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Cover design by
Mikko Reinikka

# Abstract

Enteroviruses and rhinoviruses belong to the *Enterovirus* genus of the *Picornaviridae* family. The *Enterovirus* genus is the largest genus of *Picornaviridae* and includes currently 116 enterovirus and 167 rhinovirus types that affect humans. Enteroviruses are one of the most common viruses infecting humans worldwide and they cause diseases ranging from asymptomatic illnesses to severe, even fatal conditions. Enteroviruses have also been associated with the development of some chronic diseases, such as type 1 diabetes. Type 1 diabetes is an autoimmune disease, which is characterized by the immune-mediated destruction of pancreatic β cells. A preclinical phase, which is defined by the presence of diabetes-associated autoantibodies, precedes the onset of the disease. The development of type 1 diabetes is thought to be triggered by environmental factors in genetically susceptible subjects. Enteroviruses are thought to be a significant environmental factor contributing to type 1 diabetes, but the causality of this association has not been confirmed.

The purpose of this study was to evaluate the epidemiology of enteroviruses and rhinoviruses in Finland. Serological markers of infections, as well as the presence and types of enteroviruses in stool samples were analyzed. In addition, the aim was to identify enterovirus types that could be involved in the initiation of the events leading to the disease in type 1 diabetes. The identification of the enterovirus types associated with the disease process is important, since this would create opportunities to study the mechanisms of β cell damage induced by enteroviruses and it would enable the development of a preventive vaccine in the future.

The results of the current study demonstrate that a variety of enteroviruses is found in Finland. Altogether, 7.7% of stool samples collected from healthy children were positive for enterovirus in a RT-PCR screen. The most common enterovirus types belonged to species A enteroviruses. Enterovirus 71, which has caused severe outbreaks in Asia, was detected only occasionally. Rhinoviruses were found frequently in the stool samples of healthy children; 10 % of the samples were positive for rhinovirus RNA when analyzed by RT-PCR. All rhinoviruses species were

represented. It was also shown that rhinoviruses could retain their infectivity in stool samples.

The association between enterovirus infections and the initiation of β-cell damage in type 1-diabetes was analyzed in two separate studies. First, neutralizing antibodies were measured against 41 different enterovirus types in serum samples of children who were positive for type 1 diabetes associated autoantibodies, and their matched controls. One enterovirus type, coxsackievirus B1, was identified as a potentially diabetes-associated virus type (OR 1.5, 95% CI 1.0–2.2, P= 0.04). In the other study, the presence of enteroviral RNA in stool samples was analyzed by RT-PCR. Compared with their healthy controls, case children had experienced more enterovirus infections before the appearance of the first type 1 diabetes associated autoantibodies. This was the first time when the presence of enteroviruses in stool samples was associated with type 1 diabetes long before the disease is diagnosed.

In conclusion, the results provide new information about the frequency and distribution of enteroviruses circulating in Finland. Several enterovirus and rhinovirus types are common in Finland, while some are rarer compared to other countries. In addition, the results confirm the association between enterovirus infections and the initiation of the β-cell damaging process. The identification of coxsackievirus B1 as an enterovirus type potentially associated with diabetes was a novel finding and offers possibilities to further explore its role in the pathogenesis of type 1 diabetes.

# Tiivistelmä

Enterovirukset ja rinovirukset ovat pieniä vaipattomia RNA-viruksia, jotka kuuluvat *pikornavirusten* heimoon. Tällä hetkellä tunnetaan 116 ihmisellä tautia aiheuttavaa enteroviruksen ja 167 rinoviruksen eri tyyppiä. Enterovirukset voivat aiheuttaa monia erilaisia tauteja. Suurin osa infektioista on oireettomia tai lieväoireisia, mutta enterovirukset voivat aiheuttaa myös vakavia tauteja kuten aivokalvontulehdusta ja halvauksia. Lisäksi enterovirukset on liitetty joihinkin kroonisiin tauteihin kuten tyypin 1 diabetekseen. Tyypin 1 diabetes aiheutuu haiman insuliinia tuottavien beetasolujen tuhoutumisesta. Taudin puhkeamista edeltää oireeton vaihe, jonka merkkiaineita ovat veressä esiintyvät diabetekseen yhdistetyt autovasta-aineet. Tapahtumaketju, joka johtaa sairastumiseen on vielä osin epäselvä. Geneettinen alttius määrittelee taipumuksen sairastua mutta myös ympäristötekijöillä on tärkeä merkitys. Enteroviruksia on jo pitkään pidetty yhtenä todennäköisimmistä ympäristöperäisistä diabeteksen riskitekijöistä.

Tämän tutkimuksen tarkoituksena on kartoittaa entero- ja rinovirusten esiintyvyyttä Suomessa tutkimalla uloste ja verinäytteitä lapsilta, joita on seurattu syntymästä lähtien. Lisätavoitteena on tutkia enterovirus 71:n esiintymistä ja sen aiheuttamaa sairastavuutta Suomessa. Lisäksi tutkimuksessa pyritään selvittämään, liittyykö enterovirusten ja tyypin 1 diabeteksen välinen yhteys joihinkin virusten alatyyppeihin. Tällaisten enterovirustyyppien tunnistaminen on tärkeää, sillä se avaisi uusia mahdollisuuksia tautimekanismien selvittämiselle ja mahdollisesti myös suojaavan rokotteen kehittämiselle.

Tutkimustulokset osoittivat, että useita enterovirustyyppejä kiertää Suomessa ja osa niistä on hyvin yleisiä (erityisesti A lajiin kuuluvat). Kaiken kaikkiaan 7,7 %:ssa ulostenäytteistä todettiin enteroviruksen genomia RT-PCR-menetelmällä. Toisaalta osa enteroviruksista oli harvinaisempia kuin muualla maailmassa. Esimerkiksi enterovirus 71:n aiheuttamia infektioita todettiin vain vähän, vaikka tämä virus on aiheuttanut laajoja epidemioita Aasiassa. Toisaalta rinovirukset, jotka aiheuttavat hengitystieinfektioita, esiintyvät runsaslukuisina ulostenäytteissä. Kymmenen prosenttia ulostenäytteistä oli positiivisia rinoviruksen RNA:lle PCR menetelmällä.

Enterovirusten ja tyypin 1 diabetes tautiprosessin yhteyttä tutkittiin kahdessa tutkimuksessa. Ensimmäisessä tutkimuksessa määritettiin neutraloivat vasta-aineet 41:tä enterovirustyyppiä kohtaan diabeetekseen sairastuneiden tai esidiabetesvaiheessa olevien lasten ja heidän verrokkiensa seeruminäytteistä. Tulokset osoittivat, että coxsackievirus B1 on yksi mahdollinen tautiprosessin käynnistymiseen liittyvä virus (OR 1.5, 95 % CI 1.0–2.2, P= 0.04). Toisessa tutkimuksessa enterovirusten RNA genomia monistettiin PCR menetelmällä ulostenäytteistä. Tyypin 1 diabetekseen sairastuneilla tai esidiabetesvaiheessa olevilla lapsilla oli enemmän enterovirusinfektioita, ennen kuin ensimmäinen diabetekseen yhdistetty autovasta-aine havaittiin.

Yhteenvetona voidaan todeta, että entero- ja rinovirusten aiheuttamat infektiot ovat yleisiä suomalaisilla lapsilla. Toisaalta, jotkin enterovirukset ovat harvinaisempia kuin muualla maailmassa. Lisäksi tämän tutkimuksen tulokset vahvistivat aiemmin saatuja tuloksia enterovirusten yhteydestä tyypin 1 diabetekseen johtavan prosessin käynnistymiseen. Tässä tutkimuksessa saatuja tuloksia voidaan tulevaisuudessa hyödyntää enterovirusinfektioilta suojaavien rokotteiden kehittämisessä

# Contents

# 1 List of original publications

The study is based on the following original publications, referred to in the text by their Roman numerals, I-IV:

I        Honkanen H, Oikarinen S, Pakkanen O, Ruokoranta T, Pulkki MM, Laitinen OH, Tauriainen S, Korpela S, Lappalainen M, Vuorinen T, Haapala AM, Veijola R, Simell O, Ilonen J, Knip M, Hyöty H. Human enterovirus 71 strains in the background population and in hospital patients in Finland. J Clin Virol. 2013 Apr;56 (4):348-53.

II       Honkanen H, Oikarinen S, Peltonen P, Simell O, Ilonen J, Veijola R, Knip M, Hyöty H. Human rhinoviruses including group C are common in stool samples of young Finnish children. J Clin Virol. 2013 Mar;56 (3):250-4.

III      Laitinen OH*, Honkanen H*, Pakkanen O, Oikarinen S, Hankaniemi MM, Huhtala H, Ruokoranta T, Lecouturier V, André P, Harju R, Virtanen SM, Lehtonen J, Almond JW, Simell T, Simell O, Ilonen J, Veijola R, Knip M, Hyöty H. Coxsackievirus B1 is associated with induction of β-cell autoimmunity that portends type 1 diabetes. Diabetes. 2014 Feb;63 (2):446-55. * The authors contributed equally to this work.

IV       Honkanen H, Oikarinen S, Nurminen N, Laitinen OH, Huhtala H, Lehtonen J, Ruokoranta T, Hankaniemi M, Lecouturier V, Almond JW, Tauriainen S, Simell O, Ilonen J, Veijola R, Viskari H, Knip M, Hyöty H. Detection of enteroviruses in stools is associated with the initiation of the β-cell damaging process. Submitted to Diabetologia in August 2016

# 2   Abbreviations

| | |
|---|---|
| A-549 | carcinomic human alveolar basal epithelial cells |
| ATCC | American Type Culture Collection |
| BB | bio-breeding |
| CAR | coxsackievirus-adenovirus receptor |
| CNS | central nervous system |
| CPE | cytopathologic effect |
| CTLA | cytotoxic T-lymphocyte antigen |
| CV-A | coxsackie A virus |
| CV-B | coxsackie B virus |
| DAF | decay-accelerating factor |
| DCM | dilated cardiomyopathy |
| DIPP | the Finnish Diabetes Prediction and Prevention study |
| E | echovirus |
| EIA | enzyme immunoassay |
| ELISA | enzyme-linked immunosorbent assay |
| EV | enterovirus |
| FBS | fetal bovine serum |
| GADA | glutamic acid decarboxylase antibody |
| GMK | green monkey kidney cells |
| HeLa | carcinomic human cervix epithelial cells |
| HFMD | hand, foot and mouth disease |
| HLA | human leukocyte antigen |
| IA-2A | islet antigen 2 antibody |
| IAA | insulin autoantibody |
| ICA | islet cell antibody |
| IFIH1 | interferon-induced helicase C domain-containing protein 1 |
| IRES | internal ribosomal entry site |
| LLC | monkey kidney epithelial cells |
| MHC | major histocompatibility complex |
| NA | neutralization analysis |
| NAB | neutralizing antibody |

| | |
|---|---|
| NOD | non-obese diabetic |
| ORF | open reading frame |
| PTPN22 | protein tyrosine phosphatase, non-receptor type 22 |
| PV | poliovirus |
| RD | human rhabdomyosarcoma cells |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcription polymerase chain reaction |
| RV | rhinovirus |
| TEDDY | the environmental determinants of diabetes in the young -study |
| T1D | type 1 diabetes |
| TRIGR | trial to reduce IDMM in the genetically at risk |
| UTR | untranslated region |
| VP | viral protein |
| ZnT8 | zinc transporter-8 |

# 3   Introduction

Enteroviruses (EVs) and rhinoviruses (RVs) belong to the *Enterovirus* genus of the *Picornaviridae* family. The *Enterovirus* genus includes 116 EV and 167 RV types by current knowledge. These viruses can bind to several cellular receptors and they differ in their ability to infect various cell types and organs. Consequently, EVs can cause a diverse spectrum of diseases.

EVs have been associated with type 1 diabetes (T1D) in several studies. The role of EVs in the pathogenesis of the disease has received increasing interest, ever since polymorphisms associated with diabetes were discovered in the innate immune system receptor for EVs (IFIH1). However, studies have reported conflicting results about the association, and a causal relationship has not been confirmed.

EV infections are endemic all over the world, but temporal and geographical differences exist. According to serological studies, EV infections are less frequent in Finland and in Sweden with a high incidence of T1D, compared to the neighboring countries Estonia and the Karelian Republic of Russia where the incidence of T1D is lower.

The purpose of this study was to evaluate the epidemiology of EVs and RVs in Finland in the child population, using a diverse set of samples. The presence and types of these viruses in stool samples and serological markers of infections were analyzed. In addition, one of the aims of this study was to identify EV types that could be associated with the initiation of the β-cell damaging process leading to T1D.

# 4 Review of the literature

## 4.1 Enteroviruses (EVs)

### 4.1.1 Classification and structure of enteroviruses (EVs)

Enteroviruses (EVs) belong to the *Enterovirus* genus of the *Picornaviridae* family. The *Enterovirus* genus is the largest in the family and contains 12 species. Originally, human EV types, including polioviruses, coxsackie A viruses, coxsackie B viruses and echoviruses, were classified into four subgroups based on their antigenic and biological properties. Later on, the characterization of the viral genome has led to another type of classification, which is based on genetic similarities between different EV types. Currently, 116 EV types that infect humans are classified into four enterovirus species (*Enterovirus A-D*) (Table 1). *Enterovirus E-J* species contain virus strains that infect non-human primates and bovines (1, 2).

The *Enterovirus A* species consist of 21 types; 11 coxsackie A viruses (CV-As), and 10 numbered EVs, whereas the *Enterovirus B* species consist of 28 echovirus types (Es) and six coxsackie B virus (CV-Bs) types. The prototype EV, poliovirus 1-3, belongs to the *Enterovirus C* species, along with nine CV-As and 11 EVs. The *Enterovirus D* species consists of five EVs (EV-D68, EV-D70, EV-D94, EV-D111 and EV-D-120 (Table 1). In contrast to other EVs, EV-D68 is sensitive to acids. Therefore, it also has epidemiological and biological features similar to those of RVs (3).

**Table 1.** Enterovirus types affecting humans.

| Species | Types |
|---|---|
| *Enterovirus A* | CV-A2, CV-A3, CV-A4, CV-A5, CV-A6, CV-A7, CV-A8, CV-A10, CV-A12, CV-A14, CV-A16, EV-A71, EV-A76, EV-A89, EV-A90, EV-A91, EV-A92, EV-A114, EV-A119, EV-A120, EV-A121 |
| *Enterovirus B* | (CV-B1), CV-B2, CV-B3, CV-B4 (incl. swine vesicular disease virus 2 [SVDV-2], CV-B5 (incl. SVDV-1), CV-B6, CV-A9, E-1 (incl. E-8), E-2, E-3, E-4, E-5, E-6, E-7, E-9 (incl. CV-A23), E-11, E-12, E-13, E-14, E-15, E-16, E-17, E-18, E-19, E-20, E-21, E-24, E-25, E-26, E-27, E-29, E-30, E-31, E-32, E-33, EV-B69, EV-B73, EV-B74, EV-B75, EV-B77, EV-B78, EV-B79, EV-B80, EV-B81, EV-B82, EV-B83, EV-B84, EV-B85, EV-B86, EV-B87, EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107 |
| *Enterovirus C* | PV-1, PV-2, PV-3, CV-A1, CV-A11, CV-A13, CV-A17, CV-A19, CV-A20, CV-A21, CV-A22, CV-A24, EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116, EV-C117, EV-C118 |
| *Enterovirus D* | EV-D68, EV-D70, EV-D94, EV-D111 (from both humans & chimpanzees), RV 87 has been reclassified as a strain of EV-D68 |

CV=coxsackie virus, EV=enterovirus, E=echovirus, PV=poliovirus

* the types currently designated to RV-C, according to the http://www.picornaviridae.com in19.5.2016

Like other picornaviruses, EVs have a simple structure consisting of a small icosahedral protein coat that surrounds a positive-sense-single-stranded RNA genome. Sixty copies of each of the proteins VP1, VP2, VP3 and VP4 form an icosahedral protein coat (Figure 1) (4). VP1, VP2 and VP3 are the major proteins of the capsid. They share an eight-stranded β-barrel, and the loops that join the barrels vary in length and sequence between the EV types. The loops project onto the surface of the capsid and contain the antigenic sites, which define the diversity of the serotypes in the *Enterovirus* genus. (5)
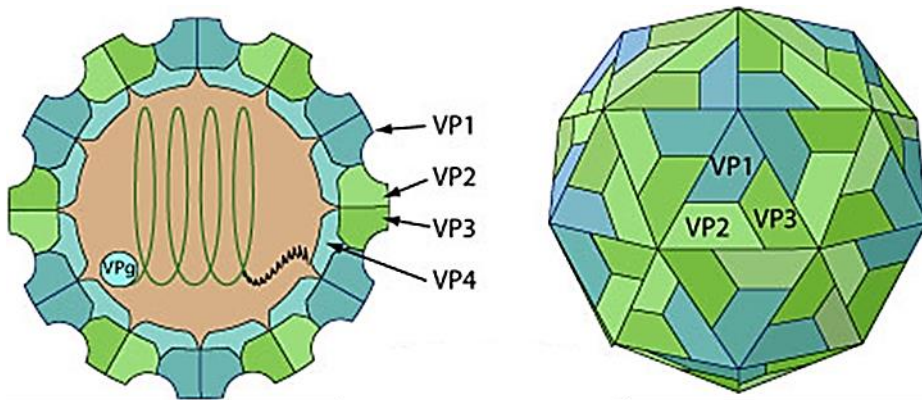
**Figure 1.** The structure of an enterovirus. Single-stranded positive-sense RNA genome is surrounded by a capsid consisting of four structural proteins VP1-VP4. (The figure is presented at http://viralzone.expasy.org)

The RNA genome of EVs is about 7.5 kb in length, uncapped and packed into a 30 nm icosahedric capsid (4). An internal ribosomal entry site (IRES) is located in the 5' untranslated region (UTR) and is necessary for translation. The RNA contains a single open reading frame (ORF). The long UTR is covalently linked to the VPg protein and precedes the ORF and a much shorter 3' UTR linked to the polyA tail of the genome (6) (Figure 2.). The ORF is divided into three parts. The first part (P1) encodes for the capsid proteins VP1-VP4, and other parts (P2 and P3) give rise to nonstructural proteins (2A-2C and 3A-3D). The polyprotein is cleaved by proteases to produce the final virus proteins (4). Structural proteins (VP1-VP4) form the capsid and non-structural proteins (2A-C and 3A-C) take part in RNA synthesis and in protein processing, and they modify the properties of the host cell to facilitate viral replication and suppress the host defense (7). 3Dpol is the viral RNA polymerase protein, which does not contain a proofreading activity and is therefore prone to errors. The polymerase uses negative sense RNA as a template to create new copies of the genome. Finally, the capsid is built around the genome to create a mature particle, which is then released by lysis (7) or possibly by the non-lytic release of lipid-enriched vesicles packed with virions (8).

The VPg protein appears to be cleaved from the genomic RNA at an early stage in the replication cycle. The protein is thought to prime the viral genome for replication, but recent results have shown that translation and replication are not

affected by the presence of the peptide. The reason for the cleavage of the protein from the genomic RNA after its release into the cytoplasm is still unknown. It has been speculated that VPg may be necessary for the encapsulation of synthesized genomic RNAs into particles, as only viral RNA containing VPg is found in virions (9).

Several steps in virus replication and release require cell specific host proteins. The varying repertoire of proteins in different cells may influence which cell types are infected, in e.g. the mucosal or lymphoid tissues of the pharynx or in the small intestine. The uptake of the virus can be mediated by clathrin-dependent or – independent endocytosis or via micropinocytosis (10). A conformational change in virions is caused by a drop in pH or by binding to a receptor, which eventually leads to the release of the genome into the cytoplasm. The genome of the virus is replicated in association with cytoplasmic membranes, and the host cell's ribosomal machinery mediates cap-independent synthesis of the polyprotein (8).

During the viremic phase EVs can spread to various organs, such as the heart, central nervous system and pancreas causing infections in multiple organs (11). Consequently, EVs use a variety of different receptors to enter different cell types. These receptors include intracellular adhesion molecule 1 (ICAM-1), decay-accelerating factor (DAF), integrins α2β1 and αvβ3, poliovirus receptor and coxsackievirus-adenovirus receptor (CAR). In addition, several other molecules on the cell membrane might contribute to viral entry (10).
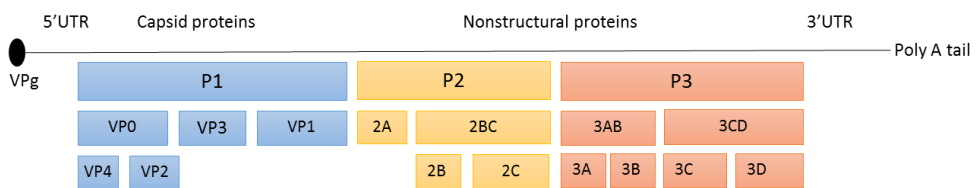


**Figure 2.** The genome of enterovirus and viral proteins coded by different genome regions. (Modified from Linden et al. *Viruses.* 2015 Aug 10;7(8):4529-62 (8)

## 4.1.2    Diagnosis of enterovirus infections

Measurement of neutralizing antibodies (NABs) is a commonly used method for analyzing EV infections. These antibodies can neutralize the infectivity of the virus *in vitro* and their levels can stay elevated for years after the infection. Hence, the antibodies reflect the infection history of the individual. On the other hand, transient antibody responses also occur, and NABs may not arise, especially if the infection has been associated with low virus titer (12, 13). In addition to NABs, also other kinds of antibodies can be analyzed from blood or serum samples. Acute infections are typically diagnosed by the presence of a virus-specific IgM (or by an elevation in IgA and IgG levels between two different time points), and past infections can be studied by measuring IgG class antibodies with EIA or RIA. Noteworthy is that the IgG antibodies detected by EIA can be transient and remain in the blood for a few months.

Several types of samples (tissue, blood, stool, cerebrospinal fluid, respiratory secretions and vesicular fluid) can be used to isolate EVs, but the virus titer is highest in stool samples and in respiratory secretions. After infections, EVs are shed to stools for three to four weeks (14). Thereby, stool samples are often used for the isolation of EVs by culturing them in susceptible cell lines.

The methods described above are labor-intensive, and for example the sensitivity of the isolation procedure is relatively low. Therefore, molecular methods, especially RT-PCR, have largely replaced other methods in EV diagnostics. PCR is also typically extremely sensitive compared to other methods used for the detection of EVs. Sensitivity is a desired feature, as the amount of the virus can be very low in clinical samples such as blood, cerebrospinal fluid and tissue samples. On the other hand, the sensitivity of the PCR methods in different laboratories may vary considerably. Most of the RT-PCR methods take advantage of the conserved sequences in the 5' untranslated region of the genome, and EVs have been distinguished from each other by using a virus-specific primer pair. The identification of the EV types is achieved by sequencing the capsid protein region of the genome (VP1-VP4) (15-17).

## 4.1.3 Epidemiology of enteroviruses

EVs have a seasonal pattern in both temperate and tropical climates, but this is more prominent in the former. Infections peaks in the summer and fall months in the northern hemisphere (18). More than 30 years of passive laboratory surveillance of hospitalized patients showed that over 50 EV serotypes were circulating in the US during 1970-2005 and 15 of these accounted for almost 85% of all reported detections of EVs (18). The most common types were E-9, E-11, E-30, E-6 and CV-B5. During the surveillance period, the most common types varied over time, and some serotypes appeared constantly, whereas others were detected at a certain time, but were less common at other times. The circulation of EVs appeared in two forms; as endemic and as epidemic. EV types with an epidemic pattern fluctuated in circulation over time, including large peaks (e.g. E-9 showed large epidemics every 3-5 years). The endemic EV types (e.g. CV-B4) circulated at stable and low-levels (18). This type of circulation is typical for EVs. E.g. in Finland, E-30 caused an outbreak in 2009, which was preceded by years of low prevalence of the virus (19).

Most of the surveillance data in Europe is obtained from hospitalized patients or from sewage samples, and the circulating types are same as in the US. Between 1996 and 2011 the most prevalent EVs in the Netherlands belonged to *Enterovirus B* species, including E-11, E-6, E-30, E-7 and E-13 along with CV-B4 and CV-B5 (20). Similarly, in France between 2000 and 2004, the most prevalent types were E-30, E-13, E-6, CV-B5, E-11, CV-B4, E-9, E-7, CV-B1 and CV-B2 (21). *Enterovirus B* species were also most prominent in the UK, Spain, Slovakia and in Hungary, E-30 being the most common type in the UK, Spain and Hungary and CV-B5 in Slovakia (22-25). In addition, the increased detection rates of certain EV types in a given year was observed simultaneously both in the US and in Europe; e.g. E-11 in the US and in the Netherlands in 1999, E-13 in the US and in the Netherlands (18, 20), England and Wales, Spain in 2000/2001 (22, 23). On the other hand, the most prevalent types may differ greatly even between two neighboring countries during the same period of time. For example, in Hungary more variation was observed in circulating echovirus types compared to Slovakia, and the most prevalent virus type differed between these countries being E-30 in Hungary and CV-B5 in Slovakia (24, 25). Furthermore, in these surveillance studies, CV-As have been a minority among the circulating types. However, several epidemics of hand, foot and mouth disease (HFMD) have been caused by *Enterovirus A* species (26) and a recent study in Norway showed that CV-As were the most common virus types found in the stool

samples of healthy children, and EV-A71, CV-A6 and CV-A4 were the most frequently detected individual EV types (27). This implies that the sample type (CFS, nasal swab, stool or vesicular fluid) and source of samples (clinical symptom vs. healthy surveillance) as well as the detection method (virus isolation or PCR) may give different pictures of the circulating types. Furthermore, certain EV types may be missed because the type of infection they cause and the primary place of replication may differ (respiratory vs. gastrointestinal).

Previous surveillance studies in Finland have been based on sewage samples and clinical samples. Altogether 24 different EV types were detected in sewage samples in 1971-1992 (28). The EV positive sewage samples peaked in September tailing until January and the nadir was observed in May. The most prevalent types were CV-B4, CV-B5, E-11, E-6, CV-B2 and CV-B3. These types accounted for 87% of all typed isolates. CV-A9 and E-9, E-22 and E-30 were relatively more abundant in clinical samples than in sewage samples. The serotype distribution only poorly followed the distribution of the clinical samples collected at the same time, but the circulating types were similar as in clinical samples in the US, although some virus types were more common (CV-B5, CV-A9, E-22) or less common (E-4, E-9) in Finland (28). The most prevalent types in the following surveillance of the sewage samples in 1994-2003 were coxsackie B viruses (CV-B1-5) and echoviruses (E-6, E-7, E-11, E-25, E-30) (29), which were also among the most common types in clinical samples in the US and in the Netherlands at same time, although E-11 circulation was at its highest in the US before 1992 and after a quiescent period it peaked again in 1999 (18, 20). In another study the distribution of different EV types was quite similar in sewage samples and clinical samples collected during the years 2000-2007, the most prevalent being E-11, E-6, CV-B4 and CV-B5 (60% of the analyzed samples came from Finland and 40% from other European countries) (30). Consequently, the circulating EVs are quite similar all over the world. However, some geographical fluctuations in the circulating types can be seen.

