Endocrine cells (alfa, beta, delta, gamma and epsilon cells) are producing various hormones needed in keeping the blood sugar levels in balance. (Modified from Ellis *et al.* 2017 by utilizing Servier MedicalArt Powerpoint image bank).

T1D cases have been gradually increasing globally. During 1960 to 1996, the annual increase was 3.0% (Onkamo *et al.* 1999). Interestingly, according to the

various statistical studies, Finland is registered with the highest number of new T1D cases per year (The DIAMOND Project Group 2006.; Onkamo *et al.* 1999; Green *et al.* 2001; Patterson *et al.* 2005). As a comparison, the lowest annual amount of incidences per 100 000 people were in China (0,1) and the highest in Finland (40,9) during the years 1990–1999 based on The DIAMOND Project Group (2006). However, it should be noted that the T1D case registration, let alone the amount of information, have been improved during the years. In addition, in the developing countries with high child mortality, diabetes can remain undiagnosed even after death. Therefore, the increasing trend and the differences between countries might not reflect the actual situation (The DIAMOND Project Group 2006).

The active research question is how beta cells become immunogenic. First of all, genetic factors play an important role. Especially genes in HLA (Human Leukocyte Antigen) region have been associated to the development of T1D (Concannon *et al.* 2005). The proteins coded by the genes of this region are activating the immune-mediated destruction of the pancreatic islets by binding to the key peptides of the autoantigens against beta cells (Pociot *et al.* 2010). However, the gene pool of the population does not change enough between generations to provoke such an increase in T1D incidences as noticed, for example, in a cohort study surveyed by Harjutsalo *et al.* (2008). Secondly, genetics alone do not explain the significant differences in T1D incidence trends between European countries (Green *et al.* 2001) since the gene pool of Europeans is somewhat homogenous (Cavalli-Sforza and Piazza 1993). In addition, individuals with genetic susceptibility for T1D do not always get the disease. Actually only 10% of new patients have a relative with T1D (Steck and Rewers 2011). Research on monozygotic twins has revealed that only in 25% to 50% of the cases both of the twins have the T1D (Kaprio *et al*. 1992; Metcalfe *et al.* 2001; Knip and Simell 2012). Furthermore, Danish and Finnish twin studies have estimated that T1D is

approximately 70% hereditable (Kyvik *et al.* 1995; Hyttinen *et al.* 2003), leaving non-genetic factors guilty of the rest of the cases.

It has been implicated that several environmental factors can trigger the autoimmunity against beta cells and initiate the development of T1D. Various studies from around the world have given contradictory results regarding vaccines, excessive hygiene, cow's milk and gluten, to mention a few (reviewed in Åkerblom *et al.* 2002; Rewers *et al.* 2017). However, the diversity of the intestinal microbiota and the exposure to enterovirus infections have been more positively linked to the development of T1D. The association between enteroviruses and T1D is further discussed in the following chapter.

1.4 Diabetes and enteroviruses

Enteroviruses are the prime viral candidates causing the development of T1D (Filippi and von Herrath 2008). However, not all of the HEV types are inducing T1D (Oikarinen et al. 2014). From the nearly 200 HEV types, only a few are suspects, including Coxsackievirus B (CVB) serotypes. Already in late 1960s Gamble *et al.* resulted that T1D patients had higher titers of CVB antibodies in their sera than healthy controls, and since then various studies have supported these results (Morgan and Richardson 2014). Oikarinen *et al.* (2008) presented that in the Aab positive pancreas the enterovirus capsid protein VP1 was mainly detected only in pancreatic islets, whereas exocrine tissue was enterovirus free. As a conclusion, virus targets the pancreatic islets and especially the insulin producing beta cells. Two reasons for this have been revealed (Reviewed by Richardson and Morgan 2018). Firstly, the beta cells have receptors necessary for the virus binding and internalization. They express the Coxsackie and adenovirus receptor (CAR), which is one of the enterovirus receptors, used specifically by CVB serotypes (Ifie *et al.* 2018). The receptor is strongly produced in the pancreatic

islets but not in the exocrine tissue, which supports the findings that enteroviruses are associated with the beta cell destruction (Oikarinen *et al.* 2008). Secondly, beta cells contain specific host factors, which are needed to mediate important reactions associated with viral life cycle. Especially, IRES-mediated translation and RNA replication require several protein-protein and RNA-protein interactions where host factors are involved (Lin *et al*. 2009).

Reviewed by (Szopa *et al*. 1993; Hyöty and Taylor 2002; Filippi and von Herrath 2008), there are two possible ways how enteroviruses could cause beta cell destruction. They can induce the destruction directly during the course of an acute infection or they can provoke an autoimmune response against beta cells leading to persistent infection. Direct cytolysis of beta cells can occur when replicated viruses are exiting the host cell or when the host cell is eliminating the pathogen by apoptosis as an antiviral response. Anyhow, autoantibodies and inflammatory cytokines are introduced to boost the immune response and inflammation. This could later induce the production of virus-specific cytotoxic T-lymphocytes (CTL), which could cause further damage by killing infected beta cells. Interestingly, molecular mimicry could take place, since some sequence and epitope homologies between virus and beta cell proteins could induce cross-reactive CTLs generating immune responses against beta cells. In addition, beta-cell damage can also be induced by bystander activation of pre-existing autoreactive T-cell clones with heterologous antigenic specificity to the enterovirus.

If HEV can avoid the defense mechanisms of the host cell, it could establish a persistent, lower level infection (Hyöty and Taylor 2002). In this case, the host cell lysis is minimized but the virus is still present affecting to the functions of the host cell and the whole host organism. In the case of beta cells, constant replication of the virus can directly damage and distract their function. Alternatively, during

persistent infection viral and beta cell antigens can be presented to the CTLs hereby maintaining the immune responses of the host.

However, relatively small portion of the enterovirus infections lead to T1D; it was evaluated that less than 5% of CVB1 infected children develop the disease (Laitinen *et al*. 2014). This seems contradictory since various *in vitro* studies have demonstrated that HEVs can cause drastic damage on beta cells (Flodström-Tullberg *et al.* 2019). One reason could be that not all of the CVB serotypes are inducing T1D at the same intensity. Frisk *et al*. (1992) resulted that CVB4 is the most commonly detected serotype in T1D patients whereas CVB1 is the rarest. Indeed, CVB4 have been separated from the T1D patients by several researchers, for example in 1978 by Yoon *et al*. The other explanation can be the release of the virus defense proteins called interferons (INFs): cytokines, which have been found to promote beta cell surveillance (Chehadeh *et al.* 2000). Moreover, the risk for developing T1D may coordinate with the amount of the IFNs released during early in the infection and with the beta cell antiviral defense efficiency (Tsai *et al.* 2003; Chehadeh *et al.* 2000). Low IFN concentration can even increase the virus infectivity of the beta cells, whereas high concentration aids beta cells to organize essential antiviral response. However, it has been noted that the efficiency of the beta cell antiviral defense may naturally differ, which could explain why some individuals are more susceptible to the viral infection (Tsai *et al.* 2003).

1.5 Previous clinical and experimental studies

The relationship between HEVs and T1D has been under investigation in recent years and many different research methods have been introduced. *In vivo* the connection between HEVs and the disease has been broadly studied in various mice models (Jun and Yoon 2001). Mouse is considered to be an informative model to evaluate the pathogenesis of the virus but it is not always comparable to human

(Roep 2007). The mice models do not have the similar genetic basis as humans, and even the models, which are transgenically expressing human gene products, do not develop autoimmune diabetes. In addition, the epitopes of the autoantigens and their processing are not identical in mice and men.

Human samples are generally difficult to get since the pancreas tissues of T1D patients are valuable and pancreas biopsies are anatomically difficult to collect (Coppieters and von Herrath 2009). The possible studied tissue samples are therefore *post-mortem*, which might decrease the reliability of the results. However, virus can be detected *post-mortem* and the histology of the organ can be observed. Fortunately, programs such as the Network for Pancreatic Organ Donors (nPOD), a collaborative T1D research project sponsored by the JDRF (https://www.jdrfnpod.org/), are providing pancreas and related organ tissues relevant to T1D research.

Despite of the lack of the human pancreas tissue samples, the behavior of the enterovirus can be observed in human cell cultures. These *in vitro* studies have been focusing on the enterovirus infection of the various cells related to the T1D (Hober *et al*. 2013). By infecting the beta cells, the molecular pathways of the virus induced cell death have been studied. In addition, the ability of beta cells to produce insulin during infection has been examined. The effects on to the morphology of the pancreatic islets during infection has been examined likewise the effects of the release of inflammatory signaling proteins. Apart from pancreas, the studies concentrated on the infection of thymus have been achieved. Thymus has a role in T-cell development which may be interfered by enterovirus infection further inducing the immune destruction leading to T1D.

The imaging techniques are useful to map the pathology behind T1D and study different processes and kinetics of the immune surveillance initiated by virus infection (Roep 2007). Already in 1950s, histopathological changes of pancreas

tissue have been imaged with light and electron microscopy (Richardson 1951; Vuorinen and Kallajoki 1989). Nowadays, immunohistochemistry and in situ hybridization are used to detect viral RNA and protein in the different parts of the pancreas tissue of T1D patients (Hober *et al*. 2013).

Studies in humans have been focusing on to the detection of enteroviruses with different methods. Serological studies have been conducted to identify antibodies in the serum, mainly using the neutralizing antibody tests and immunoassays such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) (Green *et al.* 2004). In many studies enterovirus antibodies have been more prevalent in diabetic patients than in healthy children (Stene and Rewers 2011). For example, Oikarinen *et al.* (2014) measured neutralizing antibodies against different CVB serotypes in children diagnosed with T1D from five European countries and antibodies against CVB1 were more commonly detected in diabetic children than in the healthy controls. However, it is argued that many serological studies have been based on methods that are not specific enough to separate the HEV serotypes from each other and therefore non-diabetogenic HEVs are possibly detected (Oikarinen *et al.* 2014). In addition, serological studies have given inconsistent results as Green *et al* (2004) compared the results of 26 case-control serological studies but concluded that there is no reliable connection between CVB infections and T1D. This may be because, the compared studies were from 1969-1995 and the methods nowadays are more sensitive.

With reverse transcription polymerase chain reaction (RT-PCR) viral RNA can be detected in the blood, stool or tissue samples. Bergamin and Dib (2015) reviewed that with this method 24 selected studies had concluded significant connection between enterovirus infection and autoimmunity or T1D. Compared to the serological methods, PCR methods are more sensitive and can be used to identify the diabetogenic HEVs by RNA sequencing (Yin *et al.* 2002; Green *et al.* 2004).

Serological methods are also indirect in a sense that can only detect previous virus infection whereas PCR methods can indicate ongoing infection lasting a few days (Hyöty and Taylor 2002). Some possible drawbacks are still associated with virus detection with PCR methods. Contamination risk and repeated freezing and thawing of samples can interfere with the results leading to false conclusions.

The cell-mediated immunity to enterovirus antigens has been studied with cytokine and proliferation assays (Hyöty 2002). As mentioned earlier, INFs are released during the virus infection and may correlate with the development of the T1D. Chehadeh *et al.* (2000) studied ongoing virus infection by detecting IFNs in the plasma of 70% of T1D patients but healthy control examinees were clear. In addition, virus infection induces the development of CTLs, which can destroy the infected beta cells. Indeed, T-cell proliferation rate has been noted to increase in response to enterovirus antigens in T1D patients (Jones and Crosby 1996; Juhela *et al.* 2000).

These mentioned methods are retrospective, based on the virus detection in diagnosed T1D patients. However, prospective systematical studies have been accomplished by following initially non-diabetic children until possible development of T1D (Hyöty and Taylor 2002). This way the beta cell damaging process can be observed before the clinical diagnosis and the possible viral triggers could be traced. The appearance of autoantibodies, viral RNA or proteins in the examinee's blood or stool could be followed and the connection between viral infections and the initiation of the beta cell damage could be analyzed. However, viral RNA can be detected in the serum and stool samples only in the time frame of three months, and therefore frequent sampling is important (Salminen *et al.* 2004). In addition, prospective studies have received criticism because of the heterogenic study designs, the small number of examinees and the low sensitivity of the methods used to detect enterovirus infection (Bergamin and Dib 2015).

1.6 Aims of the thesis

The idea was to study possible signs of enterovirus infection in human pancreas tissue with fluorescent labeling at light microscopy level and with immuno-gold labeling at ultrastructural level by using Tokyuasu's immuno-EM technique. Three different gold sizes were used enabling the simultaneous observation of three different targets in the pancreas tissue. Cell type markers, capsid and viral RNA were studied in pancreas tissues from healthy, autoantibody positive and Type 1 diabetic individuals. The different cell types were separated and enterovirus and cell marker labels were calculated.

Hypothesis of the thesis:

1. With the help of marker proteins, it is possible to distinguish reliably the major cell types in the human pancreas (alpha, beta, delta, PP and acinar cells).

2. Enterovirus capsid and/or dsRNA may be detected with Tokyuasu's immuno-EM technique in the T1D pancreas tissue proven positive for enteroviruses by other techniques, but not from the control tissue without virus infection.