Recombination seem to be the driving force behind the epidemic profile of certain EV types (31). Increased genetic diversity of the EV-D68 was also the probable reason for the large epidemic that occurred in 2014 in the US and in some other countries. This epidemic was the largest since the identification of the virus in the 1960s (32). The virus type has been associated with severe respiratory tract infections and pneumonia, but before the large epidemic in 2014, it has caused minor outbreaks and had actually been one of the rarest identified EVs. EV-D68 shares features with

EVs and RVs. For example, it differs from other EVs in its temperature sensitivity, and hence grows in cell culture at 33°C, at the temperature of the nose (3). EV-D68 has been isolated from respiratory samples, and has very rarely been reported in stools. In 2014, a high number of people (mainly children) across the USA, Canada and in some parts of Europe were hospitalized due to severe lower respiratory disease (33, 34). In addition, probably millions of people suffered from mild symptoms but did not require medical care. It remains to be seen whether the increased incidence of EV-D68 in 2014 was a single epidemic or whether the increased incidence in recent years indicates that EV-D68 is establishing itself as an emerging pathogen.

Also other factors, such as the genetic background of the population and climate conditions, may influence the worldwide distribution of EVs. For example, frequent recombination is typical for EV-A71 and several different EV-A71 strains have caused severe outbreaks with neurological symptoms in the Asia-Pacific region (35-40). The simultaneous circulation of different EV-A71 populations could favor recombination and further contribute to the diversity of the strains. EV-A71 strains have also been detected in Europe, but with milder outcomes and the circulating strains have been different (41-49) than in Asia. It seems that at least until now, EV-A71 has not been able to cause epidemics of this magnitude elsewhere. Point mutations in circulating strains or other factors can limit the spread of EV-A71 into other populations. (Discussed further in chapter 4.1.4.1)

The tendency to cause epidemics and the potential for endemic transmission and frequent recombination indicates a need for the surveillance of EVs as potential pathogens that can cause severe diseases worldwide.

## 4.1.4    Spectrum of enterovirus diseases

EVs are one of the most common pathogens infecting humans worldwide and the major routes for transmission are by the fecal-oral route or by intranasal or conjunctival routes. The primary replication site of EVs is in the lymphoid tissue of the pharynx and small intestine.

Most of the infections caused by EVs are asymptomatic or mild and do not require medical care. However, EVs can also cause severe and even life-threatening diseases.

Annually, several millions of symptomatic EV infections occur in the United States (18). They are associated with diverse clinical syndromes including common cold, herpangina, hand, foot and mouth disease, pneumonia, aseptic meningitis, encephalitis, pleurodynia, myocarditis, neonatal sepsis and paralysis. In addition, EVs have also been associated with the development of chronic diseases such as T1D and chronic dilated cardiomyopathy. Male gender and young age increase the risk of severe disease (14).

Poliomyelitis is one of the best characterized EV disease. It is caused by three EV-C serotypes named poliovirus 1, 2 and 3. These serotypes have a tropism for motor neurons in the spinal cord. The tropism is at least partly explained by the expression of the PV receptor in these cells. The most typical manifestation of the PV infection in humans is the symptomless replication of the virus in the gastrointestinal tract and the shedding of PVs into the stool. PV enters the human body through the oral route and first infects the cells of the mouth, nose and throat mucosa, which are susceptible to the virus. Primary viremia occurs in most infected individuals, which allows the virus to spread to the systemic reticuloendothelial tissue. At this stage the infection still lacks a clear clinical manifestation. In less than 10% of infected individuals, a second viremia causes symptoms such as headaches, a sore throat and fever. A paralytic disease is caused by the destruction of motor neurons in the spinal cord in only about 1% of infected individuals (50). Thus, neurological symptoms represent a rare inadvertent event of an enteric infection and are not obligatory for the replication of PV.

One of the most common EV disease nowadays is hand foot and mouth disease (HFMD), which was first reported in 1957 and ever since several outbreaks have been described worldwide. HFMD typically affects children of the age of five and under but is seen in adult population fairly often as well. . The disease usually resolves spontaneously with mild symptoms such as fever, blister-like eruptions in the mouth and/or skin rashes on the hands and feet. But sometimes, the symptoms are severe and can include aseptic meningitis, brainstem encephalitis, acute flaccid paralysis, or can even lead to death (51) EVs are the most common pathogens causing this disease, and certain coxsackie A and B viruses along with some echoviruses and EV-A71 have been associated with HFMD (52). EV-A71 and coxsackievirus A 16 (CV-A16) have been the most important cause of HFMD, since they usually cause also the most severe symptoms, but also CV-A4, CV-A6 and CV-A10 can cause HFMD (52).

In the 2000s, EV-A71 has occurred with elevated epidemic activity in Asia and Oceania and several severe outbreaks have been reported. For example, in China, more than 1.7 million cases of HFMD caused by EV-A71 were reported in 2010, and 27 000 patients suffered from neurological complications and 905 died (53). In other parts of the world, smaller epidemics have been observed. Outbreaks of EV-A71 have been reported in many countries in Europe, including Bulgaria and Hungary in 1970 (43, 44), and in the UK, France, Germany, Denmark, Hungary and the Netherlands in the 1990s and 2000s (24, 41, 42, 47, 48, 54). The circulation of this virus can also be asymptomatic as has been observed in Norway (49). EV-A71 is divided into three major genotypes (A-C), which are further divided into subgenotypes A, B1 to B5, C1 to C5.  Genotype replacement has been documented for EV-A71. For example, in Malaysia, the B genotype predominated in 1997, C in 1998, B in 2000, C in early 2003 and B in late 2003 and 2005-2006 (55). In other parts of Asia, the B and C genotypes have fluctuated in predominance over time. B genotypes were detected in Europe before 1988 and since then C1, C2 and C4 have been the predominating types in Europe (42, 46-48).

CV-A6 has been associated with severe HFMD in children especially in Europe and USA. Already in 2008, CV-A6 along with CV-A10 caused an epidemic of HFMD affecting both children and adults in Finland (56, 57).  Since then, many other studies have been published in Europe. Additionally, Asia and other parts of the world have also been affected by CV-A6 and CV-A6 is one of the major pathogens in HFMD (26, 58). It seems that CV-A6 can cause atypical HFMD and lead also to severe diseases such as aseptic meningitis, encephalitis and other neurological diseases (58).

Several candidate vaccines against EV-A71 have been developed and three of them have gone through phase III trials in China with good efficacy and safety profiles (59-61). The protective efficacy against EV-A71-associated HFMD was over 90%. On the other hand, these vaccines were based on local EV-A71 types and may not protect against all EV-A71 subgenogroups (62). In addition, these vaccines do not prevent HFMD when caused by another EV types, such as CV-A16 or CV-A6.  To tackle this problem a multivalent vaccine including EV-A71 and CV-A16 has been tested in preclinical trials (63) and it seems that it might be possible to develop a multivalent vaccine against EVs causing HFMD.

Another example of an EV disease is acute myocarditis and chronic dilated cardiomyopathy (DCM). Myocarditis is a heart disease, which involves inflammation

of the myocardium accompanied by the necrosis of cardiomyocytes. Many myocarditis cases are asymptomatic and patients with myocarditis may recover with little or no permanent damage. On the other hand, myocarditis may cause sudden death or lead to DCM, which is a chronic condition, where the heart becomes weakened and enlarged (64). Coxsackie B viruses are a significant cause of both acute myocarditis and DCM (65). The exact mechanisms of the development of DCM after EV are not known, but the persistent presence of the virus in a slowly replicating form may be a contributing factor. Coxsackie B viruses are known to establish persisting infections in the heart tissue, and the development of viral persistence seems to be associated with deletions in the 5' UTR of the viral genome (64, 66). The CV-B virus enters cells using the Coxsackie and adenovirus receptor (CAR) as its primary receptor (67) and the strong expression of this receptor in heart tissue may explain part of the cardiotropism of these viruses (68, 69).

The humoral immune response is important for the outcome of an EV infection. It provides protection and life-long immunity. NABs develop after a natural infection or vaccination and protect against the disease and the systemic spread of the virus. However, the virus is able to replicate in mucosal cells even when NABs are present at high titers in the blood (14). The neutralization sites are formed by nonsequential antigenic sites on the surface of the virion (5). The role of cell mediated immunity is not yet well known. There are several ways how T cells may protect from an EV infection. They might help humoral responses, or cytolytic T cells might clear the virus directly by lysing the virus-infected cell or through cytokine release (70). In addition, natural killer cells might play a role in the eradication of the virus (71). In immune-mediated diseases, such as in T1D, the immunological cross-reactivity between EV and pancreatic islets might have an effect on the β-cell damaging process (72, 73). Furthermore, antibodies that target the virus but do not neutralize it (enhancing antibodies), could actually enhance the severity of the disease by increasing the replication of EVs in immune cells and by spreading viruses to other parts of the body via immune cells. Moreover, this might activate the immune system to shift from the Th1 type immune response to the Th2 type response (74).

There are several antiviral drugs tested against EVs, among which a capsid binding compound pleconaril has been demonstrated to have a broad-spectrum activity against EVs, and had some beneficial effects in clinical trials (8). In addition, other compounds such as ribavirin (a nucleoside inhibitor) and fluoxetine (anti-depressant)

have shown to have an effect on EV propagation but none of the several compounds have been licensed (8).

## 4.2    Rhinoviruses (RVs)

Rhinoviruses (RVs) belong also to the *Enterovirus* genus of the *Picornaviridae* family. Currently, the 167 RV types that infect humans are classified into three RV species (*Rhinovirus A–C;* Table 2) based on their anti-viral drug sensitivity pattern (75) along with molecular analyses and partial sequencing of the genomes of the virus strains (30). The new group of C RVs was discovered in 2006 by molecular methods. These viruses seem to differ from species A and B as much as they do from other EVs (76).  They do not grow in standard cell culture and have larger antigenic variation than RV-A and RV-B species, reflected by larger sequence divergence in the capsid of the RV-C species (77) The RV-A and RV-B species have distinct sequences in their 5' untranslated region, and some C species resembles species A in this genome region. The remainder of the RV-C variants cluster into a separate group, which differs from both RV-A and RV-B, along with all other EV species (78).

**Table 2.**  Rhinovirus types affecting humans.

| Species | Types |
|---------|-------|
| *Rhinovirus A* | RV A1, A2, A7, A8, A9, A10, A11, A12, A13, A15, A16, A18, A19, A20, A21, A22, A23, A24, A25, A28, A29, A30, A31, A32, A33, A34, A36, A38, A39, A40, A41, A43, A45, A46, A47, A49, A50, A51, A53, A54, A55, A56, A57, A58, A59, A60, A61, A62, A63, A64, A65, A66, A67, A68, A71, A73, A74, A75, A76, A77, A78, A80, A81, A82, A85, A88, A89, A90, A94, A96, A100, A101, A102, A103, A104, A105, A106, A107, A108 and A109 |
| *Rhinovirus B* | RV B3, B4, B5, B6, B14, B17, B26, B27, B35, B37, B42, B48, B52, B69, B70, B72, B79, B83, B84, B86, B91, B92, B93, B97, B99, B100, B101, B102, B103, B104, B105 and B106 |
| *Rhinovirus C\** | RV C1, C2, C3, C4, C5, C6, C7, C8, C9,C10,C11, C12,C13, C14, C15,C16, C17, C18, C19, C20, C21, C22, C23, C24, C25, C26, C27, C28, C28, C29, C30, C31, C32, C33, C34, C35, C36, C37, C38, C39, C40, C41, C42, C43, C44, C45, C46, C47, C48, C49, C50, C51, C52, C53, C54 and C55 |

RV=rhinovirus
* the types currently designated to RV-C, according to the http://www.picornaviridae.com in19.5.2016

The RNA genome of RVs is approximately 7.2 kb in length and it consists of a single gene which translated protein is cleaved by virally encoded proteases to produce 11 proteins similar to EVs. Also, the structure of RVs is identical to EVs and the same proteins VP1-VP4, make up the viral capsid that encases the RNA genome and the remaining nonstructural proteins are involved in viral genome replication and assembly (Figure 1 and Figure 2 and chapter 4.1.1). RV virion has an icosahedral structure that consist of capsid proteins, with a canyon in VP1 that serves as the site of attachment to cell surface receptors. Most *Rhinovirus A* and *Rhinovirus B* types utilize intercellular adhesion molecule (ICAM)-1 as a receptor for cell entry, and the other virus types bind to the low density lipoprotein receptor (LDL-R) (79), whereas *Rhinovirus C* apparently utilizes a different receptor molecule, cadherin-related family member 3 (CDHR3) (80).

Isolation of RVs in cell culture is more complicated compared to that of EVs and requires optimal conditions and cell lines, preferably of human origin (77). Hence, RVs are mostly detected by the molecular methods (RT-PCR and sequencing of the

capsid region), and the most frequently collected samples are nasopharyngeal secretions. In addition, antibodies can be are measured in both serum and nasal secretions by neutralization, complement fixation and enzyme-linked immunosorbent assays (ELISAs). However, the variety of RV types and the lack of cross-reacting antigens have hampered the routine use of serology in the diagnostics of RV infections. (14) Historically, organ cultures of fetal nasal or tracheal epithelia were used to isolate RVs and sinus organ culture has been used to grow an isolate of RV-C (77).

The studies of the epidemiology of RVs are mainly based on nasopharyngeal samples collected from hospitalized children. Some studies have also been conducted among healthy children (81-83) and in adults (84, 85). In addition, RVs have been detected in stool samples (86-90) and in sewage (91). RV infections have seasonal peaks in the fall and spring but can occur all year round (92). Their circulation can follow epidemic or endemic trends. RV-C might have a distinct trend, which peaks during the winter months (93). Furthermore, a large number of RV types can circulate at the same time. During two years, 100 different types were reported to circulate in Wisconsin, USA (94), and similar results were reported from the Netherlands, where almost 70 types were detected (82). In both studies, RV-A types predominated, followed by RV-C and RV-B (82, 94). The same phenomenon has also been observed in other studies (95-99). However, some studies have suggested that at certain occasions, RV-C types can be even more common than RV-A types (83, 87, 100). RVs are common also in Finland, and frequently detected in nasopharyngeal, middle ear, stool and sewage samples. When RV positive samples have been typed, RV-A types have been the most prevalent types in Finland (84, 89, 91, 101).

It seems that genetic drift is the main cause of the genetic diversity of RVs as opposed to EVs, which diversity is also clearly linked to intra- or intertypic recombination (102-104). Recombination among RVs seems to be rare and occurs most often between RVs belonging to the same RV species (79).

RVs are transmitted by intranasal or conjunctival routes and their primary replication site is in the nasal epithelium or in the pharyngeal mucosa. The symptoms are located at the replication site (79). The most typical disease caused by RVs is common cold. An acute RV infection is characterized by a sore throat, nasal congestion, fever and cough. The symptoms usually last from 7 to 14 days. At the age of two years, over 90% of all children have had at least one RV infection (105). RVs are easily

transmitted from person to person contact or via aerosols. RVs shedding is relatively short, approximately 2 weeks, in otherwise healthy individuals (106).

It was long believed that RVs do not infect the lower respiratory tract, but nowadays it is clear that RVs can also infect the lower airways (107) and RVs have been linked to asthma exacerbation and bronchiolitis (108). RV bronchiolitis in infancy has been associated with the later development of wheezing and asthma (109). In addition, RV infections have also been associated with otitis media (110) and pneumonia (111). Unlike most respiratory viruses, RVs may also cause allergic inflammatory responses that could explain the link to asthma exacerbations (109). The mechanism of the effect of RVs in the pathogenesis of lower respiratory tract diseases is still unknown. Recently, it has been speculated that that there could in fact be age differences in the prevalence and in the severity of infections caused by various RV species. RV-A types are more frequent in adults, while RV-C types are frequent among children and they also seem to cause more severe diseases (112). However, the study conducted in the Netherlands showed that the clinical outcome of young children was not restricted to the RV type (82). Nor were there differences in the pathogenicity of different RV types in adults (85). In general, RV-B species seems to cause a less severe illness compared to the disease caused by other RV species (113). In addition, similarly to RV-A and RV-B, RV-C is acid-sensitive. But the temperature sensitivity of the viruses differs from one type to another. Some RV-C types can seemingly grow at higher temperatures, which could explain the lower airway diseases caused by certain RVs (114).

RVs multiply best at 33°C-35°C (i.e. the temperature of the nose) but grow usually poorly in cell cultures. Currently, there is no animal model generally available for RV infection, which has hindered studies on the disease mechanisms and possible development of vaccines against these viruses. In addition, RVs show considerable antigenic diversity (115) and the immunity induced following RV infection does not significantly protect from the future infection by a different RV type (116). Moreover, an attempt to immunize with multiple RV types, did not induce significant cross-type protection. Hence, the development of a widely protective vaccine (94) seems unfeasible using conventional methods. However, virus-like particle or subunit vaccines and novel adjuvants might offer new opportunities in the future. Conserved protein antigens exits in RVs and these antigens can induce cross-reactive cellular and humoral immune responses with protective abilities in small animal models (117). There are several antiviral drugs tested against RVs, among which a

capsid binding compound pleconaril resulted in some decrease in total symptom severity, but it was not broadly effective against the multiple different strains of the virus but was rejected by the FDA in 2002 for the treatment of the common cold due to safety issues (8, 77). A newer derivative, vapendivir, is being tested in adults with rhinovirus infections and asthma (77) and several other compounds have been tested but there are currently no antiviral agents licensed for the treatment of rhinovirus infections.

After an RV infection, NABs develop in the serum and persists for several years (14). Among infected volunteers, the antibodies developed between 7 to 14 days after the inoculation of the virus, but initially antibody-free individuals developed NABs slower and they become detectable three weeks after the inoculation (118). NABs appear also in nasal secretions paralleling the appearance of antibodies in the serum (118, 119). The late appearance of the antibodies and the observation that infections continue throughout life suggest that an effective immunity does not developed or is not maintained for longer periods. In addition, there is evidence that T cells can cross-react between different RV types indicating shared epitopes in different RVs (120). In addition, T cells from tonsils responded to different RV types by CD4$^+$ cells and Th1 type cytokines (121).

## 4.3     The role of enteroviruses in type 1 diabetes

### 4.3.1    Type 1 diabetes

Type 1 diabetes is an autoimmune disease, where the immune-mediated destruction of pancreatic β cells results in insulin deficiency. A pioneering study in which the pathogenesis of the disease was described in detail was published in 1965 (122). The disease often manifests during childhood or adolescence (123).

The disease process is usually slow and initiates long before clinical T1D is diagnosed. The prediabetes phase is characterized by the appearance of autoantibodies against insulin (IAA) (124) and other autoantigens of the pancreatic

islet cells, islet cell cytoplasmic autoantibody, (ICA) (125) insulinoma-associated protein 2 antibodies (IA-2A) (126), glutamic acid decarboxylase antibodies (GADAs) (127) and zinc transporter 8 autoantibodies (ZnT8) (128) in the peripheral blood (129). The seroconversion occurs very early in life, in some cases even as early as at a few months of age (130). In 2013, in a combined study from three countries, Finland, Germany and Colorado (USA), the seroconversion age was on average 2.1 years (131) and in a recent study from 2015, 1.3 years (132). These antibodies also predict the development of the clinical disease (133-135). 69.7% of the children with multiple islet autoantibodies progressed to T1D compared to 14.5% in children with a single islet autoantibody. In addition, only 0.4% of children who had no islet autoantibodies were at risk for developing T1D (131). In the TEDDY (The Environmental Determinants of Diabetes in the Young) study, the corresponding numbers in cumulative incidence of diabetes by 5 years after the appearance of the first autoantibody are 47%, 36%, and 11%. In addition, high levels of IAA and IA-2A increased the risk by eightfold while increased levels of GADA did not have an effect on the risk (132). As persistent islet autoimmunity develops, progression into diabetes seems highly probable, although the time to diagnosis varies.

The current belief is that genes determine the susceptibility to the development of T1D, which is triggered by environmental factors (11). Approximately 60 genes have been shown to modulate the risk for the disease. The major genetic determinants are the highly polymorphic human leukocyte (HLA) genes of the major histocompability complex (MHC). They account for 40% of the familial inheritance of the disease (136). The particular combination of HLA II class DR and DQ gene regions define the risk association with T1D. Several studies have shown that a high risk HLA-DR3-DQ2/DR4-DQ8 genotype influences the progression to T1D (135, 137). In addition to HLA, several other genes have been identified in T1D. These include insulin gene locus (INS), CTLA-4 locus and PTPN22 gene. Moreover, rare variants of IFIH1 gene, which codes a sensor of double stranded RNA produced during replication of viruses including *Picornaviruses*, are strongly protective against T1D, whereas other variants are associated with risk for T1D (138). Also, the polymorphism in the PTPN22 gene regulates innate immune responses against EVs (139).

Monozygotic twin studies have revealed that about 30 to 50% of the disease susceptibility is caused by a genetic predisposition (140). However, the majority of

new cases lack a family history of T1D, indicating that environmental factors are involved in the pathogenesis of the disease (141).

In addition, studies on populations migrating from low incidence to high incidence regions and vice versa, support the role of the environment in the pathogenesis of T1D. The incidence rates of migrant populations from low-incidence countries converge rather quickly with those of the indigenous population (142). In contrast, Sardinians in mainland Italy maintain the high incidence rate of their native region (143).

Another phenomenon associated with T1D is the variation in disease incidence across nations. The incidence of T1D in the age group of 0–14 years varies more than 100-fold worldwide. Finland and Sardinia (Italy) have the highest rates, while the lowest rates have been reported in Venezuela and China, and in other Asian countries (143). Still, the geographic location does not reveal the whole truth, since the neighboring countries of Finland show up to 6-fold lower incidence rates than Finland (144, 145). Also, since the 1950's, the incidence of childhood-onset T1D has been increasing essentially worldwide (143). For example, in Europe, the average annual increase in T1D has been 3.9% from 1989 to 2003 (146). The rates of increase show considerable geographical variation and the increase is too fast to be accounted for by genetic factors (143).

Several environmental factors are thought to be involved in the disease process. Various dietary factors have been linked to T1D. The duration of breastfeeding has shown an inverse correlation with the incidence of T1D (147) and the early exposure to supplementary milk feeding has been linked to the risk of T1D (148). Specifically, the proteins in cow's milk (149-151) and cow insulin (152) have been proposed as potential triggers of the disease. In addition, gluten intake has been linked to an increased risk of T1D in some studies. However, more recent studies and a clinical trial testing the effect of avoiding cow milk proteins have suggested that neither these proteins nor gluten would be important risk factors for developing the disease.(153-155) Also some toxins such as N-nitroso compounds have been linked to T1D, whereas the intake of some vitamins, especially vitamin D, has been shown to reduce the risk for T1D (156, 157).

The possible role of gut microbiota has recently been studied using new sequencing technologies. The gut microbiota is required for the development of a normal immune system and latest evidence supports the hypothesis that dysbiosis may be

linked to the development of immune disorders (158). Furthermore, children who progressed to T1D developed less diverse and less stable microbiota than healthy control children. The same phenomenon has been observed in animal studies (159-163) However, the stool virome did not significantly differ between children who developed islet autoimmunity and closely matched control subjects (164). Moreover, the next generation sequencing method was found to be less sensitive than virus specific real-time PCR method (164). Another study did not find any significant differences in the blood virome between children who progressed to T1D within 6 months from the appearance of islet autoimmunity, and in their matched islet-autoantibody-negative controls (165).

Viral infections have been linked to the pathogenesis of T1D. These viruses include rubella, mumps, cytomegalovirus and EVs. During the last few years EVs have received particular attention, since several studies have linked them to the pathogenesis of T1D (166, 167). This evidence has accumulated from studies where EVs have been detected in the pancreatic islets of T1D patients, and where EV infections have been associated with an increased risk of T1D in prospective studies. In addition, EVs have tropism for β-cells both in systemic infections in infants and in vitro cultured pancreatic islets (168-171) and in vivo (172-174).

However, none of the suspected environmental factors have been causally linked to the disease. This reflects the complex nature of the disease and difficulties to identify critical environmental factors among all other determinants of the disease. In addition, several factors may have mutual interactions and even act differently in different subgroups of T1D patients.

### 4.3.2 Epidemiological linkage

Several epidemiological studies support a connection between EV infections and T1D. In 1969, Gamble and Taylor described this association for the first time by showing that the seasonality of T1D and EV infections follow a similar pattern, both peaking in autumn. Since then several other studies have confirmed the seasonality of T1D. In the northern hemisphere, the peak in EV infections is seen in the summer and fall months and it seems to be more significant in countries with cold winters (175, 176). Moreover, the seasonality of the appearance of T1D-associated

autoantibodies resembles remarkably the seasonal pattern of EVs (18, 177, 178). A report by Gamble and Taylor showed also that reset-onset diabetics had higher antibody titers to Coxsackie B group EVs than both healthy subjects and patients with diabetes of a longer duration (177). In addition, it has been shown that the incidence of T1D rose remarkably after a CBV5 infection (179) and a clear relationship was seen between T1D and EV IgM positivity (180). Since then, several studies have reported the analysis of EV antibodies in T1D subjects and controls. These studies have shown variable results indicating a risk association while, some failed to find such a connection (177, 181-184) (Table 3).