3. Insulin releasing beta cells have higher concentration of capsid proteins and dsRNA replication intermediate products than other pancreas cell types in infected T1D samples.

2 MATERIALS AND METHODS

2.1 Pancreas samples and antibodies

Two control, two autoantibody positive and four T1D tissues of human pancreas (*post-mortem)* were provided by nPOD (Table 1). Local ethical committee has approved the human nPOD samples to be handled in Varpu Marjomäki's project, funded by Helmsley Foundation and nPOD (Ethical exempt dated 16.4.2018). The tissue samples were first processed in Jyväskylä: they were cut into smaller cubes and immersed in 2.1 M sucrose to prevent ice crystal formation during freezing. Further sectioning was performed in Oulu Biocenter Finland. Antibodies were used to separate the various cell types of the pancreas and to detect the enterovirus capsids and replication intermediates. The used antibodies (Table 2) were targeted against insulin (beta cell), glucagon (alfa cell), proinsulin (beta cell), somatostatin (delta cell), pancreatic polypeptide (PP) (gamma cell) and cytokeratin 19 (exocrine cell). Enteroviruses were localized by targeting dsRNA and capsid protein VP1.

Table 1. The used human pancreas samples provided by nPOD.

Sample type	**Sample ID**
Control	6103-01 Pancreas head
Control	6153-01 Pancreas head
Autoantibody	6197-01 Pancreas body
Autoantibody	6197-01 Pancreas head
T1D	6212-01 Pancreas body
T1D	6243-02 Pancreas head
T1D	6337-01 Pancreas body
T1D	6362-01 Pancreas body

Table 2. The final concentrations of antibodies used in the labelings of semithin and thin sections of the human pancreas.

Antibodies used	**Isotype**	**Made in**	**Dilution**	**Origin**
Insulin	IgG	Guinea Pig	1:500	Dako, California, USA
Glucagon	IgG	Rabbit	1:500	Abcam, Cambridge, UK
Somatostatin	IgG	Rabbit	1:500	Abcam, Cambridge, UK
PP	IgG2	Mouse	1:100	Abcam, Cambridge, UK
Cytokeratin 19	IgG	Rabbit	1:200	Abcam, Cambridge, UK
Proinsulin	IgG1	Mouse	1:400	Abcam, Cambridge, UK
Bridging Antibody (proinsulin)	IgG	Rabbit	1:1500	Sigma-Aldrich, Missouri, USA
VP1	IgG2a	Mouse	1:1000	Dako, California, USA
dsRNA, J2	IgG2a	Mouse	1:12 000	Scicons, Budapest, Hungary
Isotype control Mouse	IgG2a		1:16 800 (dsRNA) 1:1400 (VP1)	Dako, California, USA
Isotype control Rabbit	IgG		1:8300 (glucagon)	Vector laboratories Inc., California, USA
α-Guinea Pig 488	IgG1		1:400	Invitrogen/Thermo Fisher scientific
α-Rabbit 555	IgG		1:400	Invitrogen/Thermo Fisher scientific
α-Mouse 633	IgG		1:400	Invitrogen/Thermo Fisher scientific
DAPI			1:40 000	Invitrogen/Thermo Fisher scientific

2.2 Methods

2.2.1 Semithin sections

Tissue samples were first cut into semithin sections to pinpoint areas more rich with studied cells and enteroviruses. The samples were double and triple labeled with antibodies and fluorescent secondary antibodies using different cell, granule and virus marker combinations. Imaging was done with Olympus IX81 confocal microscope with software Olympus Fluoview 4.0a and the favorable areas were marked. For confocal imaging, 488 Argon laser as well as 546 and 633 Helium Neon lasers were used. The information of the interesting areas for thin sectioning was then forwarded to Oulu, where the samples were placed on a specimen support grid and sent back to Jyväskylä in protecting film of gelatin and methyl cellulose.

2.2.2 Thin sections

The thin pancreas sections were examined with Tokyuasu's immuno-electron microscopy technique according to the procedure published in *Nature Protocols* by Slot and Geuze (2007) (Table 3). Triple-labelings with 5, 10 and 20 nm Protein A gold particles (CMC, Utrecth) were used to show simultaneously the virus capsid, dsRNA and a cell marker or different cell marker combinations together. First, the optimum labeling dilutions for the PAG and for used antibodies were adjusted to eliminate any background noise. The dilutions were made in 0,1 % Aurion BSA-c™ (acetylated bovine serum albumin) washing buffer to prevent any charge-based background. Finally, the antibody dilutions were inspected with the isotype antibody controls for VP1, dsRNA, glucagon, somatostatin, cytokeratin 19 and bridging antibodies. The isotope labelings were not adjusted for insulin, proinsulin and PP markers due to the lack of suitable isotype controls. However,

these markers locate distinctly in the granules and therefore odd labels in the cytoplasm and nucleus would be background.

Table 3. Tokyuasu's immuno-EM technique according to the procedure published in *Nature Protocols* by Slot and Geuze (2007).

Step	Procedure	Duration
1	Put grids on 2% gelatin plates under a lamp +40 °C	5 min
2	Incubate grids on the melted gelatin at 37 °C	20 min
3	Wash in PBS + 0,1% glycine droplets	5 x 2 min
4	Wash with 1% glutaraldehyde in PBS	5 min
5	Wash in PBS	2 x 5 min
6	Incubate in blocking solution	15 min
7	Incubate in primary antibody droplets	45 min
8	Wash in washing buffer	4 x 2 min
9	*If needed, incubate in secondary antibody droplets*	30 min
10	*Wash in washing buffer*	4 x 2 min
11	Incubate in Protein A gold droplets	30 min
12	Wash in washing buffer	2 x 2 min
13	Wash in PBS	4 x 2 min
14	Wash with 1% glutaraldehyde in PBS	5 min
	For double/triple labelling repeat steps 3-15	
15	Wash in PBS when doing double/triple labelling	2 x 5 min
16	Wash in water droplets	8 x 1 min
17	Incubate in 2 % neutral Uranyl acetate pH 4	5 min
18	Rinse quickly on a water droplet	
19	Dip grids 10 times in 2 drops of 2 % methyl cellulose/0,4 % uranyl acetate pH 4 on ice	
20	Incubate in 2 % methyl cellulose/0,4 % uranyl acetate pH 4 on ice	10 min
21	With filter paper, dry grids onto the loops	

As described in the protocol by Slot and Geuze (2007), the protecting gelatin film from sample grids were removed by placing the grids on 2% gelatin plates under lamp +40 °C for 5 minutes. After melting, the grids were incubated at 37 °C for 20 minutes. Then the grids were washed with 0,1% glycine in phosphate buffered saline (PBS) droplets for 2 minutes repeating 5 times to block free aldehyde groups. Unspecific binding was further avoided by adding extra 5 minutes incubation in 1% glutaraldehyde in PBS to the protocol. Next, the grids were washed twice in PBS for 5 minutes. Before adding the antibody, the grids were incubated in Aurion blocking solution for 15 minutes to erase unspecific binding. The grids were incubated in 5 µl droplets of primary antibody for 45 minutes and next washed 4 times with washing buffer for 2 minutes. Because the isotype of the antibody used against proinsulin (IgG1) was not compatible with PAG, secondary antibody was introduced to the samples by incubating for 30 minutes. The secondary antibody recognized the isotype of the proinsulin antibody but also bound to the PAG. After antibodies, the first PAG size was introduced for the grids in 5 µl droplets for 30 minutes. The PAGs were added on to the grids in a size order starting from the smallest (5 nm) particle. Next, the grids were washed for 2 minutes twice with washing buffer and 4 times with PBS. Finally, the grids were washed again with 1% glutaraldehyde in PBS for 5 minutes and twice with PBS for 5 minutes. Then the protocol was repeated twice in order to add the next antibodies and PAG sizes in the same grid. After last repeat, the grids were washed 8 times in water droplets for 1 minute. To enhance the contrast the grids were incubated in 2% Uranyl acetate (pH 4) for 5 minutes. The grids were rinsed quickly over water droplet and then dipped 10 times in 2 drops of 2% methyl-cellulose and 0,4% uranyl acetate (pH 4) on ice. The final incubation was done in 2% methyl-cellulose and 0,4% uranyl acetate (pH 4) on ice for 10 minutes to form a protecting film for the grids. With the filter paper, the grids were dried and attached on to the loops to properly dry overnight.

2.2.3 Imaging and Data analysis

The samples were imaged with JEOL-1400 transmission electron microscope using 80 kV with software Olympus Radius. Overall images were taken from samples with lower magnifications but also cells were separated and imaged throughout. 15 000 magnification was needed to separate all of the PAG particle sizes by eye. The main focus was on the beta cells from which the labels were calculated in 30 images from each tissue types. The gold particles were calculated from nuclei, cytoplasm, mitochondria, exocrine granules and hormone granules. Enterovirus VP1, dsRNA and cell marker labels were calculated and characterized. The results were analyzed statistically by calculating the average of labels with standard error of means using GraphPad Prism version 6. T1D samples and autoantibody positive samples were compared to the control samples without T1D by using Student's t test. The limit of statistical significance was 0.05.

3 RESULTS

3.1 Semithin sections

The aim was to detect enteroviruses from the T1D human pancreas tissue with Tokyuasu's immune-EM technique and separate the different cell types of the human pancreas. Several T1D pancreas samples that were shown to be positive for enteroviruses with other techniques were chosen for the study. In addition, control pancreas and autoantibody positive samples were studied. In order to locate the different cell types and enteroviruses the semithin pancreas sections (Figures 3-6) were first analyzed with confocal microscopy. Cell, granule and virus markers were attached with fluorescent secondary antibodies and with double or triple labelings, the positive areas were pinpointed. Most of the sections were exocrine

tissue and therefore negative for the used granule markers. However, after examining several areas from all samples, also pancreatic islets with endocrine cells were found in some sample sections. The most common endocrine cells, beta cells with insulin granules and alfa cells with glucagon granules, (Figure 3) were found in control and autoantibody positive tissues. In T1D tissue, the alfa cells were found but beta cells were rare.

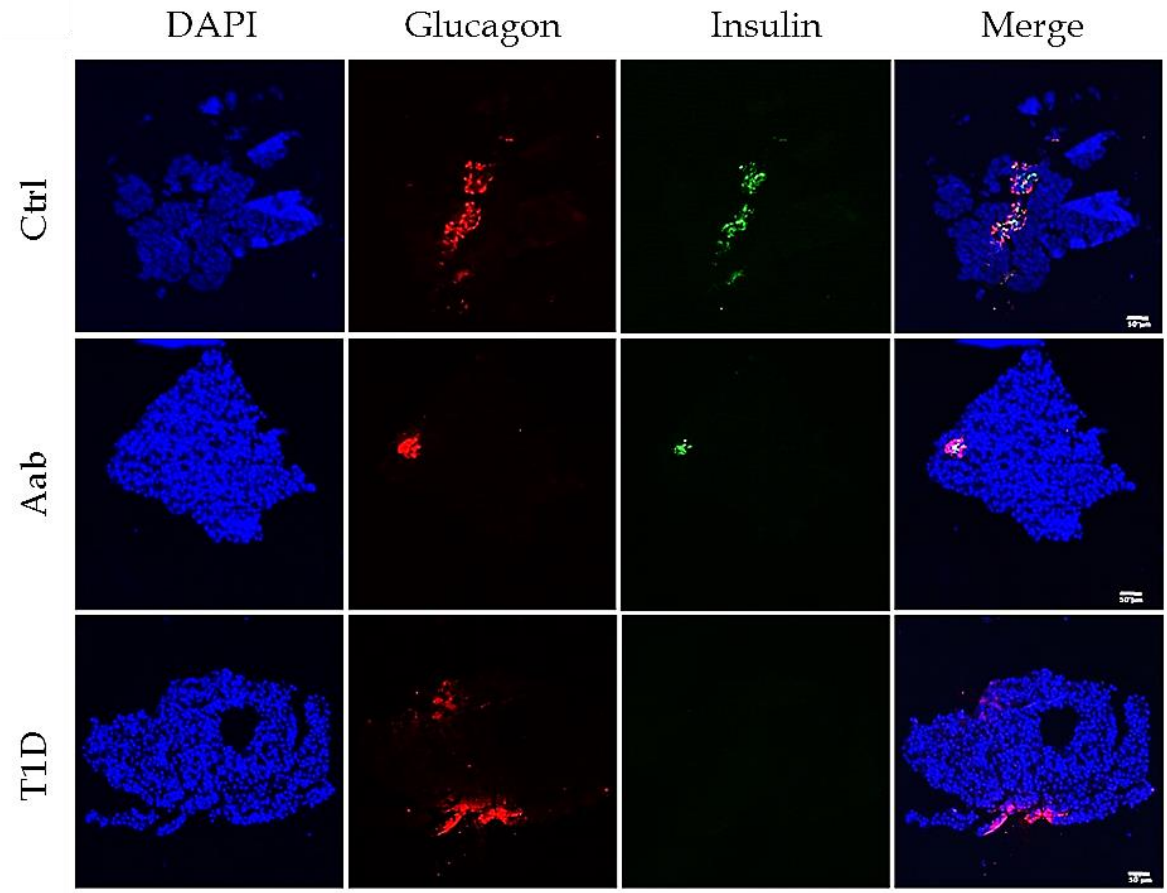

Figure 3. Alfa and beta cell location in semithin sections of pancreas. Fluorescently labeled control, autoantibody and T1D samples were imaged with confocal microscope. Glucagon (red) was regularly found in all tissue types in these sections but insulin (green) was rare in T1D tissue. Figures are representatives of the labeled 20 control sections, 14 Aab sections and 72 T1D sections. Scale bars 50 µm.

The other endocrine cell types were hardly detected in any of the tissue types. Somatostatin and PP were labeled in order to find gamma and delta cells but only a weak somatostatin signal was detected in a couple of autoantibody positive sections (Figure 4). No signal was detected in any control or T1D tissues. Moreover, insulin was labeled simultaneously with somatostatin, but the found somatostatin signal did not localize in similar areas with insulin signal as glucagon did (Figure 3).

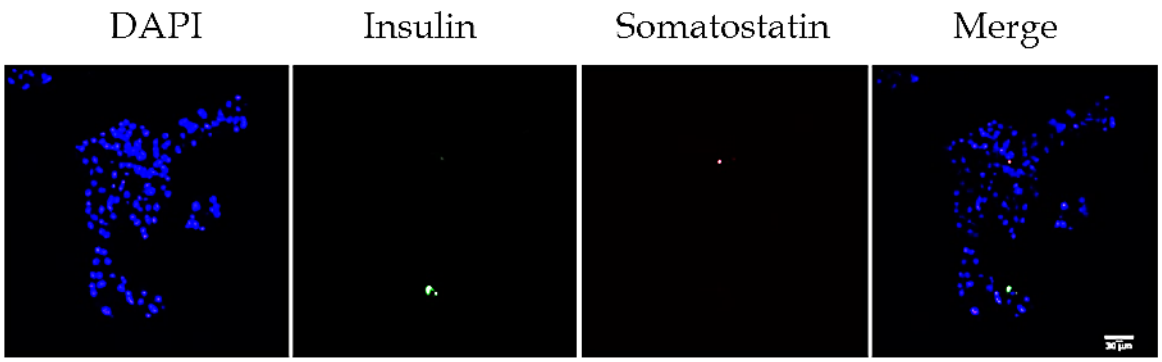

Figure 4. Somatostatin in semithin sections of autoantibody positive pancreas. Insulin (green) was labeled simultaneously with somatostatin (red) which gave positive signal only in a few autoantibody tissue sections. Figures are representatives of 5 labeled Aab sections. Scale bars 30 µm.

When viral dsRNA and capsid with cell and granule markers were labeled, it was noted that the virus was not commonly detected. The control sample sections were negative for virus, which was expected since the control samples were non-infected. This also indicated that the dsRNA and capsid backgrounds were insignificant. Virus capsid was labeled simultaneously with insulin (Figure 5). Only one autoantibody positive and one T1D sample sections gave significantly high signal of colocalization indicating that a few infected beta cells are located in the tissue.

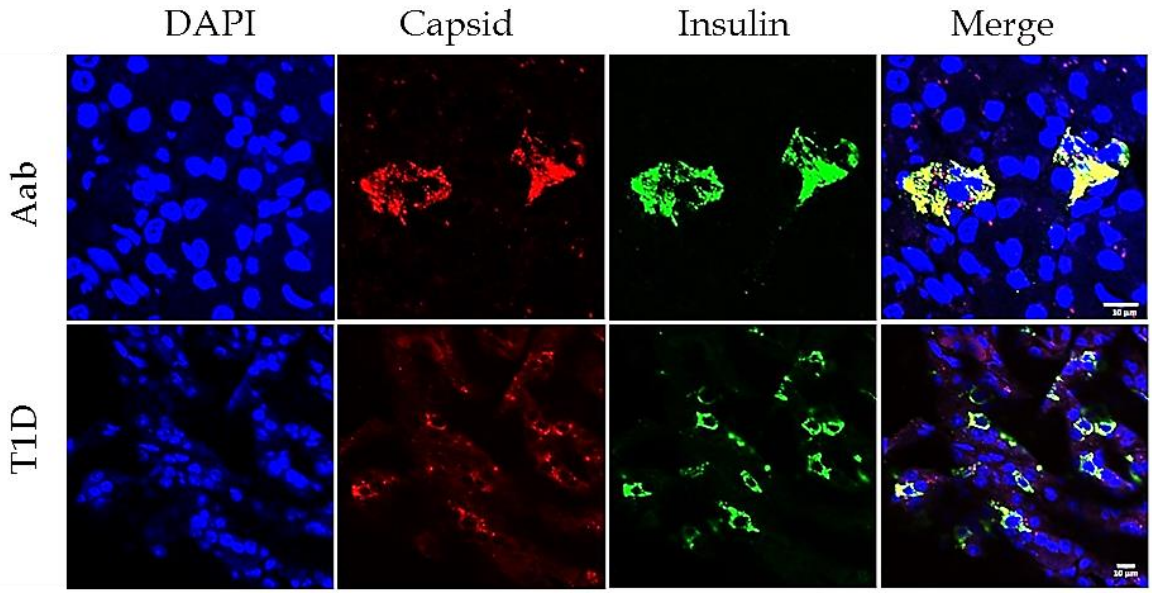

Figure 5. Insulin and virus capsid colocalization in semithin sections of pancreas. Fluorescently labeled control, autoantibody and T1D samples were imaged with confocal microscope. Significant capsid (red) and insulin (green) colocalization was found in one sample of autoantibody and T1D tissues. Figures are representatives of 5 labeled Aab sections and 10 labeled T1D sections. Scale bars 10 µm.

Viral dsRNA was labeled together with insulin and cytokeratin 19. Most of the sections were virus free but insulin and dsRNA showed significant colocalization in a few autoantibody positive and T1D tissues (Figure 6). Cytokeratin 19 was located mostly around the colocalized insulin and dsRNA but gave also a faint signal in those areas.

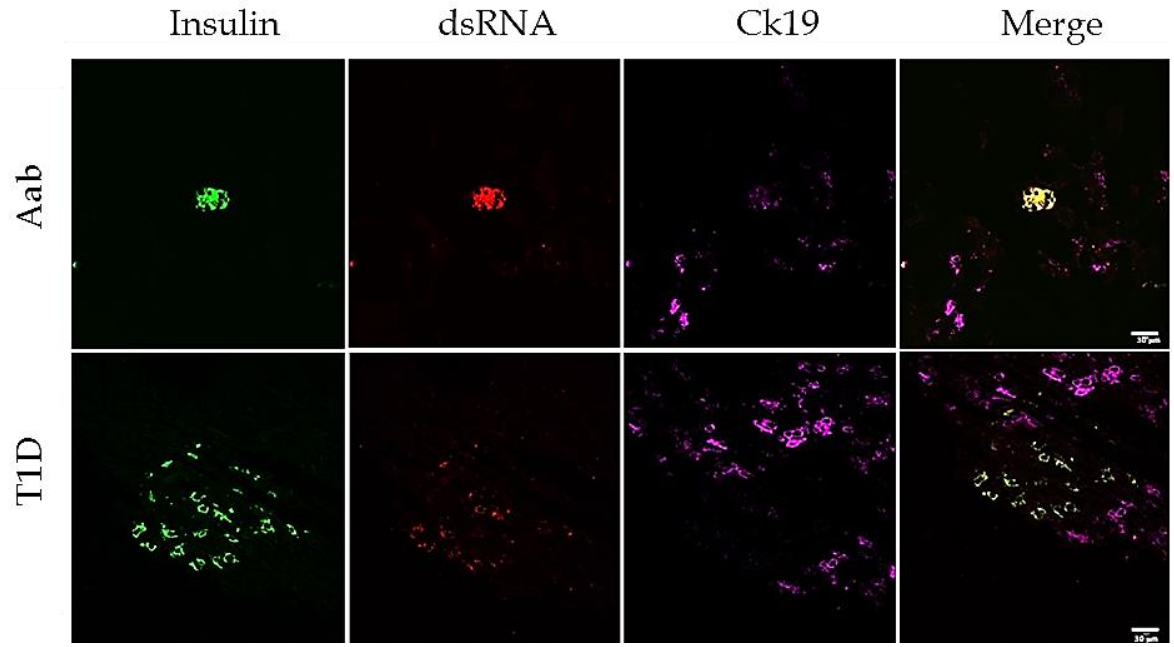

Figure 6. Insulin and viral dsRNA colocalization in semithin sections of pancreas. Fluorescently labeled control, autoantibody and T1D samples were imaged with confocal microscope. Significant insulin (green) and capsid (red) colocalization was found in a few samples of autoantibody and T1D tissues. Cytokeratin 19 (purple) was present in exocrine tissue around the insulin islets. Figures are representatives of 4 labeled Aab sections and 11 labeled T1D sections. Scale bars 30 µm.

3.2 Optimization of the PAG labeling in Tokyuasu's technique

The samples were then cut into thin sections and labeled according to the Tokyuasu's immuno–EM technique. The work was started with the optimization of the dilutions for the antibodies and PAG. The smallest PAG (5 nm) was causing significantly higher background than 10 and 20 nm particles. The PAG labeling order was changed to start with the 20 nm PAG and end with the 5 nm PAG. However, this did not sufficiently lower the background. Next we tested if an additional 1% glutaraldehyde wash at the start of the labeling protocol would reduce unspecific binding. This extra wash lowered notably the background of the 5 nm PAG but it stayed still a bit higher than the signals of the other PAG-particles

(Figure 7). Despite of the background differences the dilutions were accepted and 1% glutaraldehyde wash was added to the protocol.

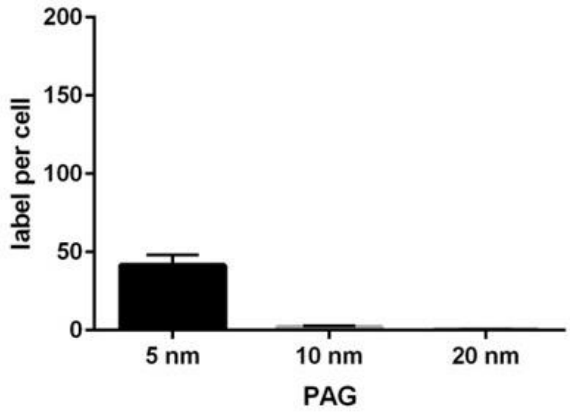

Figure 7. PAG background control. Thin sections of the pancreas were labeled only with PAG particles to observe the background noise and adjust the right dilutions. After finding the good level, the average PAG sum was calculated from 11 cells (4 endocrine and 7 exocrine cells) with standard error of means.

Isotype control antibodies were used to estimate the background of the labelings in the tissues and reduce it to the minimum (Figure 8). Especially, it was focused on isotype control labelings of virus capsid and dsRNA antibodies, which were impossible to separate from the background labels whereas granule markers were presumed to locate only in the granules. Finally, the isotype background level was adjusted low enough in all of the tissue types. However, still some low background was observed especially in the nuclei but it was considered low enough for the actual labelings.

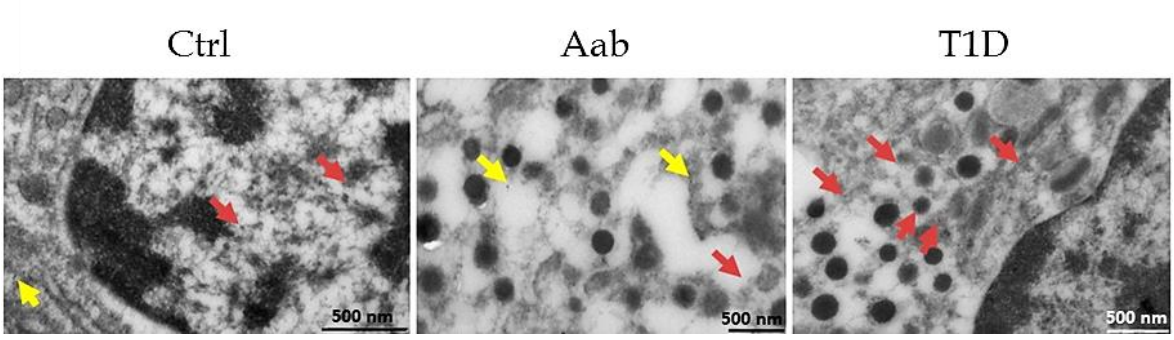

Figure 8. dsRNA, capsid and glucagon isotype control antibody labelings. Thin sections of control, autoantibody and T1D tissues of pancreas were labeled with isotype controls of the used antibodies (dsRNA (red), capsid (yellow)) to find the right dilutions. Here, some background label is observed in the nucleus (Ctrl) and in the cytoplasm (Aab, T1D). Figures are representatives of three isotype labelings. Scale bars 500 nm.