In addition to antibodies, the EV genome has been detected in the blood of T1D patients in many cross-sectional studies (185-193) (Table 3). Moreover, a meta-analysis of molecular studies showed a clear association of EVs and T1D in 33 analyzed prevalence studies (194). Strong evidence has also been obtained from cross-sectional reports, where EVs have been found in the pancreas of T1D patients more frequently than in non-diabetic control subjects (174, 195). In addition to the pancreas, EVs have been detected more frequently in the intestine of T1D patients compared to control subjects (196). Coxsackie B viruses have also been isolated from children with T1D and these isolates have induced diabetes or abnormal glucose tolerance in mice (197, 198) (This is further discussed in sections 4.2.3. and 4.2.4.). Moreover, previous studies have shown that in addition to Coxsackie B group EVs echoviruses could be associated with the induction of diabetes-associated autoantibodies (199-206)

**Table 3.** A summary of retrospective case-control studies evaluating a possible association between enteroviruses and T1D.

| Sample | Country | Method | Association | Reference |
|--------|---------|--------|-------------|-----------|
| Blood | UK | CF | Yes | (177) |
| | Canada | NT | No | (207) |
| | Sweden | RIA | Yes | (181) |
| | USA | IgM ELISA | Yes | (180) |
| | Sweden | IgM RIA | No | (208) |
| | Finland | RIA | Yes | (182) |
| | UK | PCR | Yes | (185) |
| | France | PCR | Yes | (186) |
| | UK | PCR | Yes | (187) |
| | Finland | EIA | Yes | (183) |
| | Sweden | PCR | Yes | (188) |
| | Australia | PCR | Yes | (209) |
| | USA | PCR | No | (210) |
| | Japan | PCR | Yes | (190) |
| | Germany | PCR | Yes | (191) |
| | Cuba | PCR | Yes | (192) |
| | Netherlands | PCR | Yes | (193) |
| Stool | USA* | PCR | No | (211) |
| | Germany | PCR | No | (212) |
| | Norway | PCR | No | (213) |

*rectal swab

CF=complement fixation, ELISA=enzyme-linked immunosorbent assay, EIA=enzyme immunoassay, NT=neutralization test, RIA=reverse radioimmunoassay, PCR=polymerase chain reaction

Prospective studies, based on the follow-up of initially healthy individuals, have also provided evidence for the link between T1D and EV infections. The first studies were conducted in Finland and showed that EVs were more common in children who progressed to T1D than in healthy control children. Moreover, the EV infections clustered to the time when T1D-associated antibodies appeared (182, 214). Additional studies have confirmed these results in Finland (86, 183, 215-219) (Table 4). Moreover, the offspring of a mother who was exposed to EV infections during pregnancy seem to have an increased risk for T1D, but the risk association appears to be relatively weak (182, 220-223). Prospective studies outside Finland and

studies on EVs in stools have not detected an association between EV infections and T1D (210-213). However, the number of children analyzed in these studies has been quite low.

**Table 4.** Summary of prospective studies evaluating a possible association between enteroviruses and T1D.

| Country | Study | Method of detection | Association | Reference |
|---------|-------|---------------------|-------------|-----------|
| Finland | DiMe | Serum EV antibodies | Yes | (182) |
| | DiMe | Serum EV antibodies | Yes | (214) |
| | DiMe | Serum EV RNA | Yes | (216) |
| | DIPP | Serum EV antibodies and EV RNA | Yes | (215) |
| | DIPP | Serum EV antibodies | Yes | (183) |
| | DIPP | Serum, EV antibodies and EV RNA | Yes | (217) |
| | DIPP | Serum, EV antibodies and EV RNA; Stool EV RNA | Yes (Not in stools alone) | (86) |
| | DIPP | Serum, EV RNA | Yes | (218) |
| | TRIGR pilot study | Serum, EV antibodies and EV RNA | Yes | (219) |
| USA | DAISY | Serum, saliva, rectal swab | No | (210) |

Table 4 continues

| Country | Study | Method of detection | Association | Reference |
|---------|-------|---------------------|-------------|-----------|
| USA | DAISY | EV RNA in blood | Yes | (211) |
| Germany | BABYDIAB | Stool EV RNA | No | (212) |
| Norway | MIDIA | Stool EV RNA | No | (213) |
| | MIDIA | Serum EV RNA | No | (224) |

Although many infections in developed countries have decreased over the last decades, the incidence of T1D is rapidly increasing. The increase in T1D incidence seems to correlate with a change in the epidemiology of EV infections. This has been explained by the polio hypothesis, which dates back to a similar epidemiological change observed in the epidemics of polio paralysis. The circulation of poliovirus decreased at the beginning of the 19th century, whereas the incidence of paralytic polio, as a result of the spread of the virus to the central nervous system (CNS) during an acute systemic infection, started to increase. Simultaneously, a larger proportion of children were infected with PV at an older age, when maternal poliovirus antibodies were no longer detected. The same pattern is now seen for EVs; the frequency of EV infections has decreased in developed countries (e.g. Finland and Sweden), and on the other hand, the incidence of T1D has increased (225). Also, maternal EV antibodies were shown to be higher in countries with a low incidence of T1D compared with high-incidence countries (226). This supports the hypothesis that a low frequency of EV infections in the background population exposes the children who are more susceptible to the diabetogenic effect of EVs, because the first infections occur at an older age, when children are no longer protected by maternal antibodies.

The improved standard of living leading to improved sanitation and the diminished circulation of microbes may also have many effects, on immune responses, for example by mechanisms suggested according to the hygiene hypothesis. In line with this, EV infections may have a beneficial effect by stimulating immunoregulatory mechanism and thus down-regulating autoimmune responses. The idea has been

supported by experiments in NOD mice: EVs were able to prevent mice from spontaneously developing diabetes at a young age (227, 228). This protection seems to be mediated by the induction of regulatory T cells, which however, is not specific for EVs. A variant of the encephalomyocarditis virus (EMCV-D) also prevented diabetes in NOD mice (229). In addition, EVs are also associated with a low risk of IgE-mediated allergic sensitization and atopic diseases suggesting that EVs might have immunoregulatory effects also in humans (230, 231).

### 4.3.3    Tissue studies

EVs can establish a persistent infection in susceptible cells. EVs can infect human pancreatic islets *in vitro* (168-171) and *in vivo* (173, 174, 232). Severe islet cell damage has been observed in children who died of a systemic EV infection, particularly in CV-B infections (172). This is in line with the fact that islet cells strongly express the major receptor for CBVs, CAR (233).

The association of EVs to T1D has been studied by looking for EVs in the pancreatic tissue of T1D patients. The hypothesis in these studies has been that EVs infect β-cells and remain detectable in the pancreas for a long period of time and thus can be found at the time of diagnosis of T1D, at least in some patients. Already in 1976, EV was detected by immunofluorescence from the pancreatic tissue of a child who had an acute onset of T1D (234). EV was isolated for the first time from the pancreas of a child with T1D in 1979 (234). The virus was typed to be CV-B4 by neutralization with a CV-B4-specific antibody, and it was able to cause diabetes after inoculation into a mouse (197). A similar case involving CB-V5 was reported in 1980 but the virus was detected in stools. In 1985, CV-B3 was detected in the pancreatic islets of a baby with a generalized CV-B3 infection (198, 235). However, in one other study, an EV analysis of the pancreas of a child after the onset of T1D was negative, indicating possible inter-individual differences in the role of EVs in T1D development (236). More recently, studies have shown the presence of EV proteins and RNA in the pancreatic tissues by immunohistochemistry and *in situ* hybridization methods (195). The EV capsid protein VP1 was found in the pancreatic islets of infants with fatal coxsackieviral myocarditis. VP1 was localized almost exclusively in insulin-positive cells (237). Moreover, viral VP1 was detected in pancreatic acinar cells of patients with myocarditis (238) and in T1D patients (174). Enteroviral RNA has been detected in pancreatic islets of patients with myocarditis (239) as well as in

patients with a fatal EV infection and in patients with T1D (240). In addition to the pancreas, EVs has been detected in other tissues such as in the heart of patients with myocarditis (239, 241) and in the small intestine of T1D patients (196, 242).

### 4.3.4 Animal and cell models

Difficulties in obtaining samples from the pancreas of T1D patients have led many investigators to focus on animal models for the disease. Most animal studies on the mechanisms of T1D have been done using the non-obese diabetic (NOD) mouse model (243). Although its islet histopathology is different from that of humans, the mouse model shares many genetic and immunological disease characteristics with human T1D. Like in human, diabetes in NOD mice results from the destruction of β-cells in the pancreas by the immune system leading to insulin deficiency. Under specific pathogen-free conditions, the incidence of diabetes is 80–95% in female mice and 20–40% in male mice. Variations in the MHC genes, encoded in Idd1, are the major genetic component controlling the onset of diabetes in NOD mice as well as in humans (244). Many potential treatments have been developed in the NOD mouse and the disease can be postponed, prevented, or even cured after the onset of symptoms, but none of these interventions have been effective in human (245). EV infections have been studied in NOD mice by inoculating CV-Bs into the mice, which has allowed investigations on the mechanisms of the disease. The studies have shown that inoculation of EV into NOD mice at a young age prevents islet infections, whereas an EV infection at the older age, when insulitis is manifested, results in the infection of islets and the development of diabetes is accelerated. Mouse models have demonstrated that systemic CV-B4 infection can result in complete destruction of the exocrine pancreas, while the islet cells are rarely infected with virus. Some virus types can, however, infect also islet cells of the pancreas.(246) Moreover, in SOCS-1 (suppressor of cytokine signaling-1) (247) transgenic mice, the tropism of CV-B4 for β-cells is altered, since the mice lack the interferons needed for preventing the infection in β-cells, and the virus will replicate in the β-cells resulting in T1D (246).

In addition to the NOD mouse model, also a biobreeding (BB) (248) rat model has been used to study T1D. In this model, both male and female BB rats develop autoimmune diabetes reaching 90 % of the colony by 4 months of age under specific pathogen-free conditions. An asymptomatic phase characterized by the progressive

infiltration of the pancreatic islets by immune cells, notably CD4 + and CD8 + T cells that will destroy the β-cells, precedes the disease. In addition to the BB-DP strain, the LEW.1AR1/Zmt-iddm rat is another experimental model of human T1D. This model also expresses the MHC class II genes. Spontaneous diabetes develops in association with invasive insulitis including CD4 + and CD8 + T cells, B lymphocytes, macrophages, and natural killer cells (244, 249). Rats have been used as models of virus induced T1D and several viruses have been studied, among them EV (250). EV did not trigger T1D on its own, but the pretreatment of the rats with an innate immunity activator was associated with a moderate rate of diabetes after a CV-B4 infection (250).

An experimental model of the pancreatic islets isolated from organ donors can be used to investigate the tropism of EVs for pancreatic islets and the response of islets to an EV infection. These studies have shown, for example, that several EVs have tropism for pancreatic islets including the insulin producing β-cells. Both lytic and persistent EV infections have been described under experimental conditions (168-171, 251, 252).

The use of animal and cell models has greatly influenced the study of T1D and solved many questions regarding the pathogenesis of T1D. However, since animals and islet cell cultures are very different from humans, it is crucial to concentrate on studying this disease in humans, and prospective studies with multiple types of samples might resolve unanswered questions and provide more data on the association between EVs and the disease process. This could help in evaluating if the prevention of these infections, e.g. by vaccines, could provide tools for the prevention of T1D.

# 5    Aims of the study

The principal aim of this thesis was to analyze the epidemiology of enteroviruses and rhinoviruses in Finland and their possible connection to the initiation of the β-cell damaging process leading to T1D. The specific aims were;

1.    To study the molecular epidemiology of EV infections and their possible association with T1D (Report I, III and IV)

2.    To investigate whether RVs can be detected in stool samples of young children and which RV types are circulating in the background population (Report II).

3.    To identify EV types that could be involved in the initiation of the disease process leading to T1D (Report III and IV).

# 6    Subjects and methods

## 6.1    The study populations

### 6.1.1    DIPP study subjects and sample material (Reports I, II, III, IV)

The study populations in all four reports were derived from the DIPP study
(Diabetes Prediction and Prevention Study) in Finland (253). The children in this
study have a HLA conferred genetic susceptibility to T1D and are followed from
birth in three University Hospitals in Finland (Oulu, Tampere and Turku). Blood
samples have been drawn at the ages of 3, 6, 12, 18, and 24 months and once a year
thereafter (in Turku, the samples have been collected first every 3 months and after
the age of 2 years semiannually). If a child has become positive for any of the
measured T1D associated antibodies, blood samples have been drawn at 3 month
intervals after that. In addition to blood samples, clinical and demographic data has
also been prospectively collected at the regular visits to the DIPP clinic. In a sub-
cohort of the DIPP study, stool samples have been collected at one month intervals
starting from the age of approximately 3 months and lasting until 2 to 3 years of age.
Stool samples have been collected by the family at home and the samples have been
shipped to the University of Tampere by mail at ambient temperature. In order to
identify the selected alleles (DQB1*02, *03:01, *03:02, and *06:02/3) associated with
an increased susceptibility to T1D, a HLA-DQB1 analysis was performed from the
cord blood (254). Hybridization with lanthanide-labeled oligonucleotide probes
detected with time-resolved fluorometry was used to define the genotypes (255).
Children carrying the high-risk HLA-DQB1*02/DQB1*0302 genotype or the
moderate-risk DQB1*0302/x genotype (x ≠DQB1*03:02, *06:02, or*06:03) were
invited for follow-up studies.

All children in the DIPP study are regularly screened during the follow-up for the
possible presence of diabetes associated islet cell autoantibodies (ICA) in the serum,

and if positive, tested also for biochemically defined autoantibodies including IAA, IA-2A and GADA. Since the beginning of the year 2003 all new-born children have been regularly screened for these biochemically defined autoantibodies in addition to ICA.

The case children in the Reports II, III and IV included children who turned positive for multiple (at least two) islet autoantibodies and remained positive for autoantibodies in all later tests and/or progressed to T1D (Table 5). Two (Report III) or two to five (Report II and IV) control children were selected for each case child (Table 5). All control children remained negative for T1D-associated autoantibodies at least 2 years following the earliest detection of autoantibodies in the corresponding case child and did not develop T1D. The control children were matched for time of birth (± 1 month in Report III and ± 2 month in Report II and IV), gender, HLA-DQB1 genotype and region of birth (county of residence). Sixty percent of children in all of the Reports were boys. 102 case children and 238 control children were the same in Reports II and IV, but the number of case and control children increased over the time of the study (Table 5). In addition, a more accurate HLA-DR/DQ genotyping was performed for all cases and controls in Report IV (256).

In both Reports I and II, altogether 4184 stool samples from 359 children (mean 11 samples per child) were collected during the years 1996-2008. The majority of the samples was collected from children less than three years of age. The annual number of samples varied from 251 to 522 (mean 322, except in years 1996, 2007 and 2008 during which less than 100 samples were collected).

In Report IV, 4781 stool samples were collected from children of three years of age or less (mean 11 samples per child) during 1996-2012 (Table 6). The number of stool samples per year was on average 281 (range 5-513 samples). Only a few samples were collected in years 2011 and 2012, 26 and 5 respectively.

Altogether, 5686 serum samples (collected 1994-2010) from 928 children under 11 years of age were screened for EVs by RT-PCR and a different set of 505 samples from 505 children were used to analyze NABs against EV-A71 in Report I (Table 5 and 6). The clinical symptoms reported during the children's visits to the DIPP clinic were also analyzed for the same children in Report I.

Report III was a nested case-control study and included 183 case children and 366 control children (born 1995-2006). Altogether 2409 serum samples were analyzed for the presence of NABs against 41 EV types (Table 5 and 6).

**Table 5.** Number of case children and control children in Reports II, III and IV.

|  | Case children (N of children who had progressed to T1D) | Control Children |
|---|---|---|
| Report II | 102 (63) | 257 |
| Report III | 183 (119) | 366 |
| Report IV | 129 (97) | 282 |

**Table 6.** Number of samples used in different reports.

| Sample type | Method | Report I (N of children)* | Report II (N of children) | Report III (N of children) | Report IV (N of children) |
|---|---|---|---|---|---|
| Serum | RT-PCR | 5686 (928) | - | - | - |
|  | NT | 505 (505) | - | 2409 (549) | - |
| Stool | RT-PCR | 4184 (359) | 4184 (359) | - | 4781(411) |

NT=neutralization assay, RT-PCR=reverse transcriptase polymerase chain reaction

*the stool and serum samples were only partly from same children

## 6.1.2    Hospital patients and clinical virus laboratory statistics (Report I)

In addition to the stool samples collected in the DIPP study, stool samples were obtained from four children (mean age 2 years) hospitalized due to symptoms associated with HFMD. Stool samples were taken in the hospital ward and identified as EV-A71 with methods other than those used in the current study.

Moreover, statistics from three clinical virus laboratories in Finland (HUSLAB in the city of Helsinki, Laboratory Center of Tampere University Hospital in the city of Tampere, at present Fimlab laboratories, and the Department of Virology at the University of Turku in the city of Turku, at present Tyks-Sapa) were obtained to identify EV-A71 infections diagnosed in patients at three hospital districts in Finland. The infections in these statistics were confirmed by detection of the virus using RT-PCR or virus isolation from clinical samples. The clinical virus laboratories cover approximately 70% of all viral diagnosis and they operate in different areas in Finland. The statistics of Helsinki, Turku and Tampere covered the years 1990-2007, 1996-2009 and 1996-2006, respectively.

# 6.2    Methods

## 6.2.1    Virus isolation (Report II)

Rhinovirus isolation was carried out in GMK, Hela, RD and A549 cells at two different temperatures, 34°C and 37°C. Two different media were used; a MEM-based medium for GMK, Hela and RD cells and a HAM F12-based medium for A549 cells. The cells were cultivated for 3 weeks and CPE was observed daily. The possible replication of the virus was analyzed using lysed cell preparations to detect viral RNA by real-time RT-PCR (the same methods as described in chapter 6.2.1, real time PCR for rhinovirus).

## 6.2.2   RT-PCR methods (Reports I, II and IV)

The main methods used in Reports I, II and IV includes two modifications of the RT-PCR method, which was used to detect EV and EV RNA in stool and serum samples.

In stool samples, a 10% (w/v) stool suspension was first prepared from the original stool sample in Hanks' solution including Gentamycin, Penicillin G, Amphotericin B and 4% bovine serum albumin. A MagNaPure extraction robot (Roche, Applied Science, Mannheim Germany) and the Total Nucleid Acid extraction kit (Roche, Applied Science) were used to extract the viral RNA, whereas the QiaAmp Viral RNA mini kit (QIAGEN, Germany) was used to extract viral RNA from serum samples.

Viral RNA from both serum and stool samples was reverse-transcribed and amplified with a previously described PCR method (257). This RT-PCR amplifies both EV and RV sequences, followed by liquid-phase hybridization executed in a microtiter plate format. Time-resolved fluorometry can simultaneously detect EV- or RV-specific probes carrying lanthanide chelate labels that are used to identify amplicons in a hybridization assay (see Table 7 for primers and probes). The sensitivity and specificity of the RT-PCR-hybridization method were evaluated by analyzing a representative collection of EVs and RVs (cell culture supernatants of infected cells) and by testing further its applicability in a clinical setting by analyzing CSF samples and nasopharyngeal aspirates, which were collected from patients and were confirmed by other methods. In these analyses the RT-PCR assay amplified all EVs and RVs tested, and all but one amplicon gave also a positive result in the subsequent hybridization assay (257). Later another study reported that RV probes do not detect all RVs and especially RV-C might be underrepresented when using this method (258).

2077 of the serum samples and 682 of the stool samples were first extracted with a modified Qiagen RNeasy96 kit (QIAGEN, Germany) and then analyzed with real time PCR using the QuantiTect Probe kit (QIAGEN, Germany). The real time PCR run was performed according to the instructions on the Quantitect Probe kit using Taqman chemistry using the ABI Prism 7900 HT machinery supplied by Applied Biosystems (USA). In pilot studies, the two methods were compared using serum samples spiked with different amounts of EV-infected cells, and they were found to have comparable sensitivity (data not shown) (Table 7).

The RT-PCR methods for EVs and RVs have also been tested for sensitivity and specificity by attending into a quality control program (Quality Control for Molecular Diagnostics, QCMD) annually since 2002 (the results shown below are from the year 2013 round). The aim of the QCMD program is to offer independent tools for assessing a laboratory's ability to use molecular diagnostic technologies and evaluate the sensitivity of these methods. The programs for EVs and RVs are slightly different. In the RV analysis, the program reveals the expected results, and as can be seen in Table 8, the results of our RT-PCR method (Ct values) are below the expected results for all QCMD samples. The EV program states the lowest dilution to be detected and the RT-PCR used in the present study can clearly detect even this dilution (Table 9). Every time since entering to the program, the results have been similar and all negative control samples were also negative indicating that contaminations did not occur. This proves that the methods used for RVs and EVs were sensitive.

All detected human EVs and RVs were molecularly typed by sequencing the VP1 or VP4/VP2 region of the viral genome with previously described primers (16). The obtained sequences were blasted against sequences found in the National Center for Biotechnology Information non-redundant nucleotide database. Phylogenetic analyses were carried out using the PHYLIP: Phylogeny Inference Package, version 3.69 program (Joe Felsenstein, 1993, University of Washington, Seattle, WA). A phylogenetic tree was constructed using the Protdist program with the parameters of the Kimura 2 model, and the amino acid matrix was processed with the Kitsch program. The consensus tree was created with the Consense program (Report III). The phylogenetic tree of Report I was constructed using neighbor-joining method. If the child had the same serotype in consecutive stool samples, these samples were interpreted as one infection by the given serotype ((Report IV).

**Table 7.**    Primers and probes used in the PCR assays

| | Primers | Probes |
|---|---|---|
| Enterovirus and rhinovirus RT-PCR (257) (Reports I, II, IV) | forward primer CGG CCC CTG AAT GCG GCT AA<br><br>reverse primer GAA ACA CGG ACA CCC AAA GTA | probe 1 (rhino) TAG TTG GTC CCI TCC CG<br>probe 2 (entero) TAI TCG GTT CCG CTG C<br>probe 3 (entero+rhino) AAA GTA GTI GGT ICC |
| Real time enterovirus RT-PCR (Reports I and IV) | forward primer CGG CCC CTG AAT GCG GCT AA<br><br>reverse primer GAA ACA CGG ACA CCC AAA GTA | probe 1 FAM-TCT GTG GCG AAA CCG ACT A-TAMRA<br>probe 2 FAM-TCT GCA GCG GAA CCG ACT A-TAMRA |
| Real time rhinovirus RT-PCR (Report II) | forward primer CYA* GCC T*GC GTG GC -3<br><br>reverse primer GAA ACA CGG ACA CCC AAA GTA | VIC-TCC TCC GGC CCC TGA ATG YGG C –TAMRA |

TAMRA= red-fluorescent tetramethylrhodamine dye, VIC= fluorescent dye (Applied Biosystems, USA)
* A* and T* locked nucleic acid primer by Exiqon, Denmark

**Table 8.** Results from RT-PCR analyses of the external quality control samples including various concentrations of different RVs. Samples were derived from the Quality Control for Molecular Diagnostics round (http://www.qcmd.org) and analyzed with the RT-PCR which was used to detect RV RNA in Report II.

| Rhinovirus Type | Reference lab result | Tampere University lab result | Reference lab RT-PCR Ct value | Tampere University lab RT-PCR Ct value |
|---|---|---|---|---|
| Rhinovirus A 16 | Positive | Positive | 33.69 | 30.1 |
| Rhinovirus A 16 | Positive | Positive | 39.09 | 33.6 |
| Rhinovirus A 16 | Positive | Positive | 41.04 | 36.7 |
| Rhinovirus A 90 | Positive | Positive | 38.02 | 30.3 |
| Rhinovirus A 90 | Positive | Positive | 42.23 | 34.1 |
| Rhinovirus A 8 | Positive | Positive | 35.37 | 29.0 |
| Rhinovirus B 5 | Positive | Positive | 31.08 | 30.7 |
| Rhinovirus B 42 | Positive | Positive | 33.98 | 32.0 |
| Rhinovirus C | Positive | Positive | 32.49 | 28.3 |
| Rhinovirus C | Positive | Positive | 36.13 | 31.5 |
| Enterovirus D 68* | Positive* | Negative* | 35.40* | Negative* |
| Negative | Negative | Negative | Negative | Negative |

* Enterovirus D 68 is not a rhinovirus and was expected to be negative in RV RT-PCR

**Table 9.** Results from RT-PCR analyses of the external quality control samples including various concentrations of different EVs. Samples were derived from the Quality Control for Molecular Diagnostics round (http://www.qcmd.org) and analyzed with the RT-PCR which was used to detect EV RNA in Reports I and IV.

| Enterovirus type | Reference lab result | Tampere University lab result | Reference lab sample Dilution factor* | Tampere University lab RT-PCR Ct value |
|---|---|---|---|---|
| Coxsackievirus A9 | Positive | Positive | 1.0E-06 | 34.1 |
| Coxsackievirus A16 | Positive | Positive | 1.0E-05 | 32.6 |
| Coxsackievirus A24 | Positive | Positive | 1.0E-05 | 34.0 |
| Coxsackievirus B3 | Positive | Positive | 1.0E-06 | 35.4 |
| Coxsackievirus B3 | Positive | Positive | 1.0E-07 | 38.8 |
| Echovirus 11 | Positive | Positive | 1.0E-05 | 35.1 |
| Echovirus 11 | Positive | Positive | 1.0E-07 | 39.4 |
| Echovirus 30 | Positive | Positive | 4.0E-06 | 33.5 |
| Enterovirus 68 | Positive | Positive | 1.0E-03 | 29.4 |
| Enterovirus 68 | Positive | Positive | 1.0E-05 | 36.0 |
| Enterovirus 71 | Positive | Positive | 1.0E-05 | 36.6 |
| Negative | Negative | Negative | Negative | Negative |

*Titer of the original virus s tocks before dilution, inactivation and freeze-drying at the reference lab.

## 6.2.3 Serological response by neutralization assay (Reports I and III)

In Report I, a neutralization assay was performed to measure antibodies against EV-A71 as follows. The EV-A71 strain, which was isolated from a stool suspension of a child participating in DIPP study in LLC-MK2 cells (ATCC CCL-7.1), was used as the virus strain in this assay. The virus was plaque purified in LLC-MK2 cells and then passaged several times in Vero cells (ATCC CCL-81). The genotype of the virus was confirmed by re-sequencing and end-point dilution method was used to determine the virus titer. A serum microneutralization assay, where equal volumes of serum and virus dilutions were mixed, was performed. These mixtures were incubated for one hour at 37 °C and then O/N at RT. After incubation, the mixtures were transferred into 96-well plates, which were subsequently seeded with 10 000 Vero cells per well. The CPE was examined on day 7.

A total of 44 EV strains representing 41 different types were used in Report III to measure NABs against these types. Most of these viruses were isolated from DIPP children or from hospital patients in Finland and in Sweden. All 44 strains were plaque purified and the genome region coding for the VP1 protein was sequenced to verify the serotype (17). Most of the viruses were analyzed using a standard plaque neutralization assay (178, 259), while those viruses that did not form clear plaques were analyzed using a microneutralization assay (Table 10). In the microneutralization assay, a defined dilution of a serum or plasma sample was mixed with an optimal amount of EV-A71. The used virus was titrated using the median tissue culture infective dose method (TCID50-units on a 96-well cell culture plate. The cells were added to this mixture and the presence of EV antibodies was detected by a reduction in cell death in the serum containing wells. The viability of the cells was compared to cells that were not infected with the virus. Inhibition was considered to be significant when the serum reduced the number of plaques more than 75% (plaque assay) or inhibited the ability of the virus to kill cells by 80% (microneutralization assay).