3.3 Electron microscopy

Thin sections of the human pancreas were double and triple labeled against different cell, granule and virus marker combinations for the EM imaging. First, the overall tissue architecture of the pancreas was evaluated (Figure 9). All of the tissue types were dominated by exocrine cells whereas the endocrine islets were located in between here and there. In the control tissue, the endocrine and exocrine areas were easily distinguished but autoantibody positive and T1D tissue seemed more ruptured and broken down. The membranous structure of the endoplasmic reticulum was spread apart in many of the cells. Cell junctions were loosened up, cells were ruptured and hormone granules were escaped from the cytoplasm, which made it sometimes difficult to separate the individual cells.

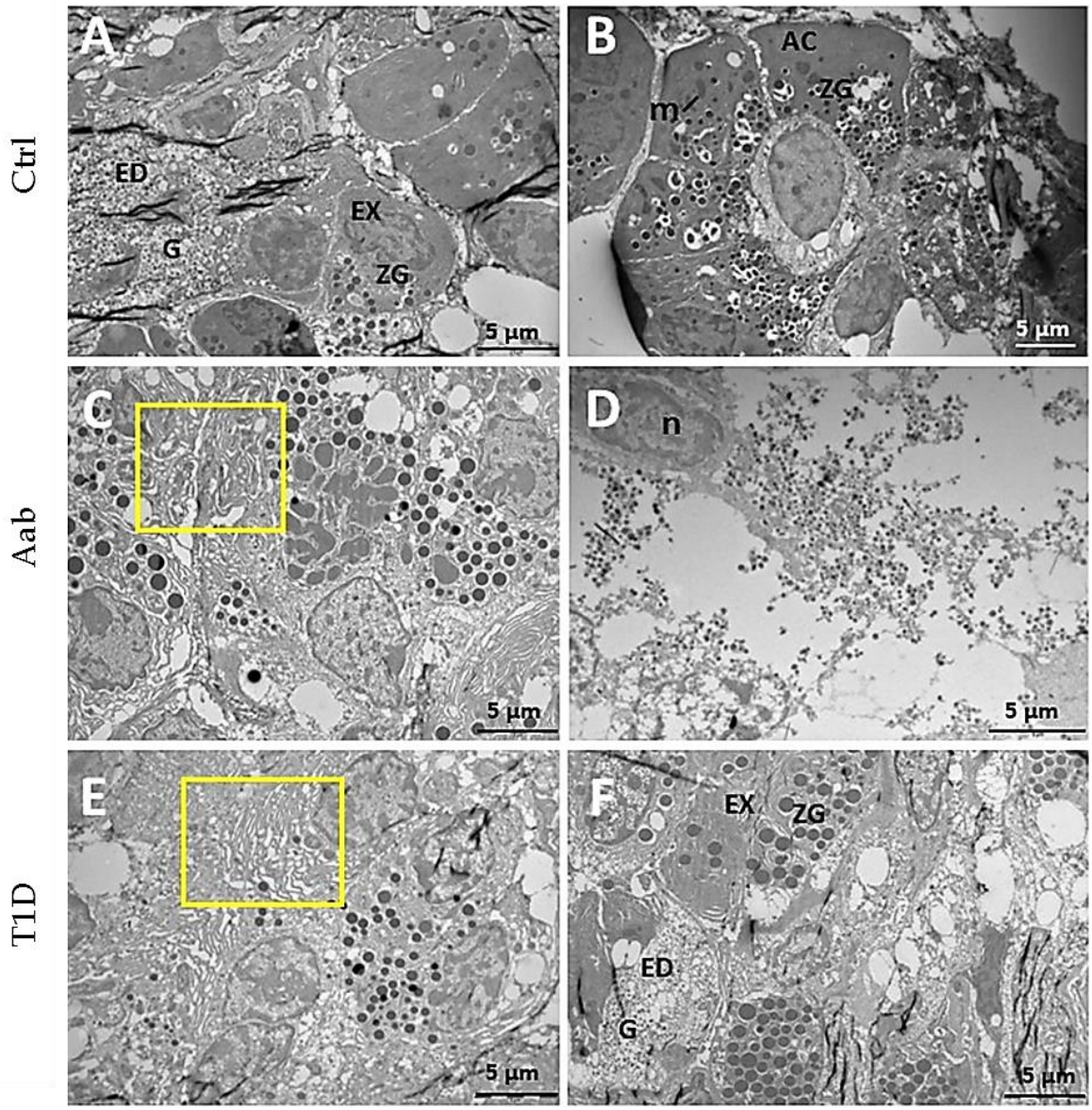

Figure 9. Observation of the human pancreas thin sections by EM. A, B) The presentation of the control tissue. Endocrine (ED) and exocrine (EX) cells are located side by side and distinguished by the granules (G) and zymogen granules (ZG). Acinar cells (AC) are forming the acini. Plenty of mitochondria (m) are present. C, E) Autoantibody and T1D tissues are more broken down than the control tissue. With the yellow boxes are highlighted the dilated ERs. D) Granules are spread around the nucleus (n) of a broken endocrine cell of Aab tissue. F) The location of the endocrine and exocrine cells of the T1D tissue. Scale bars 5 µm.

The original plan was to calculate the viral dsRNA and capsid signals per beta or alfa cell from all of the tissue types. However, the shortage of the cells, which were

labeled for the virus and insulin or glucagon, left the data low in cell number (Figure 10). Other problem was the size difference of the cells. Larger cells had inevitably more labels than the smaller cells despite of the infection level. Therefore, the absolute gold numbers per cell could not be directly compared. However, the ratio of different gold particles and antibodies within those areas could still be compared. This was taken into the consideration and the calculation method was changed to the labels per image area. The images were taken with the same magnification and therefore the calculated areas were similar size and comparable.

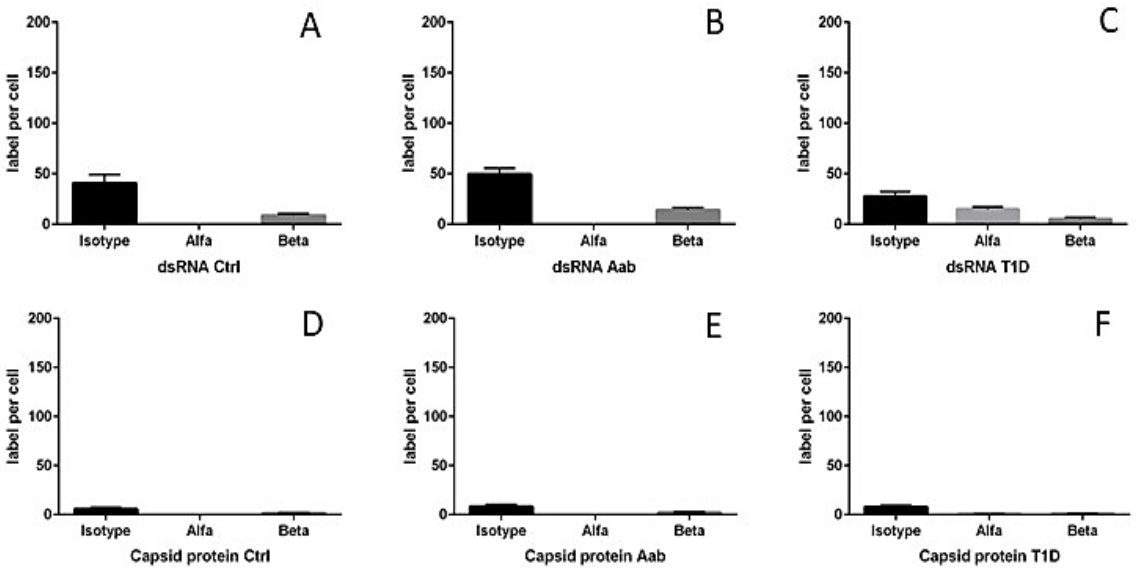

Figure 10. The average gold counts of viral antibodies from different cell types. The mean values of viral dsRNA (5nm PAG) and capsid protein (10 nm PAG) labels in alfa and beta cells from control, Aab positive and T1D tissues were calculated with standard error of means. The isotype controls of dsRNA and capsid protein were added as a comparison. A, D) From control tissue, 9 isotype controls and 16 beta cells were calculated (the amount of alfa cells was poor). B, E) From Aab positive tissue, 5 isotype controls and 7 beta cells were calculated (the amount of alfa cells was poor). C, F) From T1D tissue, 7 isotype controls, 19 alfa cells and 6 beta cells were calculated.

3.3.1 Electron microscopy of control cells

The incidence of the different cell types varied. In the control samples the alfa and beta cells were detected easily (Figure 11). Often those cells were located in the areas where the hormone granules stood out from the exocrine tissue. The cell markers were needed to distinguish the endocrine cells from each other. The alfa and beta cells could be found located side by side but the insulin and glucagon granules were mostly similar in their morphology. However, in some cases, the insulin granules were circled by white halo structures and in the other cases, both insulin and glucagon granules were possibly found inside of the same cell (Figure 11B). The beta cells also gave clear signal for the proinsulin. When proinsulin was labeled coincidently with insulin (Figure 11C) the labels could be seen located in the same granules but also separately. In a couple control sample sections somatostatin markers were localized in a few granules but only one clear delta cell was distinguished (Figure 11D). Simultaneously with somatostatin was labeled glucagon, which did not give any signal in the granules indicating that the cell was not an alfa cell. Cytokeratin 19 was localized in the thin filaments inside of the exocrine cells as expected. However, the marker signal was weak and therefore the filament identification was mainly done by eye. Despite of the ambitious attempts the PP was not found in any of the tissue types.

The control tissue was virus negative as expected. In Figures AB, only background dsRNA labels are located in the cytoplasm. Also, according to the isotype control labelings (Figure 8), dsRNA was showing little background level. In addition, dsRNA was labeled with 5 nm PAG which gave also significant background (Figure 7).

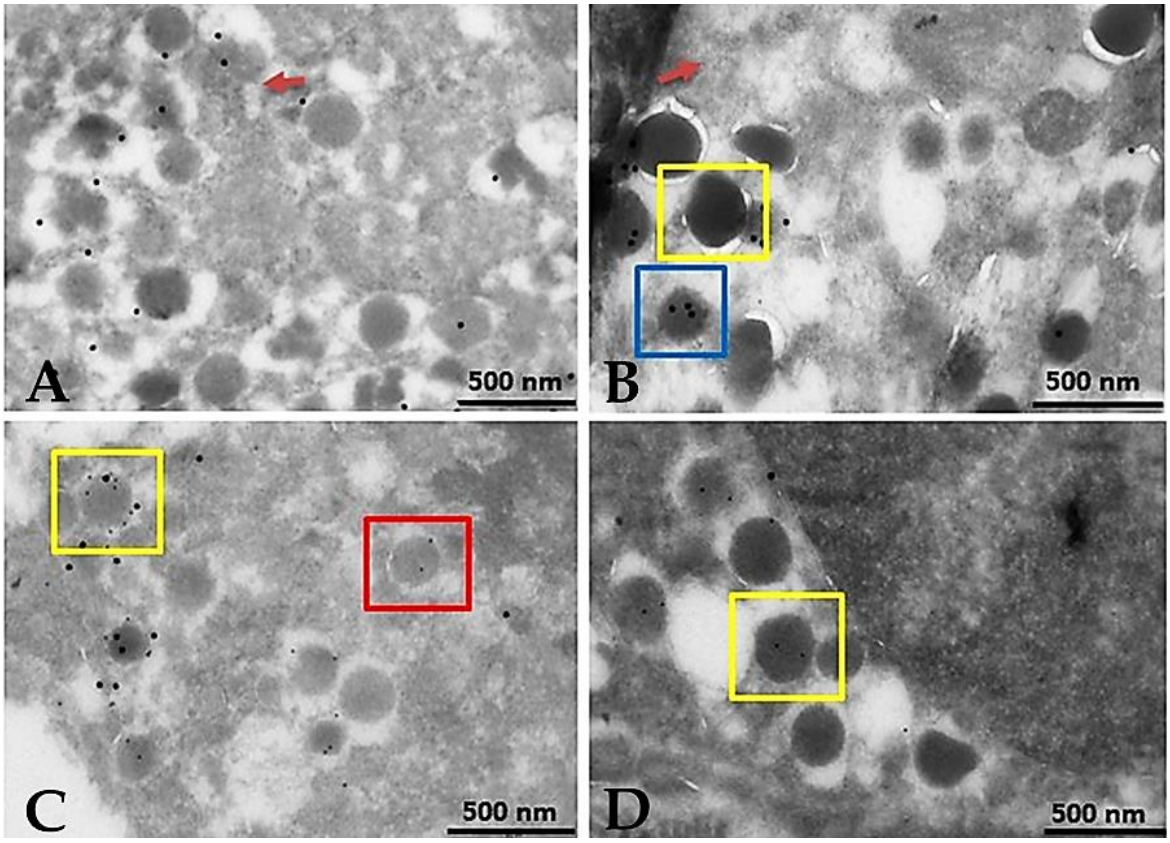

Figure 11. Control human pancreas tissue imaged with EM. A) Presentation of a beta cell labeled for dsRNA (5 nm PAG), viral capsid (10 nm PAG) and insulin (20 nm PAG). B) Presentation of an alfa cell labeled for dsRNA (5 nm PAG), viral capsid (10 nm PAG) and glucagon (20 nm PAG). With the blue box is highlighted a glucagon granule. With the yellow box is highlighted a label free granule with the white halo. C) The location of proinsulin and insulin. Proinsulin (10 nm PAG) and insulin (20 nm PAG) are located in the same granules (highlighted with the yellow box) or separately (granule with proinsulin highlighted with the red box) D) Presentation of a delta cell. Somatostatin (10 nm PAG) is located in a few granules (highlighted with the yellow box). Glucagon (20 nm PAG) was labeled simultaneously. Scale bars 500 nm.

3.3.2 Electron microscopy of autoantibody positive cells

In the Aab positive samples, alfa and beta cells were found with similar frequency as in the control tissue. However, in some sections, the cells were broken down and therefore the cell outlines were difficult to distinguish. Despite of a few virus positive areas found in semithin sections (Figures 5 and 6), virus signal was low in thin Aab sections (Figures 12AB). The results from 30 images revealed that the

autoantibody positive beta cells gave no different virus signal than the control sample (Table 4). Interestingly, when one 6197-01 (Pancreas body) sample was labeled together for glucagon, virus capsid and PP, it gave significantly elevated glucagon and capsid signal in one area compared to the surrounding cells (Figure 12CD). Distinguished with the glucagon granules the area was most likely a broken down alfa cell. Because the elevated signal was focused on one particular area, it did not seem to be background noise or gold particle interference. This sample was cut deeper into thin sections in order to find more virus positive areas but no signal from virus labels was found. Cytokeratin 19 filaments were localized inside of the exocrine cells but no gamma or delta cells were found in the autoantibody positive tissue.

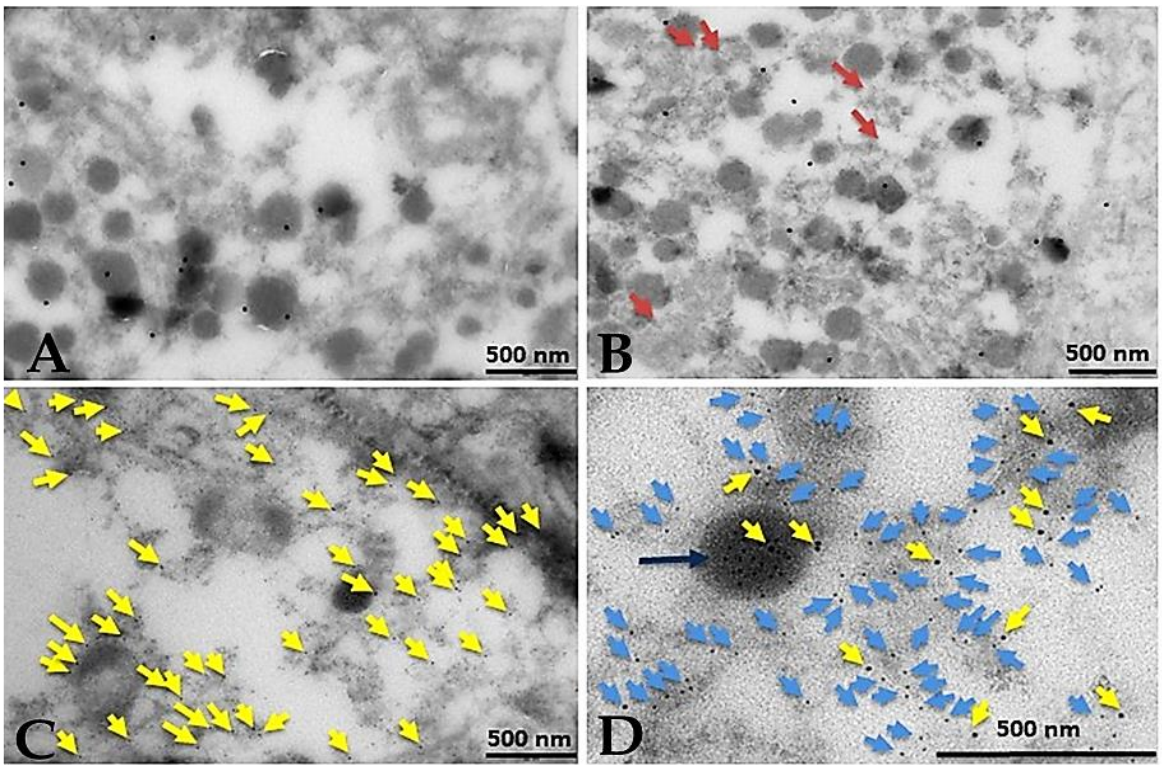

Figure 12. Autoantibody positive human pancreas tissue imaged with EM. A) Presentation of a beta cell labeled for dsRNA (5 nm PAG), viral capsid (10 nm PAG) and insulin (20 nm PAG). B) Presentation of a beta cell labeled for dsRNA (5 nm PAG), viral capsid (10 nm PAG) and insulin (20 nm PAG). C, D) Virus positive area. Glucagon (5 nm PAG), virus capsid (10 nm PAG) and PP (20 nm PAG) were labeled in the same sample and glucagon (blue) and capsid (yellow) gave elevated

signal in the cytoplasm in one area. Glucagon labels were also concentrated in the granules (dark blue arrow). Scale bars 500 nm.

Table 4. T test comparing viral dsRNA and capsid labels in 30 set of images from beta cells of control, autoantibody positive and T1D tissues.

t test	T1D vs Ctrl	T1D vs Aab	Aab vs Ctrl
dsRNA	3.59E-09	1,38E-09	0,163
Capsid protein	1.09E-05	8,23E-06	0,323

3.3.3 Electron microscopy of T1D cells

In the T1D samples, alfa cells were found with similar frequency as in control and autoantibody positive tissues. Beta cells in turn were difficult to find. With the help of the fluorescent labeling of the semithin sections some beta cells were able to be detected also with EM. In addition, significant insulin and viral dsRNA colocalization was found in the 6362-01 (Pancreas body) semithin sample, which was then cut into thin sections for EM. Finally, beta cells with clear elevated viral dsRNA and capsid signal were found in the cytoplasm and associated with the granules (Figure 13B). Quantification of the 30 images showed that virus signal was significantly elevated in T1D beta cells compared to the autoantibody positive and control beta cells (Table 4). Interestingly, in those cells the insulin signal was lower in the insulin granules compared to the beta cells of control samples (Figures 11A) and virus negative T1D samples (Figure 13A). Similarly, the insulin positivity was lower in the T1D samples than in control samples (Figure 14D) meaning that fewer insulin labels were attached to granules (Figure 13B). Instead

the insulin signal was found elevated also in the cytoplasm. When proinsulin was labeled coincidently with insulin (Figure 13CD) the labels located in the same granules but also in insulin granules alone. Insulin label was also located in the cytoplasm outside of the granules. One T1D sample, 6212-01 (Pancreas body), had also particularly many immune cells, which were rare in the other T1D or autoantibody tissues and not found at all in control tissues.

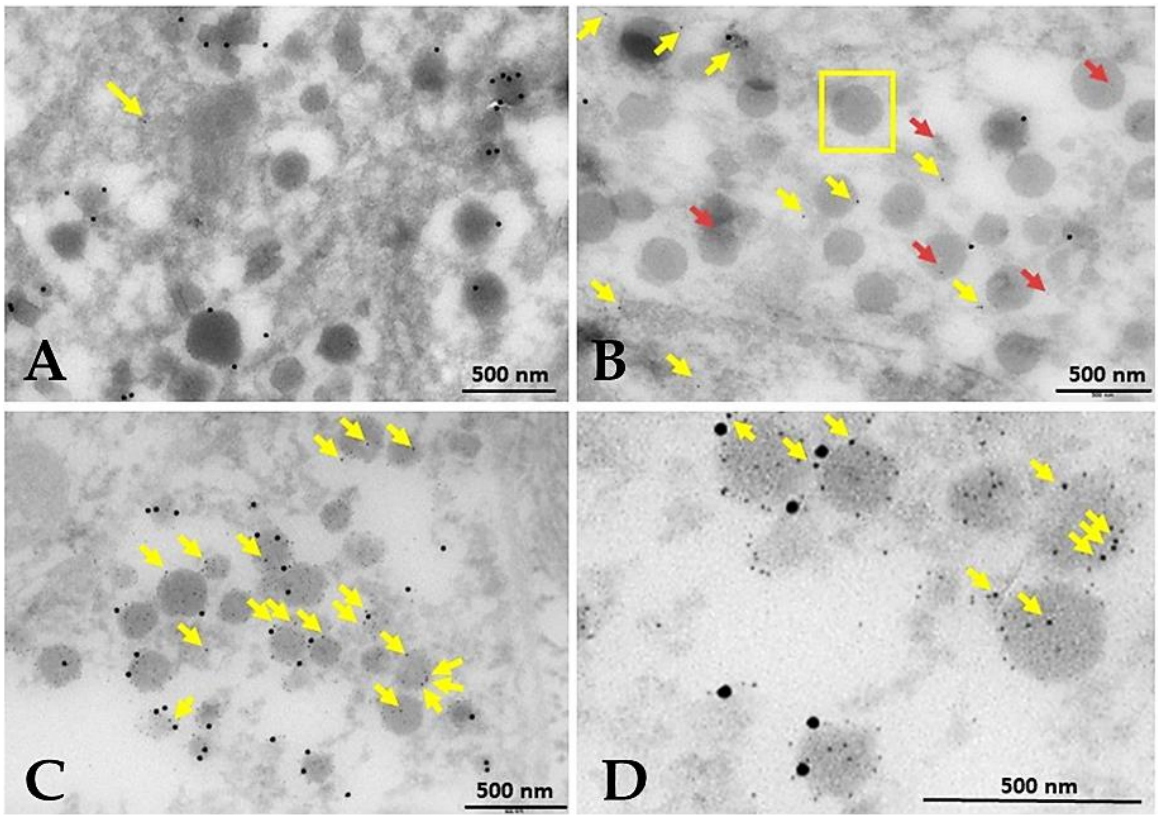

Figure 13. T1D human pancreas tissue imaged with EM. A) Presentation of a virus free beta cell labeled for dsRNA (5 nm PAG), viral capsid (10 nm PAG, yellow) and insulin (20 nm PAG). B) Presentation of a virus positive beta cell labeled for dsRNA (5 nm PAG, red), viral capsid (10 nm PAG, yellow) and insulin (20 nm PAG). Insulin marker free granules are common (yellow box). C, D) The location of proinsulin and insulin. Insulin (5 nm PAG), virus capsid (10 nm PAG) and proinsulin (20 nm PAG) were labeled simultaneously. Capsid (yellow) gave positive signal in the cytoplasm and granules. Scale bars 500 nm.

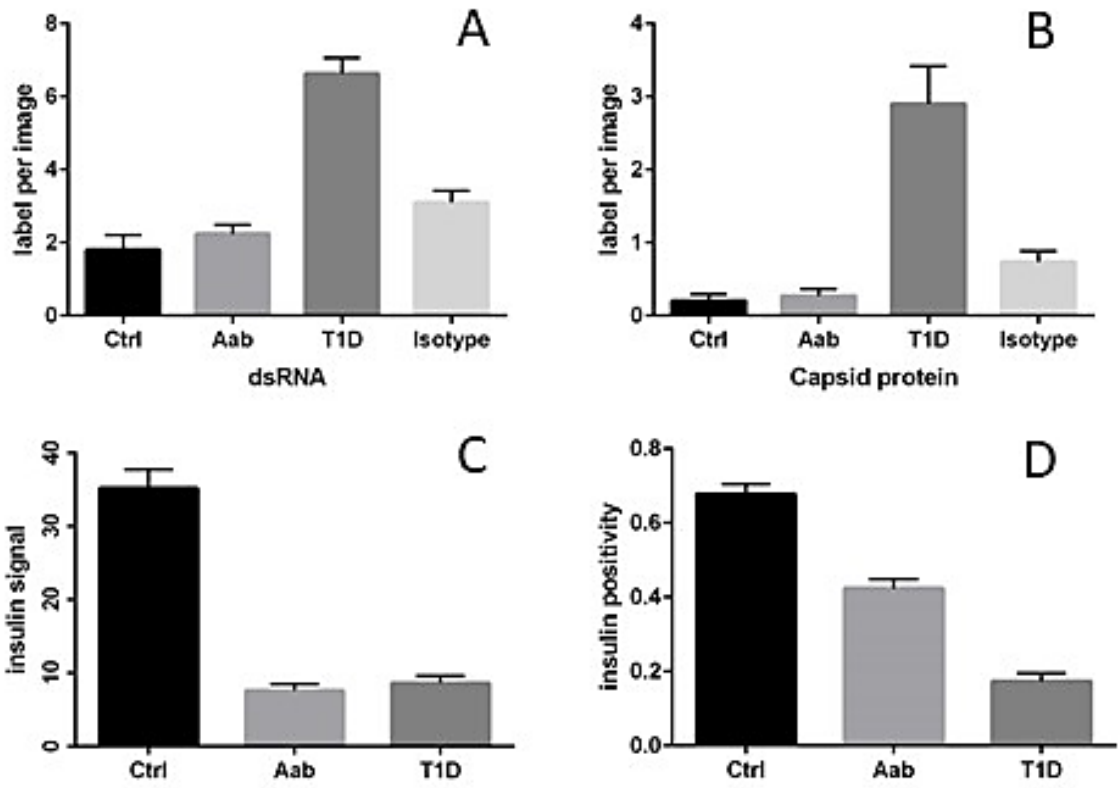

Figure 14. Mean values of virus signals and insulin of beta cells per image. A, B) Viral dsRNA (5nm PAG) and capsid protein (10 nm PAG) labels in beta cells from control, Aab positive and T1D tissues were calculated from 30 images. The results show average values with standard error of means. The isotype controls of the labels were added as a comparison. C) The average insulin signal per image was calculated from cytoplasm, cell organelles and granules with standard error of means. D) The average insulin positivity was calculated comparing the granules with insulin label to the total amount of the granules per image with standard error of means.