**Table 10.** The 44 EV strains used to measure antibodies using the neutralization assay

| No | Virus isolate | Source | Extraction date | Strain | NT method | cell line used in NT assay |
|---|---|---|---|---|---|---|
| 1 | CV-A4fi | DIPP Study | 2001 | isolate 10433 | plaque | RD |
| 2 | CV-A5fi | DIPP Study | 2001 | Isolate P-550/CA/Kanagawa/2000 | MN | RD |
| 3 | CV-A6fi | DIPP Study | 2001 | isolate CSF-1739/07 VP1 | MN | RD |
| 4 | CV-A10fi | DIPP Study | 2002 | P-2206/CA10/Kanagawa/2003 | MN | RD |
| 5 | CV-A16fi | DIPP Study | 2001 | W42-44/01 | MN | RD |
| 6 | EV-A71fi | DIPP Study | 2002 | isolate 03784-MAA97 | MN | Vero |
| 7 | CV-A9fi | DIPP Study | 2005 | FR-08-2005-149 | plaque | GMK |
| 8 | CV-B1 | HUSLAB | NK | isolate CVB1 Nm | plaque | GMK |
| 9 | CV-B2 | Laboratory center | 2000 | FR-CASE4 | plaque | GMK |
| 10 | CV-B3fi | DIPP Study | 2001 | CVB3-18219-02 from Moldova polyprotein | plaque | GMK |
| 11 | CV-B4fi | DIPP Study | 2002 | isolate P234pak92 | plaque | GMK |
| 12 | CV-B4rs | HUSLAB | 2007 | Tuscany | plaque | GMK |
| 13 | CV-B5fi | DIPP Study | 2006 | isolate CVB5-CSF1841/BLR/2003 | plaque | GMK |
| 14 | CV-B6rs | ATCC | 1953 | Schmitt [1-15-21] | plaque | GMK |
| 15 | E-1 | Laboratory center | 2001 | isolate 10429 | plaque | GMK |
| 16 | E-2 | Virology, UTu | NK | 152-77 | plaque | GMK |
| 17 | E-3fi | Virology, UTa | 1998 | PicoBank/DM1/E3 | plaque | GMK |

Table 10 continues

| No | Virus isolate | Source | Extraction date | Strain | NT method | cell line used in NT assay |
|----|------|------|------|------|------|------|
| 18 | E-3 | HUSLAB | NK | Morrisey | plaque | GMK |
| 19 | E-4rs | ATCC | 1951 | Pesascek | plaque | GMK |
| 20 | E-5rs | ATCC | 1954 | isolate Noyce | plaque | GMK |
| 21 | E-6 | Virology, UTa | 2003 | Germany/ 120/2003 | plaque | GMK |
| 22 | E-7 | Laboratory center | 1997 | FR-07-2000-55 | plaque | GMK |
| 23 | E-9 | Laboratory center | 1997 | clone:No.66 | plaque | GMK |
| 24 | E-11fi | DIPP Study | 2003 | NET/2000-10025 | plaque | GMK |
| 25 | E-12 | Laboratory center | NK | isolate:120-98 | plaque | GMK |
| 26 | E-13fi | DIPP Study | 2002 | isolate FR-06-2000-93 | plaque | GMK |
| 27 | E-14rs | ATCC | 1954 | Tow | plaque | GMK |
| 28 | E-15 | HUSLAB | NK | CH6-51 | plaque | GMK |
| 29 | E-17rs | ATCC | 1954 | CHHE-29 | plaque | GMK |
| 30 | E-18fi | HUSLAB | NK | Metcalf | plaque | GMK |
| 31 | E-19fi | HUSLAB | NK | isolate 87SD140 | plaque | GMK |
| 32 | E-20 | HUSLAB | NK | isolate 10465 | plaque | GMK |
| 33 | E-21 | HUSLAB | NK | Farina | plaque | GMK |
| 34 | E-25 | Laboratory center | 1996 | isolate SE-97-80688 | plaque | GMK |

Table 10 continues

| No | Virus isolate | Source | Extraction date | Strain | NT method | cell line used in NT assay |
|----|---------------|--------|-----------------|--------|-----------|---------------------------|
| 35 | E-26 | HUSLAB | NK | Coronel (11-3-6) | plaque | GMK |
| 36 | E-27rs | ATCC | 1953 | Bacon | plaque | GMK |
| 37 | E-29rs | ATCC | 1958 | JV-10 | plaque | GMK |
| 38 | E-30fi1 | Laboratory center | 2000 | isolate CF2191-01 | plaque | GMK |
| 39 | E-30fi2 | Laboratory center | 1996 | Bern7/ch1996 | plaque | GMK |
| 40 | E-32 | HUSLAB | NK | PR10 | plaque | GMK |
| 41 | E-33rs | ATCC | 1951 | Toluca-3 | plaque | GMK |
| 43 | EV-B74 | SMI | NK | FRA99-130 | plaque | GMK |
| 43 | EV-B78 | SMI | NK | Human enterovirus78 polyprotein gene | MN | RD |
| 44 | EV-D94 | SMI | NK | isolate 19/04 from Democratic republic of Congo | MN | A549 |

fi=field isolate, rs=reference strains

NK=not known
MN=microneutralization
Laboratory center=Laboratory Center. Tampere University Hospital, Tampere Finland
HUSLAB=Laboratory Center, Helsinki University Central Hospital, Helsinki, Finland
SMI=Smittskyddsinstitutet, Stockholm, Sweden
UTa=University of Tampere, Tampere Finland
Utu=University of Turku

## 6.3     Statistical analysis (Reports II, III, IV)

The Chi-Square test was used to analyze the statistical significance of possible differences between different study groups (T1D cases and their controls, HLA risk groups and genders) in Report II. In Reports III and IV, conditional logistic regression was used to analyze the differences in age, gender, HLA and region matched case-control groups. In Report III, data was analyzed using Stata 8·2 (StataCorp, College Station, TX, USA) and conditional logistic regression was used to estimate the odds ratios (OR) with exact 95% confidence intervals (CI) to analyze the association between EV antibodies and diabetes-predictive autoantibodies. In addition, the P values, which have been corrected for the number of comparisons (Bonferroni's correction) were calculated. In Report IV each stool sample represented a one-month follow-up time. The enterovirus shedding to stools is about 3 to 4 weeks (14). Based on this, an infection criteria was created: If the consecutive stool samples had the exactly same EV type, these two samples were interpreted as a one infection caused by this EV type. The number of infections per 10 follow-up years was used to present the data and to carry out statistical analyses. In time-dependent analyses the time of sample draws in each child was adjusted to the time of birth or to the time of collection of the first autoantibody positive sample in the corresponding child. A conditional logistic regression analysis was performed using Stata 13.1. (StataCorp LP, USA) and R 3.2.2 (www.r-project.org) to compute OR with their 95% CI.

## 6.4     Ethics

Since the subjects in this study are children, we should consider whether diabetes is such a demanding disease in general, that is it necessary to use children in the study setting and possibly cause discomfort to them (260).  At least at the moment, there are no animal or cell models, which alone could provide relevant information about the pathogenesis of T1D, and studies in humans are necessary. In addition, the onset of T1D occurs mostly in childhood or in adolescence, and a study conducted in adults might not reveal the reason for the onset at a younger age. Therefore, children are the appropriate target group to study.

The DIPP study started in 1994 and runs currently in three clinics in Finland, in the cities of Turku, Oulu and Tampere. In the DIPP study, families of all newborns in the University Hospitals of these cities have been offered the possibility to participate the study. According to the study protocols newborn babies are first screened for T1D-associated genes and children with an increased genetic susceptibility for T1D (about 12% of all infants) are invited to take part in prospective follow-up studies starting from birth. The study is conducted according to the Nuremberg code (261) following the good general practice of a medical research study, the Helsinki declaration for medical studies (262) and the guidelines given by the Finnish Advisory Board on Research Integrity (263). Written consent was obtained from each family participating in the DIPP study, and the study was approved by the Ethical Committees of the Pirkanmaa Hospital, the Southwest Hospital and the Northern Ostrobothnia Hospital districts.

Reports III and IV were carried out in collaboration with a commercial company Vactech Ltd. Therefore, a separate amendment was made to the ethical approval and a new written informed consent was signed by the families to cover these studies. All samples that were analyzed in the company laboratory were given an anonymous code, which did not allow identification of the child.

# 7    Results

## 7.1    Epidemiology of enteroviruses and rhinoviruses in Finland (Reports I, II, III, IV)

### 7.1.1    Epidemiology of enteroviruses (Reports III, IV)

NABs were screened against 41 different EV types in the serum and plasma samples collected in 1995-2006 from 549 children of less than 12 years of age (Table 10). The types tested represent the species *Enterovirus A*, *B* and *D*. The most frequently detected EVs included E-33, E-30, CV-A10, EV-B74 and CV-B1 (Table 11). Most of the children were positive for NABs against these types (range 50-80%), whereas NABs against E-4, E-26, E-13, EV-B78 and E-18 were very rare (less than 5% of the children were positive for any of these viruses). In addition, less than 10% of the children had NABs against the following types EV-A71, CV-A9, CV-B4, CV-B5, E-3 (wild type), E-2, E-6, E-9, E-14, E-17, E-25, E-27 and  EV-D94 (Report III, Table 1.).

EV RNA was detected in 7.7% of all stool samples. The detection of the EV RNA followed a clear seasonal pattern, as the samples collected in September to December gave the highest positivity rate, while the nadir was observed in samples collected during the spring months. The prevalence of EV positive samples varied slightly from year to year (Figure 3). The proportion of EV RNA positive samples did not differ between boys and girls (8.9% vs 7.0%). The most prevalent EV types detected in stools belonged to the species *Enterovirus A*, and CV-A4, CV-A16, CV-A2, CV-A10 and EV-A71 were the five most frequently detected virus types (Table 11). The prevalence of the most common *Enterovirus B* species, CV-B5, was only 5%. All other detected EV types were clearly less frequent in stools (Table 1, Report IV).

**Table 11.** The most prevalent EV types diagnosed by the detection of neutralizing antibodies in serum samples and the detection of EV RNA in stool samples.

| Neutralizing antibodies in serum samples | Viral RNA in stool samples |
|---|---|
| E-33 (81.3%) | CV-A4 (23.9%) |
| E-30 (74.2%) | CV-A16 (12.4%) |
| CV-A10 (65.8% | CV-A2 (11.9%) |
| EV-74 (59.9%) | CV-A10 (5.1%) |
| CV-B1 (54.6%) | EV-A71 (5.1%) |

The numbers (%) for serum represent the antibody prevalence in children and for stool samples the proportion of infections caused by certain EV type of all sequenced EVs.
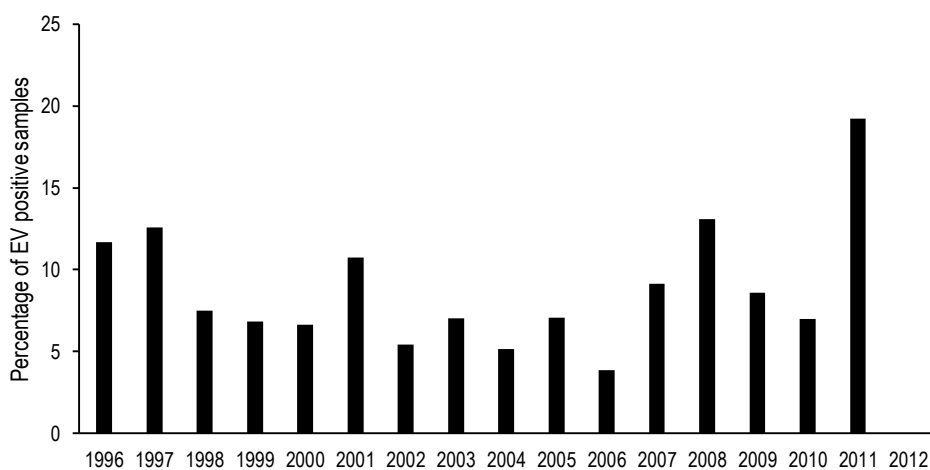


**Figure 3.** Proportion (%) of EV RNA positive stool samples collected from DIPP children in different years.

## 7.1.2    Epidemiology of EV-A71 (Reports I, III)

EV-A71 was found to be relatively rare in children in Finland. Only 12 (0.3%) stool samples and 2 (0.04%) serum samples of children of 11 years or less were positive for EV-A71 in an RT-PCR analysis (Report I). In addition, only 1.6% of the DIPP children had NABs against EV-A71 in Report I (Figure 4) and 8.2% in Report III. Plaque neutralization method was used in Report I, whereas microneutralization method was used in Report III.
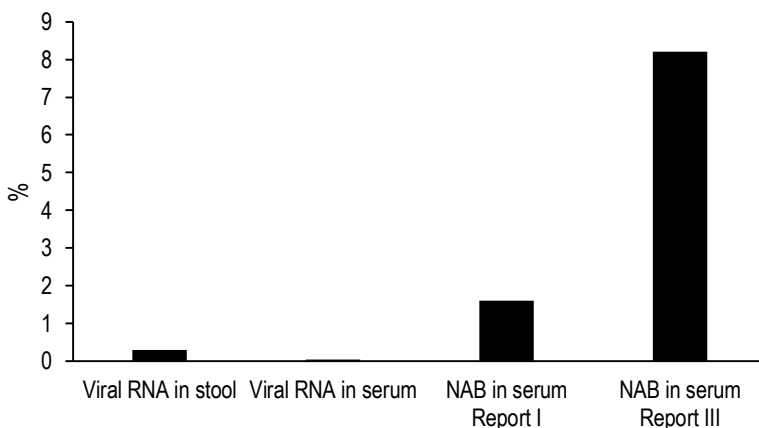


**Figure 4.**  Proportion (%) of EV-A71 positive stool and serum samples (RT-PCR) and prevalence (%) of neutralizing antibodies against EV-A71 in children in Finland.

The EV-A71 positive stool samples were collected in 1997, 1998, 2000, 2002 and 2007, and EV-A71 positive serum samples in 1999 and 2002. In addition to the DIPP study samples,   the statistics of three clinical virus laboratories in Finland were analyzed, and 11 EV-A71 infections were observed in the years 1999, 2000 and 2007 (Figure 5).
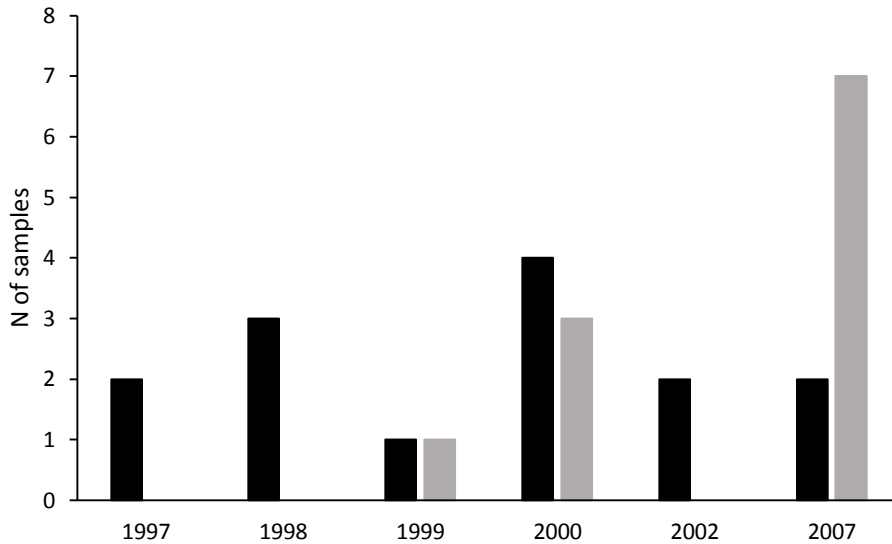
**Figure 5.**   Number of EV-A71 positive samples in the DIPP study and EV-A71 infections diagnosed by clinical virus laboratories in Finland according to the year of virus detection. (black=DIPP study, grey=clinical virus laboratories)

None of the children in the DIPP study had severe central nervous system symptoms at the time when the EV-A71 positive sample was collected. The symptoms recorded from these children included common flu (two children), chickenpox and flu (one child), otitis media (two children), gastrointestinal symptoms (one child) and HFMD (two children). In addition to the DIPP study, four stool samples from children who were hospitalized and who suffered from clear symptoms associated with HFMD were received (samples were from the HUSLAB laboratory; Table 2 in Report I).

The sequences of the 14 EV-A71 positive samples obtained from the DIPP study and of the 4 samples from hospitalized children were compared to EV-A71 strains detected worldwide using a phylogenetic analysis (Figure 1 in Report I). All EV-A71 strains detected in this study belonged to the genogroup C. Three strains detected in the DIPP study grouped with C1 strains and 7 with C2 strains. All 4 strains from hospitalized children in 2007 grouped with C2 strains. Two of the DIPP strains that were also detected in 2007 belonged to the same C2 sub-cluster, while the other C2 strains were genetically different. All four strains from hospitalized children and one DIPP child with symptoms of HFMD detected in the same year aligned together in a separate group. The strains detected in Finland did not cluster with the strains that have caused severe epidemics in Asia.

### 7.1.3    Epidemiology of rhinoviruses (Report II)

All RV types, A, B and C, were common in stool samples collected from the young DIPP children in Finland. Ten percent of all stool samples were positive for RV RNA. The percentage of RV positive samples was similar throughout the collection period, except in 1997, 2006 and 2007, when less RV positive samples were detected (Figure 6). RVs were detected throughout the year, peaking in late summer and fall (Report II, Figure 1), and they were detected in children of all ages. Seventy percent of RV positive samples were detected in stool samples of children less than one year of age.

In total, 71 RV positive samples were randomly chosen and sequenced to identify the RV types. Sequencing of the VP1 and VP4/VP2 regions of the RV genome was successful in 63 RV positive stool samples. RV-A was detected in 70%, RV-B in 8% and RV-C in 13% of these samples. RV-A61 was the most commonly detected individual RV type, and it was detected in 7.9% of successfully sequenced samples (Report II, Table 2). *Rhinovirus A* and *B* species were detected in all age groups, whereas, all RV-C species were detected in children less than 8 months of age (Figure 7).  The detection of RVs was not associated with gender, the HLA genotype or the presence of T1D-associated autoantibodies (P=0.78, P=0.17 and P=0.74, respectively).
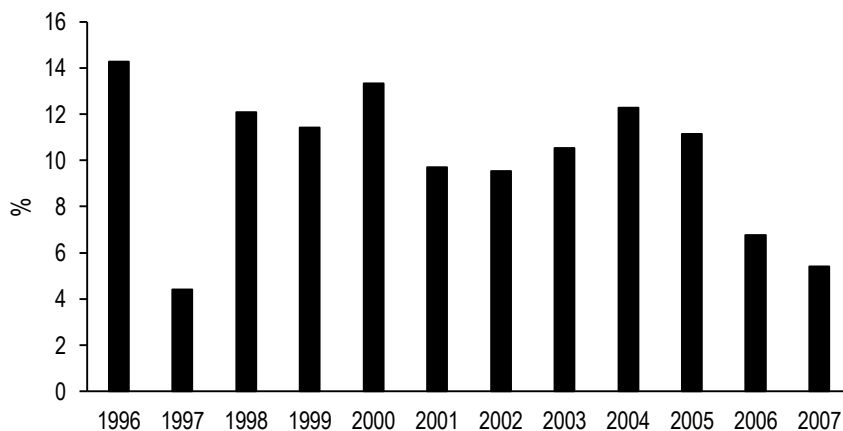


**Figure 6.**  Proportion (%) of rhinovirus positive stool samples collected from DIPP children in different years.
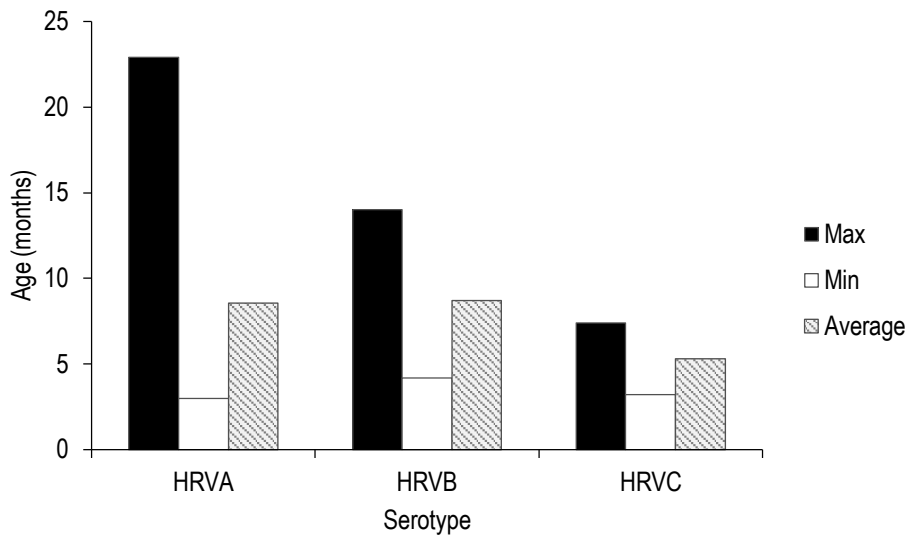
**Figure 7.** The age distribution of DIPP children positive for *Rhinovirus A, B* and *C* species in stools.

## 7.2 Rhinoviruses may retain their infectivity in stools (Report II)

Virus isolation in cell culture was attempted for six RV positive stool samples and three RV negative samples using GMK, Hela, RD and A549 cells at 34°C and 37°C. Rhinovirus A-89 was detected in stool sample by RT-PCR and by sequencing the VP4/VP2 regions of the RV genome. The positive stool sample was further passaged into GMK, RD, Hela and A549 cell lines and cultured for three weeks. RV replication was detected using real time PCR although a clear CPE was not observed. This sample was positive for RV both in cell culture medium and in the cell culture lysate giving Ct values similar to those detected in the original RV positive stool samples (Ct value 34). The type of the virus was identified by sequencing the VP4/VP2 region of the genome. The strain was the same as in the original stool sample (A-89).

## 7.3 Association between enteroviruses and T1D (Reports II, III, IV)

### 7.3.1 Neutralizing antibodies

When NABs were measured against 41 EV serotypes in 183 case children who were positive for multiple islet autoantibodies, and in 366 matched control children, CV-B1 was the only EV type which showed an association with an increased risk of β-cell autoimmunity. This analysis was based on the rate of seropositivity in the first autoantibody positive sample of the case children and in the samples taken at the same age in matched control children. In addition, two other CVBs, CV-B3 and CV-B6, were associated with a decreased risk (Table 12). However, the statistical significances were lost when the P values were multiplied by the number of tested serotypes (N=41). None of the other strains showed any statistical significance (Report III Table 1). The risk association of CV-B1 was clear when the child had experienced CV-B1 alone without the serotypes CV-B3 and CV-B6 with a decreased risk phenotype (OR 2.5, 95% CI 1.4–4.7, P =0.003), whereas those children who were infected by both CV-B1 and one or more of the protective serotypes were not at risk. The risk association of CV-B1 and the protective association of CV-B3 and CV-B6 was also seen in the 119 children who progressed to T1D (OR for CV-B1 was 1.8, 95% CI 1.1–2.9, P =0.025).

The effects of CV-B1 and CV-B3 remained significant after an adjustment for the duration of breast-feeding (CV-B1; OR 1.6, 95% CI 1.0-2.4, P=0.03, CV-B3; OR 0.3, CI 0.1-0.6, P=0.002) and the number of older siblings (CV-B1; OR 1.5, CI 1.0-2.3 P=0.032, CV-B3; OR 0.3, CI 0.2-0.7, P=0.005), whereas the effect of CV-B6 became nonsignificant (OR 0.7, CI 0.4-1.1, P=0.092, OR 0.8, CI 0.5-1.2, P=0.288).

**Table 12.** Prevalence (%) of neutralization antibodies against CV-B1, CV-B3 and CV-B6 in case and control children in the DIPP study. The analysis was carried out from the first autoantibody positive sample in case children and from samples collected at the same age in control children.

|        | Cases | Controls | OR (95% CI)   | P value |
|--------|-------|----------|---------------|---------|
| CV-B1  | 59.0  | 50.1     | 1.5 (1.0-2.2) | 0.04    |
| CV-B3  | 5.8   | 12.8     | 0.4 (0.2-0.8) | 0.01    |
| CV-B6  | 26.6  | 35.3     | 0.6 (0.4-1.0) | 0.04    |

Next, the longitudinal samples collected before the first T1D -associated autoantibody positive sample were analyzed for the presence of NABs against CVB group EVs to determine the time of the infection and to study the time-relationship between infections and the appearance of islet autoimmunity. The result showed an increased risk of autoantibody positivity when the CV-B1 infection preceded the appearance of autoantibodies, and was strongest when the infection preceded the first autoantibody positive sample by a few months (OR 2.0, 95% CI 1.1-3.6, P=0.03). The association was also seen in the children who progressed to T1D (OR 2.0, 95% CI 1.0-4.2, P=0.05) (Report III, Table 3.). Moreover, the maternal antibodies modulated the risk effect of CV-B1. The risk association was strongest in the group who experienced CV-B1 without protective maternal CV-B1 antibodies (OR 2.6, 95% CI 1.1–5.9, P=0.02) (Report III, Table 4). In addition, the order of infections seemed to play a role: children who experienced CVB1 before other CVBs were at an increased risk (Report III, Table 2.).

## 7.3.2 Detection of enteroviruses in stools

Altogether 4781 stool samples from 129 case children (1673 stool samples) and 282 control children (3108 stool samples) were screened for the presence of enteroviral RNA by PCR in the DIPP study series. All samples, which were positive for EV RNA in PCR analyses, were genotyped by sequencing the VP1 region of the viral genome. Almost 8 % of all samples were EV RNA positive. The rate of EV positivity did not differ between boys and girls (8.9% vs 7.0%) or between different HLA-DR/DQ genotypes (7.4% in the high-risk genotype group, 8.7% in the moderately increased risk genotype group, and 7.4% in the slightly increased genotype group).

The rate of EV infections was similar in children younger than 6 months of age and in those aged 6-18 months or 18-24 months (5.2, 5.2 and 5.9 infections per 10 follow-up years), but declined to 2.0 infections per 10 follow-up years in older children (Report IV, Figure 2.).