4 DISCUSSION

T1D is chronic autoimmune mediated disease caused by destruction of the insulin producing beta cells in the pancreas (American Diabetes Association 2014). Insulin production is therefore decreased causing the increase of glucose concentration in the blood. This is associated with long-term damage and even failure of different

organs, requiring the patient to be engaged with lifelong medical treatment. The trigger of the development of T1D can be genetic or it can be caused by environmental factors (Richardson and Morgan 2018). Recently, the involvement of the human enteroviruses has been confirmed by various studies (Reviewed by Stene and Rewers 2011). However, detection of the infection has been difficult, as virus load seems to be low and found only in insulin containing islets. Furthermore, those islets are lost when the disease proceeds. In this thesis, it was tested whether enteroviruses can be detected with a sensitive immuno-EM imaging technique in *post-mortem* human pancreas samples, which were confirmed positive for viral capsid proteins or RNA by other methods such as immunihistochemistry or PCR. The results showed that, indeed, virus was found in some of the diabetic samples while control samples were negative for virus infection.

The examination of the pancreas micrographs demonstrated that the incidence of the different pancreatic cell types varies. The tissue was dominated by exocrine cells whereas the endocrine islets were low in number. Indeed, it is referred that islets can occupy 1-2% of the pancreatic volume in humans (Seino and Eds; Powers 2014). Exocrine cells were indicated with cytokeratin 19 markers and with the presence of zymogen granules that were easily distinguished without further labeling. Cytokeratin 19 is absent from islet cells but especially characteristic for the ductal cells (Bouwens 1998). For EM, the marker signal was weak making the identification difficult and impractical but for fluorescent labeling, on the contrary, cytokeratin 19 was optimal for highlighting the location of the exocrine areas. What comes to pancreatic islets, our observations showed that alfa and beta cells are the most common cells. We noted that the morphology of insulin and glucagon granules is mostly similar but insulin granules can also be circled by white halos. The halo structures have been also noted in earlier studies in rodents (Klumperman *et al.* 1998; Mohamed *et al.* 2018) where they have been even more

characteristic for the insulin granules. Tokyuasu's immune-EM method does not require typical osmium or lead citrate staining as Epon embedded samples but instead, the contrast is enhanced with uranyl acetate (Mercer 1963). The staining technique affects to the intensity of image contrast thus making the halo structures possibly more notable in other examinations. In some cases, alfa and beta cells were found located side by side which is also documented in research of Mohamed *et al* (2018). More interestingly in some areas, both insulin and glucagon granules were possibly found inside of the same cell. This is also observed by some other groups (Thorel *et al.* 2010; Spijker *et al.* 2013; Mezza *et al.* 2014) when they studied Type 2 Diabetes. The presence of endocrine cells containing both glucagon and insulin granules may be due to the conversion of couple beta cells to alpha cells or vice versa (Mohamed *et al.* 2018). In addition, zymogen granules and a few insulin or glucagon granules have been reported to locate in the same cell in human pancreas (Masini *et al.* 2020). This proposes that beta cells may regenerate from exocrine cells after destruction.

Control and autoantibody positive tissues were abundant with alfa and beta cells but in diabetic tissue, beta cells were rare. This is in line with the characteristics of the T1D: insulin producing beta cells are gradually destroyed. By directing first the positive areas with fluorescent labeling, some beta cells could be also detected with EM. The diabetic tissue is not necessarily totally absent of beta cells and the presence is also noted in earlier studies (Gepts 1965; Gepts and Mey 1978; Butler *et al.* 2007). Nevertheless, the number of beta cells decreases as the duration of the disease increases. Studies on T1D patients with recent onsets showed that the beta cell count is only 10% of normal (Gepts 1965; Butler *et al.* 2007). When the disease is prolonged, the amount of the beta cells is approaching to zero. This could also be seen in our study when observing the donors of the examined tissues. The only diabetic tissue positive for beta cells was 6362-01 which was donated by patient who had suffered from T1D less than a year. The other tissues were from patients

who had had T1D diagnosis for five years. Other interesting aspect was demonstrated by Gepts and Mey in 1978 as the beta cells of T1D patients were difficult to identify with the immunocytochemical method because of the degranulation. With Tokyuasu's method, the identification of the pancreatic cells was based on the granules, which therefore left the possible insulin granule free beta cells without observation. We noticed that in some beta cells of T1D tissue, the insulin signal and positivity were decreased in granules but instead, the markers were found elevated in the cytoplasm. It might be possible that the insulin had escaped from the granules to the cytoplasm or that insulin was not packaged into the granules as normal. This could be a sign of a beta cell dysfunction caused by the presence of the virus.

We also labeled proinsulin coincidently with insulin. The labels located in beta cells in the same granules but also granules with either insulin or proinsulin alone were detected. What comes to earlier studies, Orci *et al.* (1986); Klumperman *et al.* (1998) had the same observation in rodents and Ifie *et al.* (2018) in human samples. The maturation of proinsulin to insulin occurs inside of a granule after acidification, which explains the possible simultaneous presence of the hormones. In some cases when only the insulin was labeled, beta cells had also empty granules among insulin positive granules. Were those granules empty or were they immature filled with proinsulin? Similarly, if the observed tissue had a cell with granules without any label it left us wondering the identity of that cell. We could not label all interesting granule and viral marker combinations in the same section, only up to three markers simultaneously. This is, however, the great advantage of Tokyuasu's technique in comparison to other immuno-EM methods. In theory, more different sized gold particles could have been introduced to the tissues simultaneously but the labeling protocol would have then streched easily over twelve hours and the availability of the different sized protein A gold particles is somewhat limited.

The other cell types of the pancreatic islets were found rarely in different sample types indicating that the gamma and theta cells are low in number making them difficult to locate. Cabrera *et al.* (2006) reviewed that based on various earlier studies, islets are composed of 70% beta cells, 20% alfa cells, 10% delta cells and 5% gamma cells. However, the number of beta cells is argued to be lower and the number of alfa cells higher (Seino and Eds; Brissova *et al.* 2005; Powers 2014). What comes to T1D tissue, Gepts and Mey (1978) examined pancreatic islets without any beta cells left with immunocytochemical technique and revealed that the proportional composition of other islet cells was the same: 2/3 of alfa cells and 1/3 of delta cells, with occasional gamma cells. In addition, different regions of the pancreas could have different islet cell composition (Gersell *et al.* 1978; Cabrera *et al.* 2006) which could have affected to our results, even though we had sections from both head and body of pancreas. Since the focus in this study was on alfa and beta cells, sections chosen for EM imaging were picked based on the incidence of insulin and glucagon. Therefore, fluorescent mapping of semithin sections was not comprehensive leaving the other cell types for less attention. However, some somatostatin signal was found with fluorescent imaging and with EM, one clear delta cell was distinguished along with occasional somatostatin granules. EM results also implied that delta cells would located near to beta cells, which raised a question if somatostatin and insulin hormones would have a close connection. Since the islet cells are regulating the hormone secretion of each other, they need to have a tight communicative network around them (Vergari *et al.* 2019). The communication is performed via cell-to-cell connection or via paracrine signaling which suggest that the arrangement of the different cell types is important (Brereton *et al.* 2015). However, Cabrera *et al.* (2006) concluded that beta, alfa and delta cells are distributed in no particular order in the islets and randomly organized side by side with blood vessels. Nevertheless, in the future, it would be

worthwhile to label insulin and somatostatin together, in order to examine the arrangement of the cells more.

Our main research question was if elevated enterovirus signal could be found in T1D tissue with Tokyuasu's method. However, majority of the cells showed no indication of virus infection. Only a few beta cells from Aab positive and T1D tissues gave significant virus signal compared to the surrounding cells. Virus was detected only in islets cells, not in exocrine cells. Likewise, in earlier T1D studies (Klingel *et al.* 2004; Oikarinen *et al.* 2008; Richardson *et al.* 2009) some pancreatic islets were stained positive for capsid protein VP1 or dsRNA while exocrine cells were negative. However, in all of these earlier studies the intensity of the positively stained islets was low. It can be assumed that the infection is not necessarily covering all insulin positive cells in the area, but damage even in some islets may interfere with the normal functions of the rest of the pancreas. On the other hand, acute infection could have occurred earlier simultaneously destroying majority of the beta cells, while currently when the disease is ongoing, the infection may have changed into low-grade persistent infection. Therefore, new virus particles are not actively produced explaining why virus is not easily detected in the cells.

In Aab tissue, one broken down alfa cell gave significantly elevated glucagon and capsid signal in one area compared to the surrounding cells. The amount of capsid labels in the cytoplasm and the cell lysis indicated that the infection could have been acute in that cell. In addition, Aab tissue is from a patient predicted to develop T1D, which supports the possibility of an ongoing acute virus infection. The alfa cell infection was also documented in T1D study by Oikarinen *et al.* (2008) when enterovirus was mainly found in alfa cells, whereas beta cells were typically negative. They discussed if alfa cells could survive from enterovirus infection while beta cells are destructed. Others, however, have argued if alfa cells stay

untouchable because they do not have the ideal receptors and host factors for the virus to utilize as beta cells (Reviewed by Morgan and Richardson 2014 and Ifie *et al.* 2018).

In the positive T1D tissue in our EM immunolabeling, the detected signal for virus dsRNA and capsid was low but significant suggesting that the infection was not necessarily acute but more persistent in nature. Bottazzo *et al.* (1985) noted that the studied T1D patient had low virus titer indicating past virus infection and they could not isolate any virus. It should also be discussed if the degradation of viral dsRNA during *post-mortem* changes effected to the results. Oikarinen *et al.* in 2008 faced the problem that the autopsy of the studied diabetic tissue was taken 3 days after death, and possibly pancreatic enzymes digested the RNA leaving virus proteins detectable. However, we demonstrated that viral dsRNA was found more commonly in the tissues than viral capsid protein, taken into the consideration that dsRNA was labeled with the 5 nm PAG which gave significant background according to the isotype controls. Some T1D tissues were also occupied by immune cells which are also noticed in studies conducted by Gepts (1965) and Bottazzo *et al.* (1985). Both found infiltrating lymphocytes in the diabetic pancreatic islets but especially Bottazzo *et al*. specified that majority of the immune cells were cytotoxic T-lymphocytes which are responsible for the autoimmune destruction of the beta cells.

Control samples were negative for virus, but some capsid and dsRNA labeled with 10 and 5 nm PAG were detected as a background signal. Methodologically we found more background with small gold particles than larger gold particles, which could have been solved by diluting further the small PAG sizes. On the other hand, the higher background could also be result of the labeling scheme because small particles were introduced first to the samples before numerous blocking steps. Therefore, modification of the scheme by adding an additional

blocking step before any antibody or gold particle addition clearly lowered the background to decent levels. In addition, the use of isotype antibody controls allowed us to evaluate the real background level, which was taken into account in the statistical testing. Virus detection with gold particles is not absolutely precise. It cannot be sure if an odd label in the tissue is virus or background gold. Some variation of the signal can come of course from small changes in antibody or gold concentrations or inadequate washings during the labeling. However, the method is still very quantifiable, and the possibility to use various controls, and performing the labelings with exact similar procedures, makes it reliable. In addition, PAG labeling is considered to be close to 1:1 binding to the antigen, since PAG can bind only one IgG molecule. In contrast to IgG-coated gold particles, which can bind to several sites of the antigen giving higher signal, PAG gives more realistic picture of the signal intensity.

Earlier the link between Type 1 diabetes and viruses has been examined with traditional antibody labeling techniques and PCR methodology on blood and stool samples. Here, we used the Tokyuasu's technique combining two imaging methods to detect virus on tissue samples. This technique requires hard work but can give reliable results with the help of control labelings. Different cell types can be easily separated but the infected areas are challenging to find due to their small size and low abundance. Based on our experience, the use of Tokyuasu's method should be continued in the future as well. However, it would be beneficial to use it in combination with more efficient methods to collect several islets together for EM sectioning, e.g. with modern laser capture methods.

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REFERENCES

American Diabetes Association. 2014. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*. 37: 581-590, doi: 10.2337/dc14-S081

Bergamin C.S. & Dib S.A. 2015. Enterovirus and type 1 diabetes : What is the matter? *World J Diabetes.* 6(6): 828–839, doi: 10.4239/wjd.v6.i6.828

Bottazzo G.F., Dean B.M., McNally J.M., MacKay E.H., Swift P.G.F. & Gamble R. 1985. *In situ* characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. *The New England Journal of Medicine*. 313(6): 353-360, doi: 10.1056/NEJM198508083130604

Bouwens L. 1998. Cytokeratins and cell differentiation in the pancreas. *Journal of Pathology*. 184: 239: 234–239, doi: 10.1002/(SICI)1096-9896(199803)184:3<234::AID-PATH28>3.0.CO;2-D

Brereton M.F., Vergari E., Zhang Q. & Clark A. 2015. Alpha- , Delta- and PP-cells : Are They the Architectural Cornerstones of Islet Structure and Co-ordination? *J Histochem Cytochem.* 63(8):575-91, doi: 10.1369/0022155415583535

Brissova M., Fowler M.J., Nicholson W.E., Chu A., Hirshberg B., Harlan D.M. & Powers A.C. 2005. Assessment of Human Pancreatic Islet Architecture and Composition by Laser Scanning Confocal Microscopy. *The Journal of Histochemistry & Cytochemistry*. 53(9): 1087–1097, doi: 10.1369/jhc.5C6684.2005

Butler A.E., Galasso R., Meier J.J. & Basu R. 2007. Modestly increased beta cell apoptosis but no increased beta cell replication in recent-onset type 1 diabetic patients who died of diabetic ketoacidosis. *Diabetologia*. 50:2323–2331.doi: 10.1007/s00125-007-0794-x

Cabrera O., Berman D.M., Kenyon N.S., Ricordi C., Berggren P. & Caicedo A. 2006. The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *PNAS*. 103(7):2334-2339, doi: 10.1073/pnas.0510790103

Cavalli-Sforza L.L. & Piazza A. 1993. Human Genomic Diversity in Europe : A Summary of Recent Research and Prospects for the Future. *Eur J Hum Genet.* 1(1):3-18, doi: 10.1159/000472383

Chehadeh W., Kerr-conte J., Pattou O.I.S., Alm G., Lefebvre J., Wattre P. & Hober D. 2000. Persistent Infection of Human Pancreatic Islets by Coxsackievirus B Is Associated with Alpha Interferon Synthesis in beta Cells. *J.Virol.* 74(21): 10153–10164, doi: 10.1128/jvi.74.21.10153-10164.2000

Concannon P., Erlich H.A., Julier C., Morahan G., Nerup J., Pociot F., Todd J.A., Rich S.S. & the Type 1 Diabetes Genetics Consortium. 2005. Type 1 Diabetes: Evidence for Susceptibility Loci from Four Genome-Wide Linkage Scans in 1,435 Multiplex Families. *Diabetes.* 54: 2995–3001, doi:

10.2337/diabetes.54.10.2995

Coppieters K.T. & von Herrath M.G. 2009. Histopathology of Type 1 Diabetes: Old Paradigms and New Insights. *The Review of Diabetic Studies*. 6(2): 85-96, doi: 10.1900/RDS.2009.6.85

Cordey S., Gerlach D., Junier T., Zdobnov E.M., Kaiser L. & Tapparel C. 2008. The cis -acting replication elements define human enterovirus and rhinovirus species. *RNA*. 14:1568–1578.

Cunningham L., Bowles N.E., Lane R.J.M., Dubowitz V. & Archard L.C. 1990. Persistence of enteroviral RNA in chronic fatigue syndrome is associated with the abnormal production of equal amounts of positive and negative strands of enteroviral RNA. *Journal of General Virology*. 71: 1399-1402

Ellis C., Ramzy A. & Kieffer T.J. 2017. Regenerative medicine and cell-based approaches to restore pancreatic function. *Nat Rev Gastroenterol Hepatol.* 14(10):612-628, doi: doi:10.1038/nrgastro.2017.93

Filippi G.M.& von Herrath M.G. 2008. Viral Trigger for Type 1 Diabetes: Pros and Cons. *Diabetes*. 57: 2863-2871, doi: 10.2337/db07-1023

Flodström-Tullberg M., Hultcrantz M., Maday A., Tsai D., Fine C., Silverman R., Sarvetnick N., Hultcrantz M., Stotland A., Maday A., Tsai D., Fine C., Williams B., Silverman R. & Sarvetnick N. 2019. RNase L and Double-Stranded RNA-Dependent Protein Kinase Exert Complementary Roles in Islet Cell Defense during Coxsackievirus Infection. *J Immunol*. 174(3):1171-1177, doi: 10.4049/jimmunol.174.3.1171

Flodström M., Tsai D., Fine C., Maday A. & Sarvetnick N. 2003. Diabetogenic Potential of Human Pathogens Uncovered in Experimentally Permissive β-Cells. Pancreatic. *Diabetes*. 52: 2025-2034, doi: 10.2337/diabetes.52.8.2025

Frisk G., Friman G., Tuvemo T., Fohlman J. & Diderholln H. 1992. Coxsackie B virus IgM in children at onset of Type 1 (insulin-dependent) diabetes mellitus: evidence for IgM induction by a recent or current infection. *Diabetologia.* 35:249-253, doi: 10.1007/bf00400925

Gamble D.R., Kinsley M.L., Fitzgerald M.G., Bolton R. & Taylor K.W. 1969. Viral antibodies in diabetes mellitus. *British Medical Journal.* 3: 627-630, doi: 10.1136/bmj.3.5671.627

Gepts W. 1965. Pathologic Anatomy of the Pancreas in Juvenile Diabetes Mellitus. *Diabetes*. (10):619-33, doi: 10.2337/diab.14.10.619

Gepts W. & Mey J. De. 1978. Islet Cell Survival Determined by Morphology An Immunocytochemical Study of the Islets of Langerhans in Juvenile Diabetes Mellitus. *Diabetes.* 27(1): 251-261, doi: 10.2337/diab.27.1.S251

Gersell D.J., Gingerich R.L. & Greider M.H. 1978. Regional Distribution and Concentration of Pancreatic Polypeptide in the Human and Canine Pancreas. *Diabetes.* 28(1): 11–15, doi: 10.2337/diabetes.28.1.11

Green J., Casabonne D. & Newton R. 2004. Coxsackie B virus serology and Type 1 diabetes mellitus : a systematic review of published case-control studies. *Diabet Med.* 21(6):507-514, doi: 10.1111/j.1464-5491.2004.01182.x

Green A., Patterson C.C. & Study T. 2001. Trends in the incidence of childhood-onset diabetes in Europe 1989 - 1998. *Diabetologia*. 44(3):B3-B8, doi: 10.1007/pl00002950

Harjutsalo V., Sjöberg L. & Tuomilehto J. 2008. Time trends in the incidence of type 1 diabetes in Finnish children : a cohort study. *Lancet*. 371(9626): 1777–1782, doi: 10.1016/S0140-6736(08)60765-5.

Harris K.G. & Coyne C.B. 2015. Death waits for no man – Does it wait for a virus? How Enteroviruses Induce and Control Cell Death. *Cytokine Growth Factor Rev*. 25(5): 587–596. doi:10.1016/j.cytogfr.2014.08.002

Hober D., Sané F., Riedweg K., Moumna I., Goffard A., Choteau L., Alidjinou E.K. & Desailloud R. 2013. Viruses and Type 1 Diabetes: Focus on the Enteroviruses. In: Escher A. (ed.), Type 1 Diabetes, pp. 25-69, doi: 10.5772/52087

Hyöty H. 2002. Enterovirus infections and type 1 diabetes. *Ann Med*. 34: 138-147: doi: 10.1080/713782135

Hyöty H. & Taylor K.W. 2002. The role of viruses in human diabetes. *Diabetologia*. 45: 1353–1361, doi:10.1007/s00125-002-0852-3

Hyttinen V., Kaprio J., Kinnunen L., Koskenvuo M. & Tuomilehto J. 2003. Genetic Liability of Type 1 Diabetes and the Onset Age Among 22,650 Young Finnish Twin Pairs: A Nationwide Follow-Up Study. *Diabetes*. 52(4):1052-1055, doi: 10.2337/diabetes.52.4.1052

ICTV Virus Taxonomy 2017. Picornaviridae. https://talk.ictvonline.org/ (accessed on 17.6.2019)

Ifie E., Russell M.A., Dhayal S., Leete P., Sebastiani G., Nigi L., Dotta F., Marjomäki V., Eizirik D.L., Morgan N.G. & Richardson S.J. 2018. Unexpected subcellular distribution of a specific isoform of the Coxsackie and adenovirus receptor , CAR-SIV, in human pancreatic beta cells. *Diabetologia*. 61:2344–2355, doi: 10.1007/s00125-018-4704-1

Jackson W.T. & Coyne C.B. 2018. *Enteroviruses: Omics, Molecular Biology, and Control*. Caister Academic Press, Norfolk, UK.

Jiang P., Liu Y., Ma H.-C., Paul A. V. & Wimmer E. 2014. Picornavirus Morphogenesis. *Microbiology and Molecular Biology Reviews*. 78: 418–437, doi:10.1128/MMBR.00012-14

Jones D.B. & Crosby I. 1996. Proliferative lymphocyte responses to virus antigens homologous to GAD65 in IDDM. *Diabetologia*. 39: 1318–1324, doi: 10.1007/s001250050576

Juhela S., Hyöty H., Roivainen M., Härkönen T., Putto-laurila A., Simell O. & Ilonen J. 2000. T-Cell Responses to Enterovirus Antigens in Children With Type 1 Diabetes. *Diabetes* 49:1308–1313

Jun H.S. & Yoon J.W. 2001. The role of viruses in Type I diabetes : two distinct cellular and molecular pathogenic mechanisms of virus-induced diabetes in animals. *Diabetologia*. 44(3): 271-285, doi: 10.1007/s001250051614

Kaprio J., Tuomilehto J., Koskenvuo M., Romanov K., Reunanen A., Eriksson J., Stenghrd J. & Kesäniemi Y.A. 1992. Concordance for Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland. *Diabetologia*. 35: 1060-1067, doi: 10.1007/bf02221682

Kempf B.J., Kelly M.M., Springer C.L., Peersen O.B. & Barton J. 2013. Structural Features of a Picornavirus Polymerase Involved in the Polyadenylation of Viral RNA. *J Virol*. 87(10): 5629–5644, doi: 10.1128/JVI.02590-12

Klingel P.Y.K., Otonkoski A.M.L.T. & Hovi R.K.T. 2004. Enterovirus infection in human pancreatic islet cells , islet tropism in vivo and receptor involvement in cultured islet beta cells. *Diabetologia*. 47(2):225-239, doi: 10.1007/s00125-003-1297-z

Klumperman J., Kuliawat R., Griffith J.M., Geuze H.J. & Arvan P. 1998. Mannose

6–Phosphate Receptors Are Sorted from Immature Secretory Granules via Adaptor Protein AP-1, Clathrin, and Syntaxin 6–positive Vesicles. *J Cell Biol.* 141(2):359-371, doi: 10.1083/jcb.141.2.359

Knip M. & Simell O. 2012. Environmental Triggers of Type 1 Diabetes. *Cold Spring Harb Perspect Med*. 2(7): 1–15, doi: 10.1101/cshperspect.a007690

Kyvik K., Green A. & Beck-nielsen H. 1995. Concordance rates ofinsulin dependent diabetes mellitus : a population based study of young Danish twins. *BMJ*. 311(7010): 913-917, doi: 10.1136/bmj.311.7010.913

Laitinen O.H., Honkanen H., Pakkanen O., Oikarinen S., Hankaniemi M.M., Huhtala H., Ruokoranta T., Lecouturier V., André P., Harju R., Virtanen S.M., Lehtonen J., Almond J.W., Simell T., Simell O., Ilonen J., Veijola R., Knip M. & Hyöty H. 2014. Coxsackievirus B1 Is Associated With Induction of b-Cell Autoimmunity That Portends Type 1 Diabetes. *Diabetes.* 63: 446-455, doi: 10.2337/db13-0619/-/DC1.

Leung P.S. & Ip S.P. 2006. Pancreatic acinar cell : Its role in acute pancreatitis. *Int J Biochem Cell Biol.* 38(7):1024-30, doi: 10.1016/j.biocel.2005.12.001

Lin J., Chen T., Weng K., Chang S., Chen L. & Shih S. 2009. Viral and host proteins involved in picornavirus life cycle. *J Biomed Sci*. 16:103, doi: 10.1186/1423-0127-16-103.

Li N., Yang Z., Li Q., Yu Z., Chen X., Li J., Li B., Ning S. & Cui M. 2018. Ablation of somatostatin cells leads to impaired pancreatic islet function and neonatal death in rodents. *Cell Death Dis*. 9(6): 682, doi: 10.1038/s41419-018-0741-4.

Linden L. Van Der, Wolthers K.C. & Kuppeveld F.J.M. Van. 2015. Replication and Inhibitors of Enteroviruses and Parechoviruses. *Viruses*. 7(8):4529-4562, doi: 10.3390/v7082832.