Altogether 108 infections were diagnosed in case children and 169 in control children (mean 0.84 vs. 0.60 infections per child). The infections diagnosed before the appearance of T1D-associated autoantibodies showed a similar pattern (0.61 vs 0.43 infections per child) and the difference remained when only those children who turned positive for T1D associated autoantibodies at a young age (before 3 years of age) were included (0.49 vs 0.34 infections per child).

The excess of infections in case children occurred more than 12 months before the first autoantibody positive sample was taken (6.3 vs. 2.1 infections per 10 follow-up years, OR 1.03, 95% CI 1.00 -1.06, P=0.023, Table 13). The same difference was detected in children who turned positive for T1D associated autoantibodies at a young age, and when only those samples that were collected during exactly the same calendar month in both the case child and the matched control child/children were included. In addition, the same trend was seen when the number of infections per child was adjusted for the number of samples available from the child and when only those case-control pairs were included whose completed HLA-DR/DQ genotypes matched (Table 13). In contrast, the infections that occurred later, within a year prior to autoantibody seroconversion (OR 1.0, 95% CI 0.98-1.02, P=0.973) or after autoantibody seroconversion (OR 0.99, 95% CI 0.98- 1.01, P=0.54), were not associated with islet autoimmunity.

**Table 13.** Summary of the statistical analyses of the higher frequency of enterovirus infections diagnosed by detecting viral RNA in stool samples collected 12 months or more before the appearance of the first T1D associated antibody as compared to infections diagnosed at the corresponding age in control children.

| | N of children | OR (CI) | P value |
|---|---|---|---|
| Whole study cohort | 233 | 1.03 (1.0-1.06) | 0.023 |
| Adjusted for the number of samples | 233 | 2.00 (1.0–4.00) | 0.043 |
| Autoantibody seroconversion before 3 years of age | 182 | 1.03 (1.0-1.07) | 0.035 |
| Samples matched according to calendar month of collection | 233 | 1.03 (1.0-1.06) | 0.042 |
| Complete HLA-DR/DQ genotypes matched between case and control | 176 | 1.06 (1.0-1.1) | 0.01 |

The VP1 region of the viral genome was successfully sequenced in 244 of all 370 EV-positive samples (90 case samples and 154 control samples, success rate 66 %; 73% in strong positives and 27% in weak positives. The RT-PCR analysis identified 277 infections and sequence analysis provided the exact viral genotype in 174 (63%) of them (63 infections in case children and 111 infections in control children). Cases seem to have more infections that were positive in three or more consecutive samples, but this was not statistically significant (P=0.078).

Twenty-five different EV types were detected in the whole cohort. Species A EVs were the predominating types, including the most frequent EV types CV-A4 (28% of genotyped viruses), CV-A2 (14%) and CV-A16 (11%). None of the individual EV types showed a statistically significant association with islet autoimmunity, even though some types were only detected in case children (CV-A14, CV-B2, E-13) and some only in control children (CV-B4, E-4, E-7, E-9, E-25, E-30, EV-D68, EV-90). However, the occurrence of most individual EV types in stool samples was very low

overall, which limited the statistical power of the study to detect associations with T1D.

RV positivity in stool samples was not associated with T1D. The RV positivity did not differ between case and control children (P=0.74) or between genders (P=0.78) or the HLA DQ risk groups (P=0.17) (Report II Table 1)

# 8    Discussion

## 8.1    Epidemiology of enteroviruses in Finland (Reports I, II, III, IV)

All four Reports in the current study provide important new information about the frequency and circulation of different EVs in young children in Finland. The results of Reports III and IV demonstrated that several EVs commonly circulate among Finnish children and, on the other hand, showed that some of the viruses that are common worldwide are rare in Finland. Report I demonstrates that EV-A71 has been circulating in Finland for several years in the background population, but that EV-A71 infections are still fairly infrequent. In contrast, Report II showed, for the first time, that RVs are frequently detected in stool samples in healthy Finnish children.

### 8.1.1    A wide range of enteroviruses is detected in Finnish children (Reports III, IV)

This study utilized several different methods and sample types to address the question of how frequent EV infections are in the Finnish child population. NABs are serotype-specific and long lasting, revealing the past history of encountered serotypes. The presence of the EV genome in stool samples reflects acute infections and offers the possibility to characterize the virus in detail.

NABs against 41 different EV types were measured in Report III. Only two of the 549 children who were analyzed in this study were negative for all of the tested EV types, showing that as a group EVs are very frequent in the Finnish child population (the median number of positive serotypes was 9). NABs were common against several types including E-33, E-30, CV-A10, EV-B74 and CV-B1, whereas antibodies against several echoviruses were less common (e.g. E-4, E-26, E-13, and E-18). Antibodies against E-30 (Report III, table1) were very frequent and over 95% of the studied children were seropositive for one wild type E-30 strain. Such a high

frequency was quite unexpected and therefore antibodies were measured also against another wild type strain, which gave a 70% seroprevalence. This suggests that this virus may indeed be very common in young children. However, it cannot be excluded that the NAB assay may have not worked optimally for E-30 and that it may overestimate the number of E-30 infections. In addition, there can be annual variation in the frequency of different EV types including also large epidemics of one serotype. In fact, E-30 caused a large outbreak in 2009-2010 in Finland, which was preceded by years of a low detection of this virus (19). During that epidemic, E-30 was detected both in clinical samples (CSF, stool) and in sewage samples (19, 264). EVs are secreted in feces for a long period of time and may be also prevalent in pharyngeal and stool samples during the outbreaks even in apparently healthy individuals, hence the detection of EVs solely in such samples does not definitely confirm it as a causative agent to certain infectious symptoms.

In Report IV, where EVs were detected from stools and typed using RT-PCR and sequencing, the most common EV types were *Enterovirus A* species, CV-A4, CV-A16, CV-A2, CV-A10 and EV-A71. Similar results have been obtained in Norway using the same approach and a comparable cohort of prospectively followed children (27). This is in line with the results from NAB analyses (Report III), where NABs against *Enterovirus A* species, particularly against CAV4 and CAV10, were frequently detected. The large HFMD epidemic caused by CV-A6 that occurred in Finland in 2008 (56, 57) was not however detected in the current study. CV-A6 was occasionally detected in the stools, but the number of CV-A6 positive samples did not peak in 2008 (one was found in December). The stool samples were collected according to the monthly regime from healthy children regardless of symptoms. Thus, the timing of samples probably did not coincide acute illness which may partly explain the lack of CV-A6 positive samples during that year. In addition, fewer stool samples were collected in 2008 and 2009 compared to earlier years (168 samples in 2008 and 151 in 2009 vs 467 in 2005) suggesting that the statistical power to detect this epidemic was not optimal.

The echovirus types that were rare, based on NABs analyses, in Report III, were also rare based on direct virus detection in Report IV. Echovirus 4, for example, was extremely rare in both studies. Only one E-4 positive stool sample was detected and only ~1.5% of the children had NABs against this virus. In addition, CV-Bs were also relatively rarely detected in both studies, whereas CV-A10 was one of the most common types to be found in both studies. Only a few CV-B2, CV-B3, CV-B4 and

CV-B5 positive stool samples were found in Report IV, and altogether only 24 out of 370 (6.5%) EV positive stool samples were CV-B positive. In line with this NABs against CV-B3, CV-B4 and CV-B5 were also relatively rare in Report III. However, E-33, EV-B74 and CV-B1, which were among the most common virus types in Report III, were not detected at all in Report IV and only a few stool samples were positive for E-30. The reason why CV-B1 and certain echoviruses were not detected in stool samples is not known. It is possible that these viruses are mainly respiratory in nature and stool samples are not optimal for their detection. In fact, many EVs cause respiratory disease and can be detected more frequently in nasopharyngeal swabs than in stools. Unfortunately, respiratory samples were not collected in the DIPP study. However, EV-D68, which is mainly detected in respiratory samples (3), was also detected in some stool samples in Report IV. This implies that EV-D68 could either be able to replicate in the intestine or that its detection in stools is a result of passive passage through the alimentary tract making the viral genome detectable by the sensitive RT-PCR in stools. In addition, the duration of virus excretion into stools may differ between different EV types. Thus, the collection interval of stool samples might have been too long to detect those EVs whose excretion period was short (the collection of stool samples in the DIPP study was not based on the symptoms of infections, but on a regular monthly schedule). In addition, the children in Reports III and IV were different with only a minor overlap, and the epidemiology of EV may have been different in these two cohorts. For example, the stool samples analyzed in Report IV were collected mostly before the E-30 outbreak in 2009 and therefore, which might explain why the frequency of E-30 in the current study was so rare. Moreover, a large number viruses in stool samples in Report IV remained un-typed due to a low copy number of virus in these samples. Several EVs might therefore have been missed in the current study.

Altogether, even if Reports III and IV do not provide completely consistent results of the most common virus types in Finland, they supplement each other, since they were based on different study designs and different children. There are more than 100 different EV types, while NABs against only 41 of them were analyzed in Report III. On the other hand, 25 different EV types were detected in Report IV, and 6 of them were not included in the NAB screening in Report III.

Based on surveillance data of over 30 years from the USA, the most common EV types were E-9, E-11, E-30, E-6 and CV-B5 (18). In the Netherland, surveillance data of 15 years demonstrated that E-11, E-6, E-30, E-7, E-13, CV-B4 and CV-B5

were the most common types (20). In addition, *Enterovirus B* species have been the most common EV types in European studies (22-25). Based on the surveillance data, E-30 seems to be common in many countries (18, 20, 22-24), whereas the E-6, E-9 and CV-B5, that were common in the US, in the Netherlands and in Slovakia (18, 20, 25), were relatively rare in the present study in young children in Finland (NAB in all types ~8% and the prevalence in stool samples was 1% for E-9 and 3% for CV-B5; E-6 was not detected at all).

The surveillance studies in Finland have been based on sewage samples and clinical samples with *Enterovirus B* species being the prevailing types (28-30). CV-B4, CV-B5, E-11, E-6, CV-B2 and CV-B3, which were detected in sewage between 1971 and 1992, differed from the types that were more abundant in clinical samples (CV-A9 and E-9 and E-30) (28). The most prevalent types in the following surveillance of sewage samples in 1994-2003 were coxsackie B viruses (1-5) and echoviruses (6, 7, 11, 25, 30) (29). The types that have been frequent in sewage samples were relatively rare in Reports III and IV, except E-11, CV-B1 and E-30 that were common in Report III (~30% of children had NABs against E-11, ~50% against CV-B1 and ~70% against E-30). Hovi et al. showed that the types detected in the sewage samples did not correlate with the samples of patients (28). This is also in line with the results of the current study, showing that the EV types that were detected in healthy children in the DIPP cohort were only partly the same as those detected in sewage samples during the same time-period in 1994-2003 (29). Thus, EVs which can remain detectable in the relatively hostile conditions of the sewage chain, may have been enriched for certain properties and virus types. Moreover, the collection of sewage from a larger area may dilute the virus titers to undetectable levels making it difficult to detect viruses, which are not efficiently excreted into stools. One should also note that sewage contains EVs that infect all age groups, while the present study included very young children only. Thus, the current findings concerning EVs that frequently circulate in young children cannot be generalized to whole population. On the other hand, most EV infections are asymptomatic or mild and hence they do not require special care. Therefore, many EV types may be missing from surveys that are carried out among hospitalized patients or outpatients who typically have clear symptoms or a severe disease.

Noteworthy is that the results of many of the epidemiological studies were obtained from hospital patients, whereas Reports III and IV investigated healthy children. In addition, the type of the samples and methods used in other studies worldwide varied.

For example, surveys carried out in the Netherlands were based on the analysis of stool samples using a combination of cell culturing and RT-PCR (20). Whereas in the US, the reports from different laboratories have been based on various kinds of laboratory methods used during the different decades (18). It seems that studies which have been based on the direct detection of EV RNA from stools by RT-PCR in healthy children have detected *Enterovirus A* species most frequently. This finding has been consistent in the present study and studies carried out in Norway and in Sweden (27, 212).

## 8.1.2 EV-A71 is circulating in Finland but is rare (Report I)

The results of Report I demonstrated for the first time that EV-A71 is circulating in healthy children in Finland, but that it is relatively rare. Only 0.3% of analyzed stool samples and 0.04% of analyzed serum samples were positive for EV-A71. However, the direct detection by an EV71-A specific RT-PCR using EV-A71-specific primers could have revealed more EV-A71 positive samples. On the other hand, only 1.6% of 505 children had NABs against EV-A71 supporting the rare detection of EV-A71 in stool samples. This was further supported by Report III, showing that ~8% of children had NABs against this virus, as well as Report IV, where EV-A71 was detected in 3.5% of the studied stool samples (13 EV-A71 positive samples of all 370 EV positive samples). The difference in the prevalence of NABs is explained by the use of the different methods to analyze the NABs against EV-A71. The plaque neutralization method was used in Report I, whereas in Report III microneutralization method was used. Based on the data above the rate of EV-A71 circulation seems to be lower in Finland than in many other studies. In Germany, 12% of children aged 1 to 4 years had NABs against EV-A71 and the seroprevalence rose to 49% between 5 and 9 years of age (46). In Brazil, 20% of children 0 to 3 years of age had NABs against EV-A71 (265). The studies conducted in the area where EV-A71 has caused severe epidemics, have given variable results. In children less than 2 years of age, only 1% of Singaporean children (266) but 42.5% of Thai children (267) had NABs against EV-A71. In addition, Luo et al showed that in Taiwan only 1% of children had maternal antibodies against EV-A71 at 6 months of age (268). In a very similar study from Singapore, all the levels of maternal antibodies had already diminished by 1 months of age (266). Consequently, a lack of maternal antibodies can make such young children particularly susceptible to EV-A71 infections.

EV-A71 positive samples were detected in different parts of Finland and in different years (between 1997 and 2007). However, EV-A71 was not detected in sewage samples, as part of the poliovirus surveillance system in Finland before the year 2007, when it was detected for the first time in sewage (personal communication by Merja Roivainen from the National Institute for Health and Welfare in Finland). The infections caused by EV-A71 in the present study included mild and asymptomatic diseases but also clear HFMD cases occurred. All the strains detected in this study belonged to C lineages, C1 and C2, and aligned together with the strains detected in other countries in the same year (38, 39, 41, 48, 49, 269-271). Interestingly, all strains detected in 2007, which were from samples of children with symptoms of HFMD, aligned together with a separate C2 group. This fact, together with the fact that in 2007 EV-A71 was detected for the first time in a sewage sample, implies that this particular C2 strain could have infected a larger part of the population than the EV-A71 strains that circulated during the other years. It is also possible that this strain was more virulent than the other strains detected in the study since it caused clear symptoms (HFMD). In fact, C2 was detected also in other European countries, but it was not the sole strain circulating that year (46-48, 54, 272)

The EV-A71 strains detected in the current study differed from the strains that have caused severe outbreaks in Asia (C4, C5) although they belong to the same C lineage (38, 40). Vaccines have been developed against EV-A71 in China. Three of these vaccines have gone through phase III trials and two are on the market in China. These vaccines were based on local EV-A71 (C4) strains (59-61), but they have been shown to induce protective immunity also against other strains (273). In the future, EV-A71 vaccines may be licensed also in countries other than China. However, based on the present study a vaccination against EV-A71 may not be feasible in Finland, since the virus is quite rare in Finland, and at least until now it has not caused severe central nervous system diseases or large epidemics. However, several other EV types can also cause HFMD and it seems that CV-A6 has recently caused severe symptoms (26, 58). Therefore, the development of vaccines against HFMD should not solely be based on EV-A71 and a multivalent vaccine with several EV types could be feasible in the whole world.

Our previous studies have demonstrated that the frequency of EVs is lower in Finland than in other European populations (225, 226). Since, it also seems that EV-A71 is relatively rare in Finland, it is possible that the circulation of EV-A71 depends on the same factors as that of other EVs. In addition, point mutations in circulating

strains or other factors, such as genetics, an unfavorable climate, good hygiene and low population density, could limit the spread of EV-A71. Further studies are needed to address the question why many EV types seem to be less common in Finland than in many other countries.

In conclusion, the studies carried out in Report I, demonstrate that EV-A71 has been circulating in Finland for several years in the background population, but it is quite rare and has not caused severe central nervous system diseases or large outbreaks. Monitoring the circulation of EV-A71 in different countries is important, as it seems that more virulent strains can emergence rapidly (274).

### 8.1.3 RVs are frequently detected and may retain their infectivity in stool samples (Report II)

The results obtained in Report II showed, for the first time, that RVs are common in stool samples of a group of prospectively followed children that represent a large cohort of the healthy population. Ten percent of all stool samples were RV positive. It has been previously shown that RVs can be detected in stools in a smaller cohort of children (86) and this has also been confirmed with hospitalized children (87, 88). In another Finnish study, the frequency of RVs was even higher, over 30% of stool samples were RV positive (89). In that study, stool samples were collected during a different time period than in the current study and were taken when the child had symptoms of an infectious disease (89). This may explain the higher frequency compared to the current study where the samples were collected according to a regular monthly schedule regardless of the possible symptoms.

According to previous studies, RVs are common in Finland and when RV positive samples have been typed, RV-A has been the most prevalent species (84, 89, 91, 101).

In the present study, RV positivity was detected throughout the whole sample collection period, and it followed a clear seasonal pattern. In addition, the sequence analyses indicated that a wide range of different RVs, including representatives from the RV-A, RV-B and RV-C species, infected Finnish children. However, it is possible that the frequency of RV-C was underestimated since the RV probes used in the conventional RT-PCR method (257) might miss part of these RV types (258).  In line with previous Finnish studies, the current study suggests that species a viruses

are the most commonly circulating RVs followed by RV-C and RV-B, respectively. A similar distribution of RV species has been observed also in other countries (82, 94-99). However, a few studies have demonstrated that RV-C can be more common than RV-A (83, 87, 100), and the relative proportion of different species may depend on the type of study subjects (severely ill patients vs. a random cohort representing the background population).

The detection of RV in stools at such a high frequency is still a mystery. Contamination of the samples with RV during sample collection or in the laboratory is highly unlikely, since several independent studies have shown the same phenomenon in healthy and hospitalized children. Negative control samples (PBS) were analyzed along with the actual samples in every PCR run in the present study, and the controls were always negative. Thus, the detections of RVs in stools suggests that these viruses can either replicate in the intestinal mucosa or are ingested by the children and passively pass through the alimentary tract. Consequently, the viruses are able to pass through the stomach without completely losing the integrity of their genome which, for example, allows the amplification of the relatively long PCR product. The RT-PCR method for EVs and RVs used in the present study is sensitive. This was also confirmed by analyzing samples of external quality control rounds where our method reached best possible scores (see Methods, page 43 and Table 8).

Noteworthy is that the capsid surrounds the viral genome and thus protects it from the acidic environment of the stomach. Hereby, even though the low pH could cause conformational changes in the viral capsid, the genome might be protected from degradation inside the capsid. In fact, it seems that at least in some cases the virus might retain its infectivity, since the virus possibly multiplied in cell culture under optimal conditions. This is in line with our previous study (86) and with a more recent study (89). Thus, it might not only be remnants of RV RNA that can be found in stool samples. However, in all of these studies, the number of RV positive samples that might have retained their infectivity was very low.

Since the culturing of RVs in cell lines has proven to be difficult, few efforts have been made to actively isolate all RV positive samples. This would possible shed light on the number and type of the RVs that can remain viable in stool samples. Anyway, it seems that some strains of RV-A and B species can remain infective, as RV-A was isolated in the present study and RV-B in a study by Savolainen-Kopra et al.(89). In

addition, the propagation of RV positive stool samples from adults in cell cultures would be needed to find out whether the more acidic stomach of adults can prevent the infectivity of the RV. In addition, it would have been interesting to analyze the possible symptoms of the children in the current study, especially since the study by Savolainen-Kopra et al. showed that the child, whose RV positive sample grew in cell culture, suffered also from gastrointestinal symptoms (89). This emphasizes the significance of RV as a possible agent for gastrointestinal diseases.

Several different types of RVs were detected. They showed a similar distribution as described in other studies suggesting that the RT-PCR used in the current study detected RV species with a sensitivity equal to the previous studies. Although the sensitivity of RT-PCR to HRV-A, HRV-B and HRV-C was not tested, the primers were targeted to the conserved region of the genome and the RT-PCR method was shown to detect several types described in the original study (257). In addition, virus types from all three species were detected. Regardless of this, the possibility that some types were missed in these analyses cannot be excluded. Also, the sequence of all RV positive samples was not determined, because only a subset of randomly selected RV positive samples was sequenced (N=71 samples). This strategy was used because the high cost of the sequence analysis prevented the analysis of all 438 RV positive samples. Even if the sequenced samples were randomly selected and represented all time periods it cannot be excluded that the distribution of RV species could have been different if all RV positive samples had been sequenced. Since also strains of RV-C species were detected, that have been associated with more severe disease outcomes, an analysis of the symptoms of the children could have contributed to information on the possible association of these viruses with more severe respiratory symptoms.

It would be interesting to compare the detection of RV in different sample types to identify RV types in different sample types and to find out how frequently RVs can pass through the alimentary tract. Unfortunately, nasopharyngeal swabs were not collected in the DIPP study, making these comparisons impossible to carry out. When planning prospective studies in the future it would be important to collect many types of samples to allow a wide range of analysis of several viruses and bacteria and thereby a comparison of the distribution of viruses in different sample types.

To conclude, the results in the current study show that RVs are frequently detected in the stool samples of children and that at least some of the RV types can retain their infectivity in stools, which was unexpected. This suggests that analyzing the presence of RVs in stool samples can contribute to the clinical diagnostics of RV infections in young children.

## 8.2 EVs are associated with T1D (Reports II, III and IV)

The results of Report III and IV suggest that EVs are associated with the initiation of the β-cell damaging process, whereas the results of Report II demonstrate that RVs are not associated with T1D. Report III was the first large-scale systematic study aimed at identifying EV types, which could be connected to the initiation of an autoimmune response leading to the destruction of β-cells. In addition, Report IV is the largest study so far, where the occurrence of EVs and the type of these viruses was analyzed in longitudinal stool samples in a prospective case-control study. The results of Report III showed that CV-B1 was associated with an increased risk of autoimmunity and two other types of CV-Bs, CV-B3 and CV-B6, were associated with a reduced risk of autoimmunity. The effects remained also in the children who progressed to clinical T1D.

There are several aspects that argue for a possible association between CV-Bs and T1D. First of all, the finding that the three serotypes, which were linked with T1D in the present study are closely related phylogenetically, speaks for a possible biological explanation for these results. If the results were due to chance, it would be unlikely that they would cluster together phylogenetically. Second, the results are consistent with previous reports suggesting that CV-Bs are associated with T1D (177). Third, the causative agent is probably frequent in the background population of children. Along with the prevalence of NABs against CV-B1 in the current study, CV-B1 has been one of the most frequent EVs in recent years in many countries (18, 29, 275-279). The fourth aspect is that CV-B1 can cause severe systemic infections in young infants (275, 280) and is one of the most cytolytic EV types in a human pancreatic islet model (171). Five, the protective association of CV-B3 and CV-B6 could be explained by immunological cross-protection induced by CV-B3 and CV-B6 against CV-B1. In fact, a protective association between CV-B3 and T1D has been reported in a study where patients with newly diagnosed T1D were less frequently positive for NABs against CV-B3 than control children (278). In the

present study, children who were infected by CV-B1, but none of the protective serotypes, had an increased risk of islet autoimmunity compared to children who experienced also protective CV-B types. This phenomenon was also related to the order of CV-B infections, as the children who were infected by CV-B1 before any other CV-Bs were at the highest risk of developing islet autoimmunity. The phenomenon has been described in a mouse model (281). Six, the CV-B1 maternal antibodies modulated the risk effect of CV-B1, which supports biological plausibility, since studies have demonstrated that maternal antibodies protect against EV infections (282, 283). Finally, the results were repeated with very similar OR in another study in the European populations (284).

In Report IV, none of the individual serotypes, which were detected in stool samples, was associated with the initiation of the β-cell damaging process, although some of the viruses were only detected in case children and some in control children. For example, CV-B3 which had a protective effect in Report III was only detected in control children, but the number of CV-B3 infections was low and this trend was not statistically significant. However, CV-B1, which was found to be a risk virus for developing autoantibodies in Report III, was not detected at all in stool samples. It is possible that these viruses are mainly respiratory in nature and stool samples are not optimal for their detection. In addition, the duration of virus excretion into stools may differ between different EV types. Thus, the collection interval of stool samples might have been too long to detect those EVs whose excretion period was short (the collection of stool samples in the DIPP study was not based on the symptoms of infections, but on a regular monthly schedule). In addition, the children in Reports III and IV were different with only a minor overlap, and the epidemiology of EV may have been different in these two cohorts. Other studies conducted at the same time period and near Finland have detected CV-B1 in stools with low detection rate. For example, in the Netherlands, CV-B1-6 were detected with annual rates of 0.1 to 0.4 % (20) and in Norway CBV1 was detected only during short intervals (27). Furthermore, a great proportion of EV positive samples remained un-typed because of the low copy number of the virus in these samples. The possible CV-B1 positive samples could be among these un-typed samples.

Overall, the case children tended to have more EV infections than control children. The excess of infections was detected about 1 year before the seroconversion to T1D-associated autoantibodies. None of the former studies conducted by analyzing stool samples for the presence of EVs in a prospective design have observed any

differences between case and control children (211-213). However, these studies have been much smaller than the current study and the statistical power of these studies to detect such time-dependent association between EVs and initiation of islet autoimmunity was poor. In addition, in the former studies a minority of the case children had progressed to T1D, whereas in the current study, 75% of case children were diagnosed with a clinical disease. Moreover, the circulation of EVs differs between populations and in different years, which may influence the association with T1D in different populations. Since the incidence of EV has been lower in Finland than in other studied countries in Europe (225, 226), it is possible that Finnish children are more susceptible to the diabetogenic effect of EVs. A lower frequency of EV infections in Finnish children may also make it easier to detect the association between an EV infection and T1D in Finland in this kind of cohort studies. Since the excess of EV infections was detected over 12 months before the appearance of the first autoantibody and the average age of autoantibody seroconversion was 23 months, these infections have occurred at a young age, already during the first years of life. A recent study showed that children who experienced a respiratory infection at a very young age, were associated with an increased risk of developing T1D later in life (285) suggesting possible importance of the viral infections at young age for the development of T1D.Since a large amount of EV positive stool samples remained un-typed due to a low amount of virus in these samples, these infections might possibly be respiratory infections and were passively passing thorough the gastrointestinal tract. Unfortunately, a respiratory sample was not available in the current study.

Interestingly, the time delay from the excess of EV infections to the appearance of autoantibodies was longer than that observed in Report III and in a previous study carried out by Oikarinen et al., in which the EV RNA peaked in serum samples 6 months prior to the seroconversion (218). The cohorts in these studies were different, so the lag period may vary from child to child. In addition, it is possible that the detection of viral RNA in serum reflects later stages of the infection, e.g. viral replication in internal organs such as the pancreas. The results from the MIDIA study showed that only 25% of children who had a blood sample positive for EV had a respective concomitant stool sample positive for EV also. (286). Alternatively, the association based on stool samples might not be connected directly to seroconversion, but rather demonstrates that the EV infections at a young age can activate the immune system in a way which makes a later infection by CV-B1 diabetogenic, and eventually enables the virus to initiate the disease process. For

example, EV infections at a very young age could modulate the permeability of the intestine making the child more susceptible to the diabetogenic effect of CV-B1 later in life. Further studies are needed to evaluate the time lag from EV infections to the seroconversion of T1D-associated autoantibodies and possible interactions between different EV types in this chain of events.

The association of the RV with T1D was analyzed since RV is the most commonly detected respiratory virus in all groups and is probably the most common causative agent of all acute infections in humans. Moreover, RV species belong to the same genus as EVs. The results of Report II suggest that RVs are not associated with T1D. However, the statistical analysis was performed using the chi squared analysis and not with a conditional logistic regression analysis, which takes into consideration the matching of the children as in Reports III and IV. Thus, the results of Report II should be analyzed with more advanced statistical methods to evaluate a possible association between RVs and islet autoimmunity (this work is in progress). In addition, the analysis of other enteric viruses such as rotaviruses and noroviruses could reveal important information about the specificity and mechanism of the observed association between EVs and T1D.

Both Report III and Report IV have important strengths, which increases their scientific value. First, the results in Report III are based on the analysis of NABs, which are a reliable way to detect EV infections caused by a certain EV type. In addition, the study covered a large number of different EV types, and is actually the largest such study carried out to evaluate the viral etiology of T1D. On the other hand, the number of stool samples analyzed for the presence of EVs in Report IV was high, and this study is the largest study carried out so far. In addition, both reports were conducted in a prospective birth-cohort study and a longitudinal sample series was used to analyze the timing of the infections in relation to the appearance of autoantibodies. Moreover, the case and control subjects in both reports were matched carefully for a HLA-defined diabetes risk, sex, time of birth, age at sampling, and the area of residence

 In conclusion, the results of Reports III and IV confirm the findings from previous studies suggesting an association between EV infections and the initiation of the β-cell damaging process. Putting all the published evidence of EV infections, especially CV-Bs, and T1D together, one can conclude that quite many of the Bradford Hill criteria for causality are fulfilled (287). The first criterion that was met is the strength

of association. The risk effect of CV-B1 on β-cell autoimmunity was detected in the current study and in five European countries (284) and is supported by observations from previous studies. The second fulfilled criterion is the consistency, as several epidemiological studies have demonstrated the association between EVs, particularly CV-Bs, with T1D (86, 169, 177, 179, 185, 186, 190, 195, 197, 198, 251, 259). In addition, the observation that the incidence of T1D rose after a CV-B5 outbreak supports causality (179). The third criterion is specificity, which was also met: Several other viruses have been analyzed for their possible association with T1D (including rubella, mumps, cytomegalovirus, rotavirus, retrovirus and Epstein-Barr virus) but thus far none of the analyzed viruses have shown such a strong association to T1D as EVs. In addition, the temporality criterion is fulfilled. In the current study, a CV-B1-related risk preceded the initiation of the autoimmune process. In addition, CV-B1 infections peaked a few months before the appearance of the first autoantibody, which is similar to the previously observed peak in the frequency of EV RNA in the serum 6 months before the appearance of T1D-associated autoantibodies (218). Moreover, the detection of EVs in stools peaked also before the seroconversion of the islet autoantibodies. Previous studies are in line with these observations showing that the incidence of T1D rose after a CV-B5 outbreak (179), arguing also for the temporality of EVs and T1D. The observation of the current study that the risk and protective viruses align to a small group of phylogenically close EVs, and the discovery of "protective" viruses, fits with immunological cross-protection. In addition, the observation that the maternal CV-B1 antibodies modulate the risk effect of CV-B1 infections in a child, argues for biologic plausibility. Finally, the coherence criterion is also fulfilled. Previous studies have demonstrated that EVs have tropism for the pancreas (168-171, 174, 232) and infect β-cells (174, 195, 288). Moreover, it has been demonstrated that CV-B4 isolated from human pancreas can cause T1D also in mice (197) and CV-B1 can cause a persistent infection in mice (289). Several independent studies have observed the presence of EVs in the pancreas of T1D patients (174, 195, 197, 234, 240, 288)

However, even if many criteria for causality have been fulfilled, the true causality can only be proven by eliminating or preventing the initiating factor. The elimination of EVs would be possible by a vaccine, which targets T1D-associated EVs, and such a vaccine could be used in clinical trials to test the possible causality of this association. The identification of a possibly diabetogenic type, CV-B1, was a novel finding in the current study and offers possibilities for further studying the mechanisms of EV-induced diabetes and enables the development of an EV vaccine in the future. As

argued in Cambridge bioethics (290), innovation is necessary if we wish to gain new ways to serve patients and acquire scientific knowledge, which might lead to actual clinical trials to test vaccine against EVs. In fact, the first initiative steps have recently been taken in order to develop such a vaccine as Larsson et al tested a formalin-inactivated CV-B1 vaccine in mice. This experimental vaccine was highly immunogenic and protected the mice against a CV-B1 infection. In addition, the vaccine appeared to be safe since it did not accelerate the development of diabetes in the NOD mouse model (291). This suggests that a vaccine for human use is a feasible goal.

## 8.3    Limitations of the study

The study has some important limitations, which should be considered when interpreting the results. One of these limitations is that all four Reports were conducted using samples from one population, Finnish children, and hence the study represents the epidemiology of EVs in Finland only. Moreover, this study analyzed only two virus species which are a very small fraction of the all species of the virome of the gut, let alone the entire microbiome of the gut.

In all Reports, the laboratory methods have been carefully chosen and optimized for the purposes of the present study. In addition, negative and positive controls were always used in all steps of the laboratory analyses to ensure the validity of the results. Regardless of this, the methods have weaknesses, which cannot be fully controlled even in an optimal environment. One such major weakness is the proneness of PCR to contaminations, which may lead to false positive findings. Since special attention was paid on avoiding cross-contamination between samples in all steps of the process and since negative control samples were always analyzed along with real samples, the probability of such contaminations is very low. In addition, positive findings were confirmed by the repeated testing of the sample. However, it cannot be excluded that false positive findings in a minority of samples could have occurred, especially if the sequencing of the EV positive sample by primers other than those used in the primary screening was not successful.

Furthermore, the current study lacks respiratory samples. Since many EV types often cause respiratory infections, detecting them in stools may lead to an underestimation of their true frequency in the Finnish population. This issue is relevant in Report IV. In addition, Report II would have benefited from respiratory samples, as it would have then been possible to compare the prevalence and distribution of respiratory viruses, RVs, in different sample types.

Since the prevalence of EV-A71 is rare in Finland, and only four samples were obtained from hospitalized patients and only 14 samples were positive in the DIPP study, it was difficult to obtain good statistical power for the phylogenetic analyses of these virus strains. Thus, the seemingly more virulent strain detected in 2007, might be a finding caused by chance.

The association of EVs and T1D was studied only in the Finnish child population, which limits the generalization of these findings to other populations. The circulation of EVs differs between populations and between years, and this might influence their association with T1D in different studies. Consequently, the risk effect of CV-B1 in Report III might be a strain-specific effect of this EV type, and other EV types may be associated with T1D in other populations and time periods. The virus strains used in Report III represents the most common types of EVs, but not all of them. Therefore, a possible risk or protective associations of those EV types, which were not studied in Report III might have been missed. Furthermore, the statistical power of Report III allowed the identification of viruses with major risk effects, whereas viruses with weaker effects might have been missed.

Even though the study in Report IV is the largest such study carried out so far, the relatively low number of EV-positive stool samples limited the statistical power for studying a possible association between individual EV types and T1D. In addition, a large number of positive samples remained un-typed due to a low number of virus in these samples. Thus, it is possible that these un-typed samples contain EV types, whose association with T1D might have been missed. Moreover, the prevalence of some EV types might have been underestimated because of the lack of respiratory samples.

The case and control children in Reports III and IV were matched for their sex, age, area of residence and HLA-DQ type. In spite of this rather tight matching it is possible that some other factors such as non-HLA genes could influence the detection of EVs. However, the effect of some of these factors was excluded. For

example, the risk effect of CV-B1 remained even after the results were adjusted for the duration of breastfeeding. On the other hand, this kind of tight matching might carry the risk of losing true risk-associations due to overmatching of the children. In such a scenario, the true associations would be stronger than those observed in the present study.

# 9    Conclusions and future prospects

The aim of the current study was to analyze the epidemiology of EVs in stool samples, as well as serological markers of infections in children in Finland. In addition, the EV types that could be involved in the initiation of the disease process in T1D were analyzed. Knowledge about the diabetogenic EV types would enable the development of a preventive vaccine in the future.

The outcomes of the currents study demonstrate that a variety of EVs are circulating in Finland, although it seems that EVs are rarer in Finland compared to other countries. Moreover, the results showed that RVs, which are considered respiratory viruses, are frequently detected in stool samples. In addition, it seems that these viruses can retain their infectivity in stools, against all expectations. The results of the current study confirmed that EVs are involved in the T1D disease process. The association of EVs and the β-cell damaging process was observed for the first time in a prospective study using stool samples. In addition, one type of EVs, CV-B1, was identified as diabetogenic, while two other closely related types were associated with a protective effect against T1D. These are novel findings and contribute to the development of a vaccine in the future.

The causality of EVs to T1D is difficult to prove in prospective studies, even if the number of children and stool samples are large, as was the case in the current study. International collaboration is important and the results obtained in the current study should be confirmed in other studies In addition, the analysis of other viruses could reveal the specific association of EVs to T1D. Moreover, since respiratory infections at a young age were associated with an increased risk of T1D, the symptoms of the children in the current study could be analyzed and the EV positivity could be linked to respiratory symptoms.  In addition, in future studies it is important to collect many types of samples, such as blood, stool, saliva and respiratory samples, and to use a variety of different laboratory methods to analyze the samples in order to obtain comprehensive results of the association of individual EV types and T1D. Actually, the next procedure to prevent T1D could be to develop a vaccine against CV-Bs or test antiviral medications against EVs in children with a genetic susceptibility to

T1D. In addition, with the novel method of next generation sequencing, valuable data of the possible differences in the microbiome between case and control subjects will enable novel ways to modulate the immunological responses and treat children at risk of T1D.

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# 12 Original publications

# Human enterovirus 71 strains in the background population and in hospital patients in Finland

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## ARTICLE INFO

## ABSTRACT

*Background:* Human enterovirus 71 (HEV71) is a common cause of severe outbreaks of hand-foot- and mouth disease, aseptic meningitis and encephalitis in Asian populations but has not caused such epidemics in all populations.

*Objectives:* To analyze the frequency of HEV71 in the background childhood population in Finland by screening in stool and serum samples and by measuring neutralizing antibodies against HEV71 in serum and to compare the genetic relationship of virus strains detected in asymptomatic children and those causing severe illness in Finland to the strains found in other countries.

*Study design:* 4185 stool samples and 5686 serum samples were collected and clinical symptoms recorded from children who were observed from birth. Additional stool samples were available from four children hospitalized due to EV71 infection. Samples were screened for the presence of RNA of human enteroviruses using RT-PCR and HEV71 amplicons were identified by sequencing. Phylogenetic analyses were carried out to study genetic relationships between different virus strains. Neutralizing antibodies against HEV71 were screened from 522 children.

*Results:* A total of 0.3% of stool samples and two serum samples from healthy children were positive for HEV71 genome. 1.6% of the children had neutralizing antibodies against HEV71. Most infections were asymptomatic or mild in contrast to the clear symptoms in the children hospitalized due to HEV71. All viruses were C strains.

*Conclusions:* HEV71 is circulating in Finland but it is rare. No clear difference was seen between strains circulating in the Finnish background population and those found in hospitalized patients or those causing severe outbreaks worldwide.

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## 1. Background

Human enterovirus 71 (HEV71) has been associated with outbreaks of severe hand-foot- and mouth disease (HFMD), aseptic meningitis and encephalitis and it is the most common non-polio enterovirus that is associated with poliomyelitis-like paralysis.[1,2] HEV71 has caused outbreaks worldwide since its first isolation in 1969 in North America.[3] In Europe outbreaks have occurred in Bulgaria in 1975 and in Hungary in 1978,[4,5] and severe outbreaks have been reported from Malaysia in 1997,[6] from Taiwan in 1998,[7] from Australia in 1999,[8] from Singapore in 2000,[9] from Japan in 1997 and 2000[10,11] and from China in 2008.[12] HFMD outbreaks in Asia are most likely much more frequent than currently reported.

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HEV71 includes three genogroups. The genogroup A consists of the original isolate[3] as well as viruses isolated 2008 in China.[13] The major genogroups B and C include subgroups B1–B5 and C1–C5, respectively.[14–16] C1 strains have been mainly detected in sporadic cases rather than in epidemics suggesting low-level of circulation worldwide. On the other hand C2 strains have caused outbreaks in Asia and Australia. More recently identified C strains (C3–C5) and most of the B strains have caused epidemics in Asia-Pacific region.[17] Recently a new genogroup D has been implicated.[18,19] The two major epidemics in Malaysia in 1997 and Taiwan in 1998 were caused by two quite distinct strains (genogroups B3 and C2)[6] and genogroups B4, B5, C2 and C4 have caused epidemics of HFMD in Japan.[15] Co-circulation of mixed HEV71 populations and variability in their mutual recombination frequencies can generate hybrid viruses which differ for their virulence.[20,21] In fact, a mutant form of HEV71 had attenuated neurovirulence in monkeys[22] and HEV71 C1 strains isolated in Norway from asymptomatic children differed from the strains causing severe diseases in Asia.[23] Neutralizing antibodies against HEV71 have been observed in 1–71% of children in different populations.[24–28]

## 2. Objectives

HEV71 has been widely studied especially in Asia, but we still do not know why some HEV71 strains circulate in the population without causing any disease while other strains result in severe complications. The main objective was to study the molecular epidemiology of HEV71 in Finland and compare it to HEV71 infections in other populations to obtain information on factors regulating the severity of the infection.

## 3. Study design

### 3.1. Study subjects

#### 3.1.1. The prospective DIPP study

Altogether 4184 stool samples were collected from 359 children under 6 years of age and 5686 serum samples from 928 children under 11 years of age in the prospective DIPP (Type 1 diabetes Prediction and Prevention) Study in Finland.[29] In this study children with HLA-conferred susceptibility for type 1 diabetes were invited to prospective follow-up starting from birth. The present study is based on longitudinal stool samples collected with interval of 1 month starting from the age of 3 months (on average 11 samples per child from the years 1996–2008) as well as blood samples and clinical symptoms recorded every 3–12 months in DIPP study centers (on average 6 samples per child from the years 1994–2010). Neutralizing antibodies against HEV71 were analyzed from a single sample of 505 DIPP children (mean age 2.5 years, range 5 months to 10 years). The annual number of stool samples varied from 251 to 526 per year (except years 1996, 2007 and 2008 when less than 100 samples were available per year). The number of serum samples varied from 124 to 891 per year (except years 1994, 2008, 2009 and 2010 when less than 100 samples were available per year).

#### 3.1.2. Hospital patients with acute HEV71 infection

Stool samples were collected from four children who had been hospitalized due to an acute HEV71 infection. The mean age of these children was 2 years and stool samples were taken in the hospital ward during the acute phase of their illness (all patients were from the Hospital district of Helsinki and Uusimaa).

#### 3.1.3. Statistics from clinical virus laboratories in Finland

Statistics of three major clinical virus laboratories in Finland were used to identify HEV71 infections which have been diagnosed in hospital patients or patients attending health-care centers in Finland. These statistics included infections which have been confirmed by specific detection of the virus using PCR or virus isolation from throat swab, stool or CSF samples. There were altogether 3264 confirmed enterovirus infections. These three clinical virus laboratories operate in different parts of Finland (HUSLAB in the city of Helsinki, Laboratory Center of Tampere University Hospital in the city of Tampere, and Department of Virology at the University of Turku in the city of Turku) covering about 70% of all virus diagnoses in Finland. The statistics of HUSLAB covered the years 1990–2007 and those of the two other laboratories years 1996–2009. However the statistics from Tampere are missing from years 2007 to 2009.

### 3.2. RNA extraction and RT-PCR

Stool and serum samples were systematically screened for the presence of enterovirus RNA using RT-PCR as previously described.[30] First, a 10% stool suspension was prepared from the original stool sample and viral RNA was extracted using the MagNaPure extraction robot (Roche, Applied Science, Mannheim Germany) and Total Nucleid Acid extraction kit (Roche, Applied Science). RNA from 3618 serum samples was extracted with QiaAmp Viral RNA mini kit (QIAGEN, Germany). Viral RNA from both serum and stool samples was reverse transcribed and amplified with a previously described PCR method.[30] 2077 serum samples were first extracted with modified Qiagen RNeasy96 kit (QIAGEN, Germany) and then analyzed with real time PCR using QuantiTect Probe kit (QIAGEN, Germany). The real time PCR run was performed according to the instructions on the Quantitect Probe kit using Taqman chemistry. The primers and probes used in the method were: forward primer; CGG CCC CTG AAT GCG GCT AA, reverse primer; GAA ACA CGG ACA CCC AAA GTA, probe 1; FAM-TCT GTG GCG GAA CCG ACT A-TAMRA, probe 2; FAM-TCT GCA GCG GAA CCG ACT A-TAMRA.

### 3.3. Sequencing and phylogenetic analysis

The identification of EV71 specific sequences was performed by sequencing the VP1 region of the viral genome according to Nix et al. 2006.[31] A GenBank database search for the partial VP1 sequences was performed using BLAST. Phylogenetic analysis of HEV71 is based on VP1 sequences and the phylogenetic tree was constructed using neighbor-joining method.

### 3.4. Measurement of neutralizing antibodies

HEV71 strain which was used in neutralizing antibody assay was isolated from a stool suspension of one DIPP child in LLC-MK2 cells (ATCC CCL-7.1). It was first plaque purified in LLC-MK2 cells and then passaged several times in Vero cells (ATCC CCL-81). The virus was re-sequenced to verify its genotype and virus titer was determined using the end-point dilution method. The serum microneutralization assay was performed by mixing equal volumes of serum and virus dilutions and incubating these mixtures for 1 h at 37 °C and then O/N at RT. After incubation the mixtures were transferred into wells of 96-well plates being subsequently seeded with 10 000 Vero cells per well. The cytopathologic effect (CPE) was examined on day 7.

## 4. Results

### 4.1. Circulation of HEV71 in the background population

In the series of DIPP children, altogether 4184 stool samples from 359 children and 5686 serum samples from 928 children were analyzed for the presence of enterovirus RNA using RT-PCR. Enterovirus RNA was detected in 307 stool samples (7.3%) and in

**Table 1**
Summary of samples.

|  | Stool samples | Serum samples |
|---|---|---|
| N | 4185 | 5686 |
| Enterovirus positive | 307 | 115 |
| Successfully typed | 222 | 34 |
| HEV71 positive | 12 | 2 |

115 serum samples (2.0%). The sequence analysis of VP1 region was successful in 222 (72%) enterovirus positive stool samples and in 34 (30%) of enterovirus positive serum samples. HEV71 RNA was present in12 stool samples and in two serum samples (Table 1). Accordingly, altogether 0.3% of all stool samples and 5.4% of successfully typed enterovirus positive stool samples contained HEV71. These HEV71 positive samples were obtained in the years 1997, 1998, 2000, 2002 and 2007. One child was HEV71 positive in three consecutive samples covering a total period of 2.5 months and another child was positive in two consecutive samples taken 21 days apart. None of these children had severe disease or hospital visits during their infection. The symptoms which were recorded from these children included common flu, chickenpox and otitis (Table 2), suggesting that these infections had been subclinical or very mild. However, two children had symptoms of HFMD. The other children had symptoms 2 months before the HEV71 positive stool sample was taken. We do not have information available if these children needed hospital treatment due to the HFMD symptoms. One of the HEV71 positive serum sample was obtained in 1999 and the other in 2002 (Tables 1 and 2).

### 4.2. Detection of HEV71 in clinical virus laboratories and hospitalized patients

Altogether 3264 enterovirus infections were diagnosed in the three clinical virus laboratories during the years 1990–2009. HEV71 was isolated in 11 cases (one in 1999, three in 2000 and 7 in 2007). To our knowledge four patients had severe disease: One of them suffered from acute encephalitis, one had suspected encephalitis, one had fever and suspected CNS infection and one had high fever. All these patients were diagnosed in 2007 (Table 2).

### 4.3. Molecular characterization of HEV71 strains

The genetic relationships of the detected HEV71 strains are shown in Fig. 1. (See supplementary material for the clinical isolates used in phylogenetic analysis of the complete VP1 gene of HEV71.) All HEV71 strains from both DIPP children and hospital patients belonged to the genogroup C. Three of them grouped together with C1 substrains and seven with C2. All four strains from hospital patients were detected in 2007 and grouped close to each other belonging to the C2 genogroup. Two DIPP stains from the same year belonged to this same sub-cluster while all other Finnish C2 strains were genetically different. Three DIPP strains could not be typed because of their poor sequence quality of the PCR amplicon.

### 4.4. Prevalence of neutralizing antibodies in background population

Altogether 1.6% of the 505 children had neutralizing antibodies against HEV71. In age group less than one year the seroprevalence
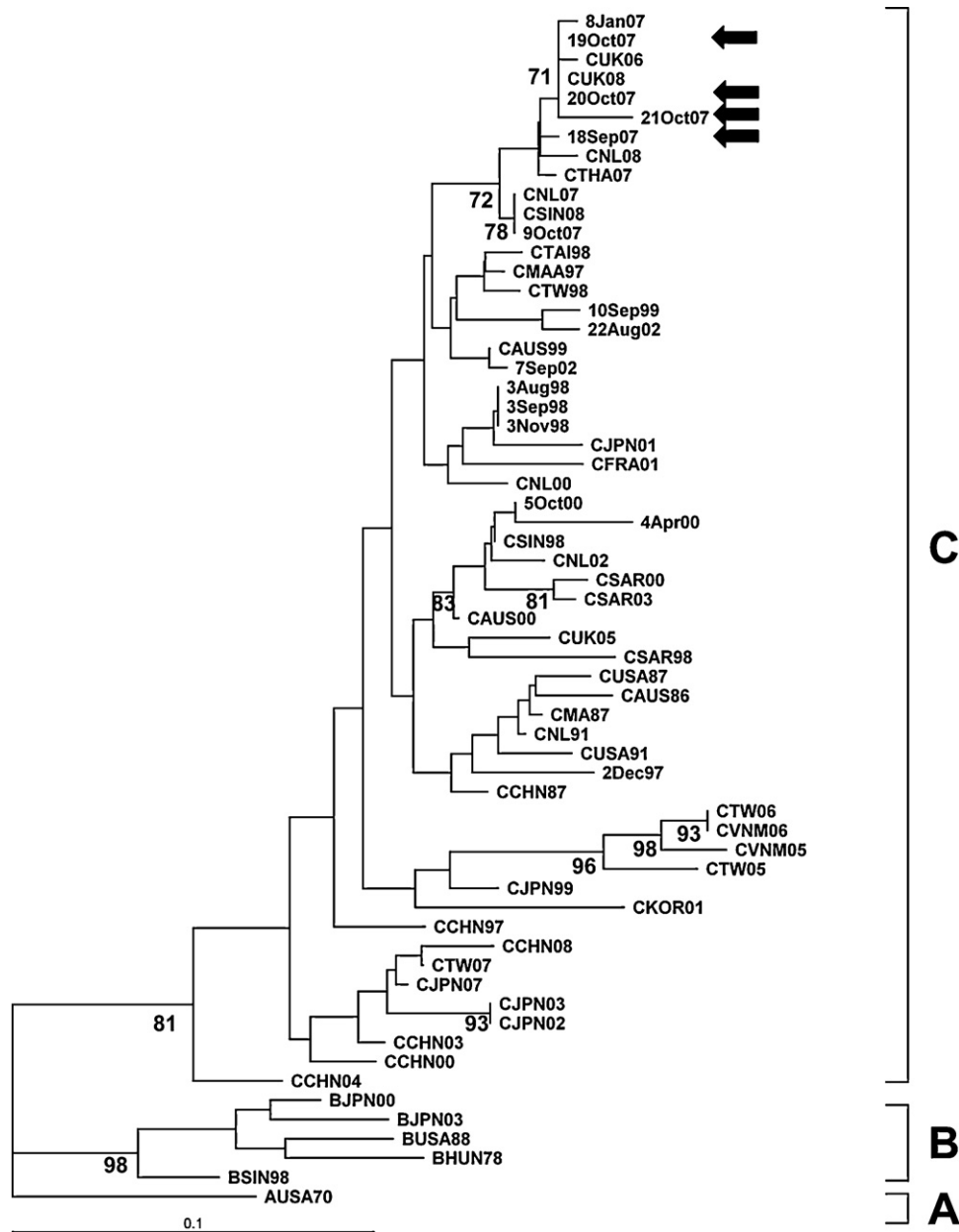
**Table 2**
Summary of HEV71 positive subjects.

| Sample type | Sample ID | Virus isolated (month/year) | Sex | Age of the child (y) | Symptoms | Virus genogroup |
|---|---|---|---|---|---|---|
| **Background population (N = 10)** | | | | | | |
| Stool | 1Sep97[a] | 9/1997 | Female | 1 | Flu | – |
| Stool | 2Dec97[a] | 12/1997 | Male | 1 | Otitis | C1 |
| Stool | 3Aug98[a,e] | 8/1998 | Female | 2 | Otitis | C2 |
| Stool | 3Sep98[a,e] | 9/1998 | Female | 2 | No symptoms | C2 |
| Stool | 3Nov98[a,e] | 11/1998 | Female | 2 | Flu | C2 |
| Stool | 4Apr00[c] | 4/2000 | Female | 2 | No symptoms | C1 |
| Stool | 4May00[c] | 5/2000 | Female | 2 | No symptoms | – |
| Stool | 5Oct00[b,e] | 10/2000 | Female | 2 | No symptoms | C1 |
| Stool | 6Oct00[b,e] | 10/2000 | Female | 2 | HFMD | – |
| Stool | 7Sep02[b] | 9/2002 | Male | 2 | No symptoms | C2 |
| Stool | 8Jan07b | 1/2007 | Male | 6 | Chickenpox, and 3 flues during the year | C2 |
| Stool | 9Oct00b | 10/2007 | Female | 2 | HFMD 08/2007 | C2 |
| Serum | 10Sep99[a] | 09/1999 | Male | | Gastrointestinal symptoms | C2 |
| Serum | 22Aug02[b] | 08/2002 | Male | 2 | No symptoms | C2 |
| **Hospitalized children (N = 11)** | | | | | | |
| Blister in finger | 11[d] | 1999 | Male | NK | NK | NK |
| Blister in tongue | 12[d] | 2000 | Female | NK | NK | NK |
| Stool | 13[d] | 2000 | Male | NK | Headache, dizziness | NK |
| Blister in hand | 14[d] | 2000 | Female | NK | HFMD | NK |
| NK | 15[a] | 2007 | NK | NK | NK | NK |
| NK | 16[a] | 2007 | NK | NK | NK | NK |
| NK | 17[a] | 2007 | NK | NK | NK | NK |
| Stool | 18Sep07[d] | 9/2007 | Male | 2 | Encephalitis | C2 |
| Stool | 19Oct07[d] | 10/2007 | Male | 4 | Suspected encephalitis | C2 |
| Stool | 20Oct07[d] | 10/2007 | Male | 1 | Fever, suspected CNS | C2 |
| Stool | 21Oct07[d] | 10/2007 | Male | 1 | Fever | C2 |

NK, not known.
[a] Turku region.
[b] Tampere region.
[c] Oulu region.
[d] Helsinki region.
[e] Samples of one child.