Marjomäki V., Turkki P. & Huttunen M. 2015. Infectious Entry Pathway of Enterovirus B Species. *Viruses*. 7(12): 6387–6399, doi: 10.3390/v7122945

Masini M., Marselli L., Himpe E., Martino L., Bugliani M., Suleiman M., Boggi U., Filipponi F., Occhipinti M., Bouwens L., Tata V. De & Marchetti P. 2020. Co-localization of acinar markers and insulin in pancreatic cells of subjects with type 2 diabetes. *PLoS One*. 12(6): 1-12, doi: 10.1371/journal.pone.0179398

Mercer E.H. 1963. A scheme for section staining in electron microscopy. *Journal of the Royal Microscopical Society*. 81: 179-186, doi: 10.1111/j.1365-2818.1963.tb02089.x

Metcalfe K.A., Hitman G.A., Rowe R.E., Hawa M., Huang X., Stewart T. & Leslie D.G. 2001. Concordance for Type 1 Diabetes in Identical Twins Is Affected by Insulin Genotype. *Diabetes Care*. 24(5): 838-842, doi: 10.2337/diacare.24.5.838

Mezza T., Muscogiuri G., Sorice G.P., Clemente G., Hu J. & Pontecorvi A. 2014. Insulin Resistance Alters Islet Morphology in Nondiabetic Humans. *Diabetes*. 63(3):994-1007, doi: 10.2337/db13-1013.

Mohamed D.I., Nabih E.S., El-waseef D.A.A., El-kharashi O.A., Abd A.A. & Samad E. 2018. The protective e ff ect of pentoxifylline versus silymarin on the pancreas through increasing adenosine by CD39 in a rat model of liver cirrhosis : Pharmacological , biochemical and histological study. *Gene*. 651:9-22, doi: 10.1016/j.gene.2018.02.001

Morgan N.G. & Richardson S.J. 2014. Enteroviruses as causative agents in type 1 diabetes : loose ends or lost cause? *Trends Endocrinol Metab*. 25(12):611-619, doi: 10.1016/j.tem.2014.08.002

Network for Pancreatic Organ Donors 2019. (https://www.jdrfnpod.org/) (accessed on 17.6.2019)

Oikarinen S., Tauriainen S., Hober D., Lucas B., Vazeou A., Sioofy-khojine A., Bozas E., Muir P., Honkanen H., Ilonen J., Knip M., Saha M., Huhtala H., Stanway G., Bartsocas C., Ludvigsson J., Taylor K. & Group S. 2014. Virus Antibody Survey in Different European Populations Indicates Risk Association Between Coxsackievirus B1 and Type 1 Diabetes. *Diabetes.* 63(2):655-662, doi: 10.2337/db13-0620.

Oikarinen M., Tauriainen S., Honkanen T. & Vuori K. 2008. Analysis of pancreas tissue in a child positive for islet cell antibodies. *Diabetologia*. 51(10):1796-802, doi: 10.1007/s00125-008-1107-8

Onkamo P., Väänänen S., Karvonen M. & Tuomilehto J. 1999. Worldwide increase in incidence of Type I diabetes ± the analysis of the data on published incidence trends. *Diabetologia*. 42(12): 1395-403, doi: 10.1007/s001250051309

Orci L., Ravazzola M., Mylene A., Madsen O. & Perrelet A. 1986. Conversion of Proinsulin to Insulin Occurs Coordinately with Acidification of Maturing Secretory Vesicles. *J Cell Biol.* 103(6 Pt 1): 2273-81, doi: 10.1083/jcb.103.6.2273

Patterson C.C., Dahlquist G.G., Gyürüs E., Green A., Soltész G. & Group S. 2005. Incidence trends for childhood type 1 diabetes in Europe during 1989 – 2003 and predicted new cases 2005 – 20 : a multicentre prospective registration study. *Lancet.* 373(9680):2027-2033, doi: 10.1016/S0140-6736(09)60568-7

Pociot F., Akolkar B., Concannon P., Erlich H.A., Morahan G., Nierras C.R., Todd J.A. & Rich S.S. 2010. Genetics of Type 1 Diabetes : What ’s Next ? *Diabetes*. 59(7): 1561–1571, doi: 10.2337/db10-0076

Powers A.C. 2014. Architecture and Morphology of Human Pancreatic Islets. In: Powers A.C. (ed.), *Cellular Endocrinology in Health and Disease.* Elsevier Inc, pp. 257-268

Rewers M., Ludvigsson J., Medicine E. & Faculty M. 2017. Environmental risk

factors for type 1 diabetes. *Lancet*. 387(10035): 2340–2348, doi: 10.1016/S0140-6736(16)30507-4

Rhoades R.E., Tabor-godwin J.M., Tsueng G. & Feuer R. 2012. Enterovirus Infections of the Central Nervous System Review. *Virology.* 411(2): 288–305, doi: 10.1016/j.virol.2010.12.014

Richardson S.J. & Morgan N.G. 2018. ScienceDirect Enteroviral infections in the pathogenesis of type 1 diabetes : new insights for therapeutic intervention. *Current Opinion in Pharmacology* 43: 11–19, doi: 10.1016/j.coph.2018.07.006

Richardson S.J., Willcox A. & Bone A.J. 2009. The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. *Diabetologia* 52(6): 1143-51, doi: 10.1007/s00125-009-1276-0

Robinson S.M., Tsueng G., Sin J., Mangale V., Rahawi S., Mcintyre L.L., Williams W., Kha N., Cruz C., Hancock B.M., Nguyen D.P., Sayen M.R., Hilton B.J., Doran K.S., Segall A.M., Wolkowicz R., Cornell C.T., Whitton J.L., Gottlieb R.A. & Feuer R. 2014. Coxsackievirus B Exits the Host Cell in Shed Microvesicles Displaying Autophagosomal Markers. *PLoS Pathog.* 10(4): 1-21, doi: 10.1371/journal.ppat.1004045.

Roep B.O. 2007. Are Insights Gained from NOD Mice Sufficient to Guide Clinical Translation? Another Inconvenient Truth. *Ann N Y Acad Sci*. 1103:1-10, doi: 10.1196/annals.1394.018

Royston L. & Tapparel C. 2016. Rhinoviruses and Respiratory Enteroviruses : Not as Simple as ABC. *Viruses.* 8(1):1-23, doi: 10.3390/v8010016.

Salminen K.K., Vuorinen T., Oikarinen S., Helminen M., Simell S., Knip M., Ilonen J., Simell O. & Hyöty H. 2004. Isolation of enterovirus strains from children with preclinical Type 1 diabetes. *Diabet. Med.* 21: 156–164, doi: 10.1111/j.1464-

5491.2004.01097.x

Seino S. & Bell G.I. 2008. *Pancreatic Beta Cell in Health and Disease*. Springer, Japan.

Slot J.W & Geuze H.J. 2007. Cryosectioning and immunolabeling. *Nature Protocols*, 2: 2480–2491, doi: 10.1038/nprot.2007.365

Spijker H.S., Ravelli R.B.G., Mommaas-kienhuis A.M., Apeldoorn A.A. Van, Engelse M.A., Zaldumbide A., Bonner-weir S., Rabelink T.J., Hoeben R.C., Clevers H., Mummery C.L., Carlotti F. & Koning E.J.P. De. 2013. Conversion of Mature Human b -Cells Into Glucagon- Producing α-Cells. Diabetes. 63: 2471-2480, doi: 10.2337/db12-1001

Steck A.K. & Rewers M.J. 2011. Genetics of Type 1 Diabetes. *Clin Chem*. 57(2):176-85, doi: 10.1373/clinchem.2010.148221

Stene L.C. & Rewers M. 2011. Immunology in the clinic review series; focus on type 1 diabetes and viruses: the enterovirus link to type 1 diabetes: critical review of human studies. *Clinical and Experimental Immunology*. 168: 12–23, doi: 10.1111/j.1365-2249.2011.04555.x

Stuart A.D., Eustace H.E., Mckee T.A. & Brown T.D. 2002. A Novel Cell Entry Pathway for a DAF-Using Human Enterovirus Is Dependent on Lipid Rafts. *J Virol*. 76(18):9307-9322, doi: 10.1128/jvi.76.18.9307-9322.2002

Su Y., Tsai A., Ho Y., Huang S. & Liu Y. 2018. Stimulation of the Internal Ribosome Entry Site (IRES) -Dependent Translation of Enterovirus 71 by DDX3X RNA Helicase and Viral 2A and 3C Proteases. *Frontiers in Microbiology*. 9: 1–17, doi: 10.3389/fmicb.2018.01324

Sun Y., Wang Y., Shan C., Chen C., Xu P., Song M., Zhou H., Yang C., Xu W., Shi P., Zhang B. & Lou Z. 2012. Enterovirus 71 VPg Uridylation Uses a Two-

Molecular Mechanism of 3D Polymerase. *J Virol.* 86(24):13662-71, doi: 10.1128/JVI.01712-12.

Szopa T.M., Titchener P.A., Portwood N.D., Taylor K.W. 1993. Diabetes mellitus due to viruses - some recent developments. *Diabetologia*. 36: 687-695, doi: 10.1007/bf00401138

The DIAMOND Project Group. 2006. Incidence and trends of childhood Type 1 diabetes worldwide 1990–1999. *Diabetic Medicine*. 23: 857–866, doi: 10.1111/j.1464-5491.2006.01925.x

Thompson S.R. & Sarnow P. 2003. Enterovirus 71 contains a type I IRES element that functions when eukaryotic initiation factor eIF4G is cleaved. *Virology.* 315(1):259-266, doi: 10.1016/s0042-6822(03)00544-0

Thorel F., Népote V., Avril I., Kohno K., Desgraz R., Chera S., & Herrera P.L. 2010. Conversion of Adult Pancreatic α-cells to β-cells After Extreme β-cell Loss. *Nature*. 464(7292): 1149–1154, doi:10.1038/nature08894

Vergari E., Knudsen J.G., Ramracheya R., Salehi A., Zhang Q., Adam J., Asterholm I.W., Benrick A., Briant L.J.B., Chibalina M. V, Gribble F.M., Hamilton A., Hastoy B., Reimann F., Rorsman N.J.G., Spiliotis I.I., Tarasov A., Wu Y., Ashcroft F.M. & Rorsman P. 2019. Insulin inhibits glucagon release by SGLT2-induced stimulation of somatostatin secretion. *Nature Communications*. 10(1):1-11, doi: 10.1038/s41467-018-08193-8.

Vuorinen T., Kallajoki M., Hyypiä T. & Vainionpää R. 1989. Coxsackievirus B3-induced acute pancreatitis: analysis of histopathological and viral parameters in a mouse model. *Br. J. Exp. Pathol*. 70: 395-403.

Vuorinen T., Vainionpää R., Heino J. & Hyypiä T. 1999. Enterovirus receptors and virus replication in human leukocytes. *Journal of General Virology* 80: 921–927,

doi: 10.1099/0022-1317-80-4-921

Ward C.D., Stokes M.A.M. & Flanegan J.B. 1988. Direct Measurement of the Poliovirus RNA Polymerase Error Frequency In Vitro. *J Virol.* 62(2): 558–562, doi: 0022-538X/88/020558-05$02.00/0

Wenzlau J.M. & Hutton J.C. 2013. Novel Diabetes Autoantibodies and Prediction of Type 1 Diabetes. *Curr Diab Rep.* 13(5):608-615, doi: 10.1007/s11892-013-0405-9.

Wilcox G. 2005. Insulin and Insulin Resistance. *Clin Biochem Rev.* 26(2):19-39.

Wimmer E., Kuhn R.J., Pincus S., Yang C., Toyoda H., Nickilin M.J.H & Takeda N. 1987. Molecular events leading to Picornavirus genome replication. *J. Cell Sci. Suppl.* 7: 251-276

Yin H., Berg A., Tuvemo T. & Frisk G. 2002. Enterovirus RNA Is Found in Peripheral Blood Mononuclear Cells in a Majority of Type 1 Diabetic Children at Onset. *Diabetes*. 51(6):1964-1971, doi: 10.2337/diabetes.51.6.1964

Yoon J.W., Onodera T. & Notkins A.L. 1978. Virus-induced Diabetes Mellitus: Beta Cell Damage and Insulin-Dependent Hyperglycemia in Mice Infected with Coxsackie Virus B4. *The Journal of Experimental Medicine*. 148: 1068-1080, doi: 10.1084/jem.148.4.1068

Yuan M., Yan J., Xun J., Chen C., Zhang Y., Wang M. & Chu W. 2018. Enhanced human enterovirus 71 infection by endocytosis inhibitors reveals multiple entry pathways by enterovirus causing hand-foot-and-mouth diseases. *Virol J.* 15(1):1-13, doi: 10.1186/s12985-017-0913-3.

Zhang H., Song L., Cong H. & Tien P. 2015. Nuclear Protein Sam68 Interacts with the Enterovirus 71 Internal Ribosome Entry Site and Positively Regulates

Viral Protein. *J Virol.* 89(19):10031-10043, doi: 10.1128/JVI.01677-15

Åkerblom H.K., Vaarala O., Hyöty H., Ilonen J. & Knip M. 2002. Environmental Factors in the Etiology of Type 1 Diabetes. *American Journal of Medical Genetics*. 115:18–29, doi: 10.1002/ajmg.10340