**Fig. 1.** Phylogenetic analysis of HEV71 based on the VP1 sequences. The phylogenetic tree was constructed by the neighbor-joining method and rooted using a prototype A strains as an outgroop. Boothstrap values more than 70% values are indicated in the figure. Finnish wild strains are named with running number and with the month and a year when the virus was isolated (e.g. 1Sep97). Multiple samples from one child are named with same running number (e.g. 3Aug97, 3Sep97 etc.). The strains of the hospitalized children are marked with an arrow.

was slightly higher (2.6%) which could be due to the presence of the maternal antibodies. In summary, the prevalence of HEV71 antibodies was very low (only 0.5% among 1–1.9 years old children).

## 5. Discussion

Our results from the prospective study on healthy children demonstrate for the first time that HEV71 is circulating in children in Finland. However, it seems to be relatively rare, since only 0.3% of the stool samples and only two serum samples collected from background children population were positive for the virus and the frequency of neutralizing antibodies was also low, being only 1.6%, respectively. The occurrence of HEV71 was even lower than that reported in Norway where HEV71 was detected by RT-PCR in 1.4% out of 1255 stool samples in a similar birth-cohort study.[23]

Luo et al. showed that the seroprevalence in mothers was strongly associated with seroprevalence in their newborn infants but only 1% of these children had neutralizing antibodies at 6 months of age.[32] In an identical study in Singapore, none of the children had maternal antibodies at 1 month of age.[27] Furthermore, in Germany, 27% of healthy children less than 4 years of age had neutralizing antibodies against HEV71 compared to 75% of persons aged 20–40 years, respectively.[24,25] A similar phenomenon was also described in Brazil.[26]

On the other hand, the prevalence of neutralizing antibodies in children less than 2 years of age has been reported to be only 1% in Singapore[27] and 4–8% in Taiwan,[28] although the virus has commonly been isolated in both countries and caused severe epidemics in Taiwan. Altogether, this suggests that HEV71 may be even rarer in Finland than in many other countries. This is in line

with our previous observations showing that other enteroviruses were also less frequent in Finland than in other European populations studied.[33] Thus, it is possible that the circulation of HEV71 depends on the same factors as that of other enteroviruses making them relatively rare in Finland.

HEV71 was detected in different parts of Finland and in different years. In spite of this, HEV71 was not detected in the national sewage sample testing during these years, except 2007 when it was found for the first time (personal communication by Merja Roivainen from the National Institute for Health and Welfare in Finland). This sewage screening is part of the national polio surveillance program.[34] This suggests that HEV71 is detectable if present in large enough quantities. Altogether, this supports the conclusion that the circulation of HEV71 has been on a relatively low level in Finland during this study. However, it is also possible that HEV71 is less resistant to the rather hostile environment in sewage than the other enteroviruses regularly found in sewage samples.

Most of the detected HEV71 infections were mild or even asymptomatic. Viral strains from both background population and hospitalized children in this study belonged to lineage C grouping together with the strains in C1 and C2 lineages (Fig. 1). Witso et al. showed that C1 was the only lineage circulating in Norway during an overlapping time period (years 2000–2003).[23] This suggests that different virus strains may circulate in close geographic areas such as these two neighboring countries. However, it is also possible that these kinds of surveys cannot pick up all circulating virus variants due to their rareness in the population. Finnish C1 and C2 strains align with HEV71 strains isolated in other countries in particular year (Fig. 1). Thus, it seems that C1 and C2 strains do circulate in Finland showing annual variation. In addition, multiple strains may have co-circulated in some years even though only one strain was detected.

Interestingly, all the strains of hospitalized children and the one strain from a child with symptoms of HFMD which all were isolated in 2007 align together in a separate group of C2 strains. This suggests that these strains may have been more virulent than the other strains detected in Finland. C2 was also circulating in other countries in Europe in 2007. It has been reported in Denmark, France and Netherlands but it was not the sole strain circulating in that year.[25,35–38] However, all of these European strains belong to C lineages different than the strains which have caused severe HEV71 infections for example in Taiwan and China in 2006 to 2008 (C4 and C5 strains)[12,16] (Fig. 1). Taking altogether, it seems that C2 was much more invasive in 2007 than in other years and was able to spread to several European countries.

Altogether, these findings show that HEV71 is rare but it has been circulating in Finland for several years without causing such severe epidemics or central nervous system diseases as described in some Asian countries. Monitoring EV71 circulation in Europe is important because of the possible importation or emergence of more virulent B and C strains. This has happened for example in Japan where C2 was the cause for outbreaks in 1998 and 1999 but already in 2000 B4 and in 2003 B5 and C4 emerged.[17] The virus strains detected in Finland (C1 and C2) do not represent the same strains that cause severe epidemics in Asia (C3–C5) although they are from the same C lineage. Thus, the reason for the lack of such epidemics in Finland may be due to point mutations in circulating strains or other factors limiting the spread of HEV71 in the Finnish population.

## Competing interests

H.Hy. and M.K. are minor (<5%) shareholders and members of the board at Vactech Ltd., which develops vaccines against picornaviruses. No other potential conflicts of interest relevant to this article were reported.

## Ethical approval

DIPP study protocol has been accepted by the Ethical committees of the participating University Hospitals and a written informed consent was obtained from all participating families.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2012.11.018.

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# Human rhinoviruses including group C are common in stool samples of young Finnish children

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## ARTICLE INFO

## ABSTRACT

*Background:* Human rhinoviruses (HRVs) are common causes of viral respiratory infections. They have been widely studied in respiratory samples in hospital patient series but only a few studies have been performed to assess their occurrence in other sample types and their circulation in healthy children background population.
*Objectives:* To analyze the frequency of HRVs in the background population in Finland by screening HRV RNA from stool samples longitudinally collected in a cohort of young children.
*Study design:* Altogether 4184 stool samples were collected regularly from a cohort of children who were observed from birth. Samples were screened for the presence of RNA of HRVs using RT-PCR. HRV specific sequences were identified by sequencing the VP1 or VP4/VP2 coding region. Virus isolation was performed using four different cell lines and the result was confirmed by real time PCR.
*Results:* A total of 9% of the stool samples were positive for HRV RNA. Sequence analysis indicated that the most prevalent species was HRV-A, and the most prevalent serotype was HRV61. HRV-B and HRV-C species were also detected. One of the six tested rhinovirus positive samples retained its infectivity and was able to grow in RD and GMK cells.
*Conclusions:* Our study shows that HRVs are frequently detected in the stool samples from the population of young children. We also show that HRV-C, which can cause severe illnesses in children, is commonly circulating in young children in Finland.

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## 1. Background

Human rhinoviruses (HRVs) belong to the *Enterovirus* genus of the *Picornaviridae* family. Enterovirus genus includes more than 100 enterovirus serotypes and more than 100 HRV serotypes. HRVs are further divided into two main species, A and B, based on their anti-viral drug sensitivity pattern[1] along with molecular analysis and partial genome sequencing.[2,3] A new and potentially more pathogenic species C of HRVs has also been discovered.[4,5,6–8] This group appears to be to as different from HRV species A and B as it is from other enteroviruses.[9] Sequence divergence in VP1 and in other external parts of the capsid of HRV-C species is also greater than that in HRV-A and HRV-B which may imply larger antigenic variation in HRV-C species. In addition, HRV-A and HRV-B species have phylogenetically distinct sequences in 5′ untranslated region while most HRV-C variants resemble species A variants in this genome region, the remaining HRV-C variants form a phylogenetically separate group which is distinct from both HRV-A, and HRV-B as well as from all other enterovirus species.[10]

HRVs are the most common cause of upper respiratory infections (URIs) and play also a role in their complications such as otitis media. The importance of HRV-C in respiratory disease is still controversial; some studies show no difference in clinical outcome between different HRV species,[7,11] whereas others provide evidence for an important role of HRV-Cs in lower respiratory tract

infections, including wheezing and asthma exacerbations.[12,13] In contrast to others, Arden et al. described a shorter duration of asthma symptoms in HRV-C infections than in HRV-A infections[14] and Calvo et al. reported that HRV-C infections were not more severe than HRV-A infections.[15] However, the frequent detection of HRV-C viruses in hospitalized children indicates that HRV-C viruses may be more pathogenic than other HRVs.

HRVs have been considered as respiratory pathogens since they are commonly detected in respiratory samples. In contrast, they are not generally believed to replicate in intestinal mucosa, since in contrast to other enteroviruses, HRVs are sensitive to low pH, which can destroy their infectivity in the stomach. This effect is thought to be due to conformational changes in the viral capsid proteins at pH 6.2, leading to loss of the VP4 subunit, which renders the virus noninfectious.[16] On the other hand, Skern et al. showed that a single amino acid change in the capsid protein of HRV-B14 is sufficient to confer resistance to inactivation when incubated in low pH suggesting that this amino acid change regulates increased stability at low pH.[17] We and others have previously shown that HRVs can also occasionally be detected in stools in young children[18–20] but large-scale systematic studies are lacking. In addition, Blomqvist et al. showed that HRVs can be detected in the sewage and that these environmental isolates are also acid sensitive, even though the physiological conditions of the sewage seem not to destroy their infectivity.[3]

In the current study we show that HRVs are frequently detected in stools in a large cohort of young longitudinally observed children. These viruses represented all HRV species including HRV-C.

## 2. Objectives

Rhinoviruses have been studied in hospitalized children but to our knowledge only few studies have been carried out to assess the epidemiology of HRVs in the background population. In addition, no systematic studies are available on the occurrence of HRVs in stool samples. The objective was to find out whether HRVs can be detected in stool samples collected from a large cohort of young children and which HRV types are circulating in the background population.

## 3. Study design

### 3.1. Study subjects

Altogether 4184 stool samples were collected from 359 children participating in the prospective DIPP study (Diabetes Prediction and Prevention Study) in Finland.[21] In this study, children with HLA-conferred susceptibility for type 1 diabetes (about 12% of all infants) were invited to prospective follow-up starting from birth. 202 of the 359 children turned positive for diabetes-predictive autoantibodies and 63 of them progressed to clinical type 1 diabetes (diabetes case children). The rest of the children have remained autoantibody negative and non-diabetic (control children) and were pairwise matched with the diabetes case children for the time of birth, gender and HLA-DQ type.[21]

Longitudinal stool samples were collected at an interval of 1 month starting from the age of 3 months, except one child who started to collect samples at the age of 1 month. Samples were collected by parents at home and were shipped to the virus laboratory by mail in ambient temperature (altogether an average of 11 samples per child was collected during the years 1996–2009). The maximum age when a sample was obtained was 76 months and 94% of the samples were collected before the age of 3 years. Sixty four percent of all samples were from boys. The annual number of

samples varied from 130 to 380, except in years 1996, 2007 and 2008 during which less than 100 samples were collected.

### 3.2. RNA extraction and RT-PCR

Stool samples were systematically screened for the presence of HRV RNA using RT-PCR as previously described.[22] First, a 10% stool suspension was prepared from the original stool sample in cell culture medium and RNA was extracted using the MagNaPure extraction robot (Roche Diagnostics, Mannheim, Germany) and Total Nucleid Acid extraction kit (Roche Diagnostics, Mannheim, Germany). RNA was reverse transcribed and multiplied with a previously described PCR method, which amplifies all rhinovirus types.[22] Cell culture isolation samples were confirmed with real time PCR. The run was performed using the QuantiTect Probe kit (QIAGEN, Germany) with Taqman chemistry and with these primers and a probe; forward primer 5'-CYA* GCC T*GC GTG GC -3' (A* and T* locked nucleic acid primer by Exiqon); reverse primer GAA ACA CGG ACA CCC AAA GTA and probe VIC-TCC TCC GGC CCC TGA ATG YGG C –TAMRA.

### 3.3. Sequencing and phylogenetic analysis

Detected HRVs were molecularly typed by sequencing the VP1 or VP4/VP2 region of viral genome with previously described primers.[23] A GenBank database search for the partial VP1 or VP4/VP2 sequences was performed using BLAST and phylogenetic analyses using Phylip program package version 3.69 (Felsenstein, J. 1993. PHYLIP: phylogeny inference package, version 3.69. University of Washington, Seattle).

### 3.4. Isolation of viruses in cell culture

Virus isolation was carried out for six rhinovirus RNA positive samples and three rhinovirus RNA negative samples in GMK, Hela, RD and A549 cells at two different temperatures (34 °C and 37 °C). MEM-based medium was used for GMK, Hela and RD cells and HAM F12-based medium for A549 cells supplemented by 2% FBS and antibiotics. Cytopathic effect was observed daily for 3 weeks and possible replication of the virus was analyzed by detecting viral RNA from lysed cells by real-time RT-PCR (same methods as described above).

### 3.5. Statistical analyses

Statistical significance of possible differences between different study groups was analyzed using Chi-Square test.

## 4. Results

Altogether 438 (10.5%) of the 4184 stool samples collected from 359 children were positive for HRV RNA in RT-PCR analyses. The detection of HRV followed a clear seasonal pattern peaking in the period from August to October (30% of all positive samples were taken during this period, Fig. 1.). HRV was seen at all ages, the youngest being 3 months of age and oldest being 61 months of age. Most of the positive samples (70%) were taken from children less than 12 months of age and the proportion of HRV positive samples peaked at the age of 4 months (12%). The detection of HRV was not associated with the gender of the child, HLA genotype or presence of diabetes-associated autoantibodies (Table 1).

We randomly chose 71 of HRV positive samples for the sequencing of the VP1 and VP4/VP2 regions of the viral genome to identify the type of detected HRVs. In 63 of these samples the sequencing was successful and the type of HRV could be identified. The most prevalent species was HRV-A but HRV-B and HRV-C species

**Table 1**
Detection of human rhinoviruses in stool samples according to gender, HLA genotype and the presence of diabetes-associated autoantibodies (T1D aab).

| | HRV positive samples (*N*) | HRV negative samples (*N*) | Total (*N*) | Proportion of positive (%) |
|---|---|---|---|---|
| **Gender** | | | | |
| Girls | 160 | 1339 | 1499 | 10.7 |
| Boys | 278 | 2407 | 2685 | 10.3 |
| | | | | *p* = 0.78 |
| **HLA-DQB1 genotype** | | | | |
| High risk[a] | 275 | 2478 | 2753 | 10.0 |
| Moderate risk[b] | 163 | 1268 | 1431 | 11.4 |
| | | | | *p* = 0.17 |
| **T1D aab** | | | | |
| aab positive | 142 | 1181 | 1323 | 10.7 |
| aab negative | 296 | 2565 | 2861 | 10.3 |
| | | | | *p* = 0.7**4** |

[a] HLA-DQB1*02/*0302 or *0301*/0302.
[b] HLA-DQB1*02/*0604 or *0301/*0302 or *02/*x* or *0302/*x* or *0302/*0604 or *0302/*0501 or *0302/*0603 or *0302/*04 or *0302/*0302.

were also commonly detected. Species A viruses were identified in 79% (50/63) of all HRV positive samples and species B viruses in 8% (5/63) of the samples. Species C viruses were more common than species B viruses being present in 13% (8/63) of the samples. The most prevalent individual HRV type was HRV61, which was detected in 7.9% (5/63) of the sequenced samples (Table 2). All C virus species were detected before the age of 8 months, whereas A and B virus species were also detected at older age.

Virus isolation was carried out for six rhinovirus RNA positive samples and three rhinovirus RNA negative samples. We were able to isolate rhinovirus in one rhinovirus RNA positive sample. Virus replication was confirmed using real-time RT-PCR in both GMK and RD cells although clear cytopathic effect was not seen. The PCR positive sample was sequenced after isolation in cell culture and we confirmed that the virus growing in cell culture was the same type (HRV-A89) as originally detected directly from stool samples in RT-PCR screening (in both cases the virus type was identified by sequencing of this sample was done from the VP4/VP2 sequencing region).

## 5. Discussion

This is the first report showing that HRVs are frequently present in stool samples from infants and children representing a large cohort from the healthy children background population. This suggests, that the generally accepted idea that HRVs are not usually detectable in stools does not hold true in young children. This also implies that stool samples can offer a useful addition to traditional respiratory samples when HRV is searched for from young children.

The reason why HRV can be detected in stools in such a high frequency is not known. Contamination of the samples with HRV during samples collection or in the laboratory is highly unlikely.
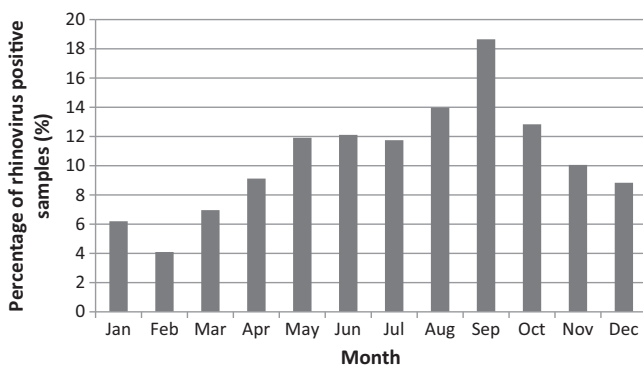


**Fig. 1.** Seasonal pattern of rhinovirus positive samples. Proportion (%) of virus positive samples in each month is shown.

Parents collected the samples from diapers at home using a special sample collection tube, which was then transported, to the laboratory by mail. The samples were processed in the laboratory in the laminar flow paying special attention to avoiding cross-contamination between HRV positive and negative samples. The sensitivity of the RT-PCR for three HRV species (A–C) have not been compared but the primers used in the RT-PCR screening are targeted in the conserved 5' non-coding region which is conserved between these species and in original article, this method was tested to detect several rhinovirus type.[22] In current study primer sequences covered different HRV-C types quite well (6 different

**Table 2**
Summary of human rhinovirus species which were identified in 63 rhinovirus positive stool samples by sequencing the VP1 and VP4/VP2 regions of the viral genome.

| Human rhinovirus A | | Human rhinovirus B | | Human rhinovirus C | |
|---|---|---|---|---|---|
| Strain | No. of samples | Strain | No. of samples | Strain | No of samples |
| HRV-A5 | 1 | HRV-B14 | 1 | HRV-C1 | 1 |
| HRV-A9 | 2 | HRV-B17 | 1 | HRV-C12 | 1 |
| HRV-A10 | 3 | HRV-B42 | 1 | HRV-C15 | 3 |
| HRV-A12 | 1 | HRV-B79 | 1 | HRV-C25 | 1 |
| HRV-A21 | 3[a] | HRV-B86 | 1 | HRV-C26 | 1 |
| HRV-A22 | 1 | | | HRV-C42 | 1 |
| HRV-A24 | 2 | | | | |
| HRV-A31 | 1 | | | | |
| HRV-A32 | 2 | | | | |
| HRV-A36 | 1 | | | | |
| HRV-A38 | 2 | | | | |
| HRV-A40 | 3 | | | | |
| HRV-A41 | 1 | | | | |
| HRV-A46 | 1 | | | | |
| HRV-A47 | 1 | | | | |
| HRV-A49 | 3 | | | | |
| HRV-A53 | 1 | | | | |
| HRV-A54 | 1 | | | | |
| HRV-A55 | 1 | | | | |
| HRV-A57 | 1 | | | | |
| HRV-A58 | 1 | | | | |
| HRV-A59 | 1 | | | | |
| HRV-A60 | 1 | | | | |
| HRV-A61 | 5 | | | | |
| HRV-A63 | 2 | | | | |
| HRV-A66 | 1 | | | | |
| HRV-A67 | 1 | | | | |
| HRV-A73 | 1 | | | | |
| HRV-A78 | 1 | | | | |
| HRV-A81 | 1 | | | | |
| HRV-A89 | 2 | | | | |
| HRV-A101 | 1 | | | | |
| Total | 50 | | 5 | | 8 |

[a] Two samples from one child. The virus was detected in two sequential samples that were taken approximately 30 days apart.

types of HRV-C species). However, in spite of this we cannot exclude the possibility that some HRV species were detected more readily than others. PBS samples were processed along with real samples and all PCR runs included also negative water controls which all were HRV negative. Furthermore, positivity was seen during the whole collection period, it followed seasonal pattern and sequence analyses indicated a wide range of different viruses. In addition, we have previously reported the presence of rhinoviruses in stool samples in a smaller cohort of young children[18] and two more recent studies have confirmed these finding in hospitalized patients.[19,20] Harvala et al. showed that HRV-A was detected in 41% and HRV-B in 13% of all samples which is in line with our findings. In contrast to our results, HRV-C was more common than HRV-A.[19] On the other hand, Lau et al. detected only few rhinovirus positive patients in their study.[20] Thus, the detection of HRV in these stool samples is a true finding.

The fact that HRVs can be detected in stools suggests that they can either replicate in the intestinal mucosa or are ingested by the children and passively passing the gastrointestinal tract. In any case, they are able to pass the stomach without losing their genome integrity completely allowing the amplification of relatively long segments by RT-PCR. Previous studies have shown that the pH of the stomach in infants is not as low as in adults which may explain this phenomenon.[24,25] Viral genome is also protected by the protein capsid which effectively prevents viral RNA from degradation. Thus, even if the low pH in the stomach may destroy viral infectivity by causing confirmatory changes in the viral capsid, it may not destroy the viral genome which locates inside the capsid. In addition, Skern et al. showed that only one amino acid change in the capsid region of HRV-B14 was associated with increased stability at low pH.[17] In fact, it seems that at least in some cases the virus even retains its infectivity, since we could propagate the virus in cell culture in one of the six rhinovirus positive samples. This finding is also in line with our previous study where rhinovirus was isolated from seven (26%) of the 27 rhinovirus positive stool samples.[18] In addition, other studies have shown that even though environmental HRV isolates are acid sensitive, the physiological condition of the sewage does not destroy HRVs and they can be isolated from sewage samples under conditions optimized for growth of most of the enteroviruses.[3]

The HRV types detected in stools may not represent the distribution of serotypes infecting these children, since certain serotypes might pass the gastrointestinal tract more readily than some others. This may be related to the resistance to acidic pH or gastrointestinal enzymes, or some serotypes may even be able to replicate in intestinal mucosa. However, there is no clear evidence that HRVs could replicate in intestinal mucosa. In spite of this limitation, the present study clearly demonstrates that all HRV species can be detected in young children. In fact, the frequency of individual HRV serotypes in stools resembles closely that pattern which has been documented previously in respiratory samples[13,26,27] suggesting that HRVs in stools can be used to study the epidemiology of different HRV species.

We were also able to show that HRV-Cs, which have been linked to severe lower respiratory tract infections, can be frequently detected in stool samples collected from children derived from the healthy children background population. This indicates that only a small proportion of infected children develop severe disease. On the other hand, the clinical symptoms were not recorded from these children in the present study and therefore we were unable to connect the presence of HRV-C to their illnesses. Most of the earlier studies evaluating the symptoms of HRV-Cs have been carried out with inpatients or outpatients and based on these studies it is not possible to estimate the morbidity rate.[4,7,12,26–30] However, at least one study has also investigated HRV-Cs in asymptomatic children but in that study the samples were nasopharyngeal aspirates.[15] In the present study HRV-C species peaked also at earlier age than other HRVs. This may partially explain the previously observed association between HRV-C species and severe forms of disease since young age is a well-known risk factor for severe enterovirus diseases.

One may question whether the current study subjects represent the general population since they were carriers of HLA genotypes conferring increased susceptibility to type 1 diabetes. Around one third of them developed diabetes-predictive autoantibodies and around 18% presented with overt type 1 diabetes. The fact that we did not see any association between HRV infections and HLA genotype, or HRV infections and autoantibody positivity, or HRV infections and progression to clinical diabetes, suggests that this cohort can be perceived as representative of the general population in this context. Previously, enterovirus infections, particularly group B coxsackievirus infections have been linked to the pathogenesis of type 1 diabetes.[31] Since HRVs were equally common in both groups, HRV infections seem not to play a role in the pathogenesis of type 1 diabetes.

In conclusion, the present study suggests that stool samples can be used to diagnose HRV infections in infants and young children. In addition, group C HRVs seem to be common in young children derived from the background population.

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## Competing interest

None declared.

## Ethical approval

The study was approved by the ethics committees of the participating university hospitals, and the parents gave their written informed consent to the participation in the study.

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# Coxsackievirus B1 Is Associated With Induction of β-Cell Autoimmunity That Portends Type 1 Diabetes

**The rapidly increasing incidence of type 1 diabetes implies that environmental factors are involved in the pathogenesis. Enteroviruses are among the suspected environmental triggers of the disease, and the interest in exploring the possibilities to develop vaccines against these viruses has increased. Our objective was to identify enterovirus serotypes that could be involved in the initiation of the disease process by screening neutralizing antibodies against 41 different enterovirus types in a unique longitudinal sample series from a large prospective birth-cohort study. The study participants comprised 183 case children testing persistently positive for at least two diabetes-predictive autoantibodies and 366 autoantibody-negative matched control children. Coxsackievirus B1 was associated with an increased risk of β-cell autoimmunity. This risk was strongest when infection occurred a few months before autoantibodies appeared and was attenuated by the presence of maternal antibodies against the virus. Two other coxsackieviruses, B3 and B6, were associated with a reduced risk, with an interaction pattern, suggesting immunological cross-protection against coxsackievirus B1. These results support previous observations suggesting that the group B coxsackieviruses are associated with the risk of type 1 diabetes. The clustering of the risk and protective viruses to this narrow phylogenetic lineage supports the biological plausibility of this phenomenon.**

Enteroviruses have been linked to type 1 diabetes in a number of previous studies, as reviewed previously (1,2). The recent discovery of diabetes-associated polymorphisms in the innate immune system receptor for enteroviruses (IFIH1) has further increased the interest

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METABOLISM

in the role of enterovirus infections in the pathogenesis of the disease (3). This association has not been observed in all studies, however, and the causal relationship has remained open.

More than 100 different enterovirus serotypes have been identified, which vary in their binding to various cellular receptors and in their ability to infect different cell types and organs. Consequently, different serotypes cause a diverse spectrum of diseases. Poliomyelitis, the classical enterovirus disease, is caused by three serotypes, polioviruses 1, 2, and 3, which have a strong tropism for motoneurons in the spinal cord. This tropism is partly explained by the expression of the poliovirus receptor (CD166) on these cells. In ~1% of infected individuals, the virus spreads to the motoneurons and causes paralytic disease. Similarly, some other enteroviruses, including the six coxsackievirus B (CVB) serotypes, seem to have a tropism for human pancreatic islets in vitro (4–7) and in vivo (8–10), possibly because islet cells express the coxsackie-adenovirus receptor (CAR), which is the major receptor for CVBs (11).

The identification of the enterovirus serotypes that may induce the disease process leading to type 1 diabetes is important because it would enable further studies on the mechanisms of enterovirus-induced β-cell damage and would pave the way for the development of a preventive vaccine. The lack of this information could also explain the variable results from previous studies that have been based on assays detecting several different enterovirus types as a group (2,12). Despite the importance of this topic, large-scale systematic studies aimed at identifying diabetogenic enterovirus serotypes have not been performed. Previous reports of data from case reports and small patient series suggest that the CVB group viruses may include diabetogenic serotypes (1) but also that certain echovirus serotypes have been linked to type 1 diabetes (13).

Here, the role of enterovirus infections was studied using the birth cohort samples systematically collected in the prospective Diabetes Prediction and Prevention (DIPP) study in Finland. By screening for the presence of neutralizing antibodies directed against a panel of 41 enterovirus serotypes, we assessed the association between each individual serotype and the appearance of diabetes-predictive autoantibodies. A study of the time-relationship between infection and initiation of the autoimmune process was thus possible. This is the first large and systematic study aimed at the identification of diabetogenic enterovirus types at the time when the process appears to start.

## RESEARCH DESIGN AND METHODS

### Subjects

The study population was derived from the DIPP study (14). Families with children carrying an increased genetic risk for type 1 diabetes, defined by cord-blood HLA typing, were invited to participate in prospective follow-up starting from birth. Blood samples were drawn at the ages of 3, 6, 12, 18, and 24 months and once yearly thereafter. Follow-up samples were screened for islet cell antibodies (ICA), and if a child seroconverted to positivity for ICA, follow-up samples were also analyzed for autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA), and the tyrosine phosphatase–related insulinoma-associated 2 molecule (IA-2A). Written consent was obtained from each family whose child took part, and the study was approved by the ethical committees of the Pirkanmaa Hospital and the Northern Ostrobothnia Hospital districts.

Our study was a nested case-control study (Fig. 1) using the following criteria to select case and control children: Case children had turned permanently positive for two or more diabetes-predictive autoantibodies and/or progressed to clinical type 1 diabetes. Two control children were selected for each case child. They all remained nondiabetic and autoantibody-negative for at least 2 years after the earliest detection of autoantibodies in the corresponding case child and were matched for time of birth (± 1 month except in 12 children ± 2 months), sex (60% were boys), HLA-DQB1 genotype, and region. The final study cohort included 183 case and 366 control children born during the period from 1995 to 2006 and who were an average age of 31 months (range 5–122) at initial seroconversion to autoantibody positivity (Supplementary Tables 1 and 2). By the end of July 2011, 119 case children had progressed to type 1 diabetes.

### HLA Genotyping

An analysis of the HLA-DQB1 genotype was performed from cord blood to identify selected alleles (DQB1*02, *03:01, *03:02, and *06:02/3) associated with susceptibility to or protection against type 1 diabetes (15). The genotyping was based on hybridization with lanthanide-labeled oligonucleotide probes detected with time-resolved fluorometry (16). Families with an infant carrying the high-risk HLA-DQB1*02/DQB1*0302 genotype or the moderate-risk DQB1*0302/x genotype (x ≠DQB1*03:02, *06:02, or *06:03) were invited for follow-up (Supplementary Table 3).

### Detection of β-Cell Autoimmunity and Clinical Type 1 Diabetes

ICAs were detected by indirect immunofluorescence, and the three other autoantibodies were quantified with radiolabel-binding assays (17). We used cutoff limits for positivity of 2.5 JDRUs for ICA, 3.48 JDRUs for IAA, 5.36 JDRUs for GADA (full-length GAD65, aa 1-585, used as construct), and 0.43 JDRUs for IA-2A (the intracellular portion of the IA-2 molecule, aa 605-979, used as construct), representing the 99th percentile in more than 350 Finnish children. The ICA assay had a disease sensitivity of 100% and specificity of 98% in the fourth
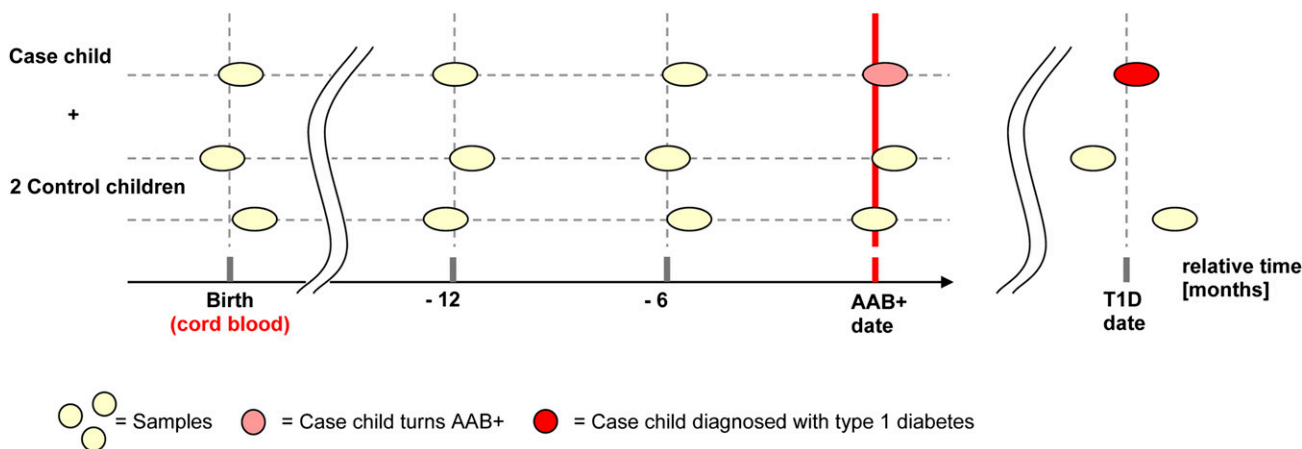
Figure 1—Study setup. The nested study consisted of 183 case/control triplets in which for each case child fulfilling the defined criteria two matched control children were selected. First, the neutralizing antibodies were analyzed in the samples where autoantibodies (AAB) were detected for the first time (AAB+ date sample) in case children and in the corresponding samples in control children (cross-sectional analysis). Next, neutralizing antibodies were screened in samples taken 6 and 12 months before AAB+ date (-12 and -6), as well as in samples taken at birth (cord blood) and at the age of 18 months, to perform longitudinal analyses for those enterovirus serotypes that were associated with the modulated diabetes risk at the cross-sectional primary screening step. However, the complete set of follow-up samples was not available from every child, which explains the small variation in the number of samples in different analyses. The information on the diagnosis of type 1 diabetes (T1D) date was used to run subcohort analyses for those triplets in which the case child progressed to type 1 diabetes.

round of the International Workshops on Standardization of the ICA assay. The disease sensitivity of the IAA assay was 58% and the specificity was 100% in the 2005 Diabetes Autoantibody Standardization Program Workshop. The same characteristics of the GADA assay were 82% and 96% and those of the IA-2A assay were 72% and 100%, respectively. The diagnosis of type 1 diabetes was based on the World Health Organization criteria.

### Cells

Viruses were isolated and cultivated, and seroneutralization assays were performed using the A549, Vero, RD, and GMK cell lines. The three first cell lines were purchased from American Type Culture Collection, and GMK was acquired from the National Institute for Health and Welfare, Finland.

### Neutralizing Antibodies Against Various Enterovirus Serotypes

Neutralizing antibodies were measured in serum or plasma against 44 enterovirus strains representing 41 serotypes. Most of these viruses were isolated from DIPP children and hospital patients in Finland and Sweden. All strains were plaque-purified and sequenced in their VP1 region for serotyping (18). Most of the viruses were analyzed using a standard plaque neutralization assay (19,20), whereas viruses that did not form clear plaques were analyzed using a microneutralization assay (Supplementary Table 4). All samples were screened using 1:4 and 1:16 dilutions. Inhibition was considered to be significant when the serum reduced the number of plaques more than 75% (plaque assay) or

inhibited the ability of the virus to kill cells (microneutralization assay).

The identification of diabetogenic serotypes was based on a step-wise strategy (Fig. 1): First, the neutralizing antibodies were analyzed in the samples where autoantibodies were detected for the first time in case children and in the corresponding samples in control children (cross-sectional analysis). All samples showing titers of 1:4 or greater were considered positive.

In the next step, neutralizing antibodies were screened in samples from earlier time points (longitudinal analyses) for those enterovirus serotypes that were associated with diabetes risk at the cross-sectional primary screening step. These longitudinal analyses made it possible to diagnose infections by virus antibody seroconversions observed between two consecutive follow-up samples (Supplementary Fig. 1). These earlier time points included samples taken 6 and 12 months before the initial seroconversion to autoantibody positivity (Fig. 1). The mean age and age range at these time points are reported in Supplementary Table 5. Some samples were collected from the children at such a young age that they possibly contained maternal antibodies. Cord-blood samples were therefore analyzed in these children, and when the presence of maternal antibodies could bias a positive result, the sample was considered negative. In addition, cord-blood samples and samples taken at the age of 18 months were analyzed from all children for CVB1 antibodies. The following definitions were used to diagnose an acute infection: the main definition was based on "sensitive diagnostic criteria," where transient and permanent antibody

seroconversions were both counted (if the child had serial transient seroconversions against the same virus only the first one was counted). The results were confirmed using more strict "specific diagnostic criteria," where acute infections were diagnosed by the following criteria: a seroconversion from a titer of <1:4 (seronegative) to ≥1:4 (seropositive), a titer of 1:16 in at least one of the following samples, and all subsequent samples were positive.

### Statistical Analyses

The primary analysis method was conditional logistic regression using the one-to-two age, sex, HLA, and region matched case-control triplets. Data from matched case-control pairs and triplets were analyzed using Stata 8.2 software (StataCorp, College Station, TX), which allows for variable matching ratios of case subjects to control subjects. Conditional logistic regression was used to estimate the odds ratios (OR) with exact 95% confidence intervals (CI) and two-sided $P$ values for univariate point estimates and multivariate modeling to assess the association between enterovirus antibodies and diabetes-predictive autoantibodies. In the first phase, a cross-sectional analysis was performed using data on the prevalence of enterovirus serotypes at the time point when the first diabetes-predictive autoantibodies were detected. The duration of exclusive and total breast-feeding was entered into a multivariate analysis to estimate adjusted ORs.

Second, to study the temporal profile of the associations detected in these cross-sectional analyses, infections occurring during all longitudinal time points before the detection of predictive autoantibodies were analyzed. The time was classified into three periods (simultaneously with the first detection of autoantibodies, 6 months before autoantibodies, and 12 months or longer before autoantibodies), and the infections were diagnosed using the sensitive and specific criteria described above.

Third, the longitudinal data were used to analyze the effect of the chronology of infections caused by different serotypes on the risk of β-cell autoimmunity.

Fourth, interactions between different serotypes were analyzed by studying the effect of different virus combinations. In addition to the raw $P$ values, the $P$ values that were corrected for the number of comparisons made (Bonferroni correction) are presented.

## RESULTS

### Seroprevalences of CVB1, CVB3, and CVB6 Show a Cross-Sectional Association With the Risk to Develop Autoantibodies

Neutralizing antibodies were initially screened against 41 enterovirus serotypes in the first sample positive for diabetes-predictive autoantibodies. The conditional logistic regression analyses showed that CVB1 antibodies were more frequent in the case children than in the control children (59.0% vs. 50.1%; OR 1.5 [95% CI 1.0–2.2]; $P$ = 0.04) suggesting that an infection with this enterovirus is associated with an increased risk of β-cell autoimmunity (Table 1). The statistical significance disappears when the $P$ value is multiplied by the number of tested serotypes ($N$ = 41). The high seroprevalence of CVB1 in the control children (50.1%) indicates that this enterovirus is a common serotype in the population studied. Only one case and one control child were negative for all 41 tested enterovirus serotypes (the median number of positive serotypes was 9 in both groups).

Neutralizing antibodies to two closely related serotypes, CVB3 and CVB6, were less frequent in case children than in control children, indicating a strong protective association for CVB3 (5.8% vs. 12.8%; OR 0.4 [95% CI 0.2–0.8]; $P$ = 0.01) and a weaker protective association for CVB6 (26.6% vs. 35.3%; OR 0.6 [95% CI 0.4–1.0]; $P$ = 0.04) (Table 1). As above, the statistical significance disappears when these $P$ values are multiplied by the number of tested serotypes. However, the fact that the protective serotypes were the closest genetically to CVB1 (Fig. 2) and no protective association was seen for more distant strains among the 41 analyzed, suggests that these findings reflect a true biological phenomenon. In fact, they support the plausible hypothesis that some immunological cross-protection exists between these closely related enterovirus types. The analysis of potential interactions between CVB1 and the other CVB serotypes indicated a clear risk effect when the child had experienced CVB1 alone without these protective serotypes (OR 2.5 [95% CI 1.4–4.7]; $P$ = 0.003), whereas children infected by both CVB1 and one or more of the protective serotypes were not at risk (Table 2 and Supplementary Table 6).

The risk association of CVB1 and the protective association of CVB3 and CVB6 was also seen in the subcohort of 119 children who progressed to clinical type 1 diabetes (OR for CVB1 was 1.8 [95% CI 1.1–2.9]; $P$ = 0.025), both among boys and girls and in different age groups (data not shown). The effects of CVB1 and CVB3 remained significant after adjustment for the duration of breast-feeding and the number of older siblings, whereas the effect of CVB6 became nonsignificant (a clear trend was observed also for CVB6; Supplementary Table 7).

### The CVB1 Risk Association was Confirmed in Longitudinal Analyses Before the Appearance of the First Autoantibodies in Case Children

The timing of infection with CVB1 was further assessed in a longitudinal analysis by detecting seroconversions in the neutralizing antibodies between consecutive follow-up samples collected before the first autoantibody-positive sample. The results showed an increased risk of autoantibody positivity when a CVB1 infection preceded the autoantibody appearance (Table 3). This association was

**Table 1—ORs for the association between neutralizing antibodies to 44 enteroviruses (41 serotypes) and signs of progressive β-cell autoimmunity (positivity for two or more diabetes-predictive autoantibodies) in 183 case and 366 matched control children**

| Virus | NAB prevalence | | OR (95% CI) | P value |
|---|---|---|---|---|
| | % Case | % Control | | |
| CVA4 | 28.7 | 31.7 | 0.9 (0.6–1.3) | 0.46 |
| CVA5 | 15.0 | 15.0 | 1.0 (0.6–1.9) | 0.96 |
| CVA6 | 17.1 | 14.7 | 1.2 (0.7–2.1) | 0.44 |
| CVA10 | 69.8 | 61.8 | 1.4 (0.8–2.5) | 0.27 |
| CVA16 | 12.5 | 16.6 | 0.7 (0.4–1.2) | 0.23 |
| EV71 | 8.4 | 7.9 | 1.1 (0.5–2.4) | 0.89 |
| CVA9 | 7.5 | 8.4 | 0.9 (0.4–1.8) | 0.72 |
| **CVB1** | **59.0** | **50.1** | **1.5 (1.0–2.2)** | **0.04** |
| CVB2 | 46.6 | 48.8 | 0.9 (0.6–1.3) | 0.61 |
| **CVB3** | **5.8** | **12.8** | **0.4 (0.2–0.8)** | **0.01** |
| CVB4-wt* | 5.2 | 8.1 | 0.6 (0.2–1.3) | 0.19 |
| CVB4-rs# | 5.2 | 7.2 | 0.7 (0.3–1.5) | 0.34 |
| CVB5 | 7.5 | 7.8 | 0.9 (0.4–1.9) | 0.81 |
| **CVB6** | **26.6** | **35.3** | **0.6 (0.4–1.0)** | **0.04** |
| E1 | 25.9 | 26.2 | 0.6 (0.3–1.3) | 0.19 |
| E2 | 9.1 | 9.9 | 1.0 (0.5–1.9) | 0.95 |
| E3-wt | 5.2 | 4.1 | 1.3 (0.6–3.2) | 0.54 |
| E3-rs | 43.8 | 39.9 | 1.2 (0.8–1.8) | 0.33 |
| E4 | 1.2 | 1.5 | 0.8 (0.2–4.1) | 0.79 |
| E5 | 36.0 | 37.2 | 0.9 (0.6–1.5) | 0.73 |
| E6 | 8.6 | 7.5 | 1.2 (0.6–2.3) | 0.65 |
| E7 | 18.4 | 17.9 | 1.0 (0.6–1.7) | 0.90 |
| E9 | 7.6 | 9.8 | 0.7 (0.3–1.5) | 0.34 |
| E11 | 32.4 | 36.3 | 0.8 (0.5–1.2) | 0.33 |
| E12 | 36.8 | 31.7 | 1.3 (0.9–2.0) | 0.22 |
| E13 | 2.3 | 4.1 | 0.5 (0.2–1.7) | 0.30 |
| E14 | 7.6 | 5.6 | 1.3 (0.6–2.9) | 0.43 |
| E15 | 8.7 | 13.0 | 0.6 (0.3–1.2) | 0.13 |
| E17 | 5.8 | 7.8 | 0.7 (0.3–1.5) | 0.31 |
| E18 | 3.5 | 3.8 | 0.9(0.3–2.5) | 0.87 |
| E19 | 9.6 | 13.1 | 0.7 (0.3–1.5) | 0.34 |
| E20 | 6.4 | 5.2 | 1.3 (0.6–2.8) | 0.50 |
| E21 | 28 | 32.4 | 0.8 (0.5–1.2) | 0.29 |
| E25 | 6.4 | 4.3 | 1.5 (0.7–3.2) | 0.34 |
| E26 | 1.7 | 3.5 | 0.5 (0.1–1.8) | 0.27 |
| E27 | 5.8 | 6.5 | 0.9 (0.4–1.9) | 0.75 |
| E29 | 9.3 | 7.4 | 1.3 (0.6–2.8) | 0.45 |
| E30-wt-1 | 98.0 | 96.8 | 0.8 (0.2–3.8) | 0.79 |
| E30-wt-2 | 72.3 | 76.1 | 0.8 (0.5–1.3) | 0.38 |
| E32 | 43.9 | 43.2 | 1.0 (0.6–1.6) | 0.97 |
| E33 | 81.9 | 80.7 | 1.2 (0.7–1.8) | 0.56 |
| EV74 | 60.1 | 59.6 | 1.0 (0.7–1.5) | 0.97 |
| EV78 | 2.3 | 4.4 | 0.5 (0.2–1.6) | 0.25 |
| EV94 | 5.3 | 5.0 | 1.1 (0.7–3.1) | 0.93 |

CVA, coxsackievirus A; E, echovirus; EV, enterovirus; and NAB, neutralizing antibody. % case represents the antibody prevalence in case children and % control represents the prevalence in control children. Data in bold type are statistically significant. *wt, wild-type strain. #rs, reference strain.
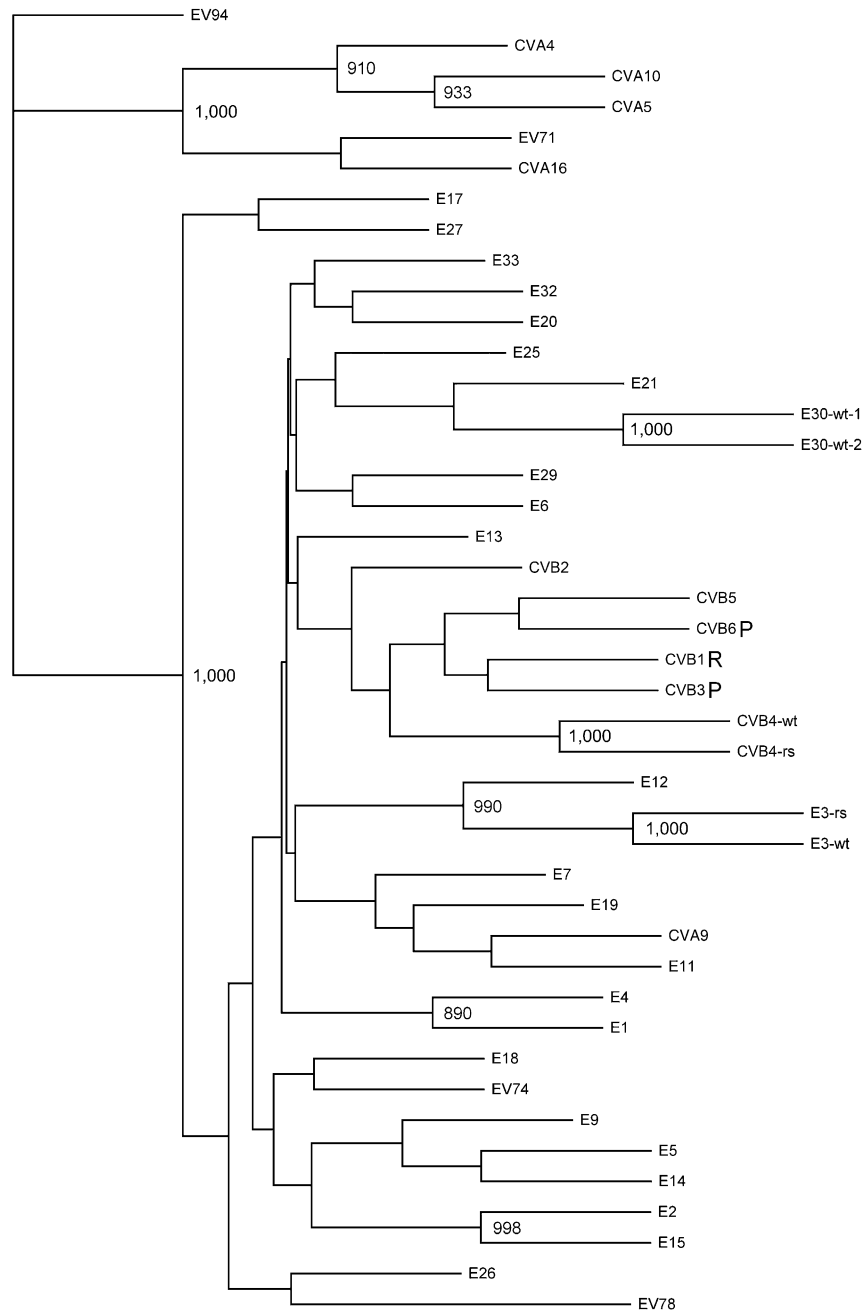
**Figure 2**—Consensus phylogenetic tree of the 44 virus strains based on 104 amino acids of the VP1 region. The part of VP1 region of all 44 viruses was sequenced, and the obtained sequences were blasted against the National Center for Biotechnology Information non-redundant nucleotide database. Phylogenetic analysis was done using the PHYLIP: Phylogeny Inference Package, version 3.69 program (Joe Felsenstein, 1993, University of Washington, Seattle, WA). The phylogenetic tree was constructed using the Protdist program with the parameters of the Kimura 2 model, and the amino acid matrix was processed with the Kitsch program. The consensus tree was treated with the Consense program. This analysis implies a close genetic relationship of the three CVB viruses that were associated with β-cell autoimmunity. The bootstrap confidence levels were analyzed with 1,000 pseudoreplicate data sets, and bootstrap levels higher than 70% were plotted onto the tree. CVA, coxsackievirus A; E, echovirus; EV, enterovirus; P, protective CVB3 and CVB6 strains; R, risk-associated CVB1 strain.

strongest when CVB1 infections preceded the first autoantibody-positive sample by a few months and was observed using both the sensitive and strict infection criteria. The association was also seen in the subgroup of children who progressed to clinical type 1 diabetes.

**Chronological Order of CVB Infections**

When the longitudinal data were analyzed to study the effect of the order of infections with CVB1 and the protective CVB serotypes, some trends suggesting a potential order effect were observed. When CVB1 was the first infecting serotype to occur, the children were at risk

**Table 2—Association of different combinations of risk- and protective-type CVB infections with the risk of β-cell autoimmunity as defined by virus antibody positivity at the time of autoantibody seroconversion (cross-sectional analysis among 180 case and 360 matched control children)**

| Antibodies against risk serotype | Antibodies against protective serotypes | OR (95% CI) | P value |
|---|---|---|---|
| CVB1 neg | CVB3 or CVB6 pos | 1 (Reference) | |
| CVB1 neg | CVB3 and CVB6 neg | 1.6 (0.9–3.1) | 0.12 |
| CVB1 pos | CVB3 or CVB6 pos | 1.5 (0.8–2.9) | 0.20 |
| CVB1 pos | CVB3 and CVB6 neg | **2.5 (1.4–4.7)** | **0.003** |

Data in bold type are statistically significant. The reference group comprises children with the lowest predicted risk being seropositive for the protective serotypes but not for CVB1.

for developing autoantibodies, whereas when CVB3 or CVB6 infection occurred first, the risk of developing autoantibodies was lower (Supplementary Table 8). This again supports the conclusion that infection by CVB3 or CVB6 provides some immunological protection from the diabetogenic effect of CVB1.

**Maternal Antibodies Modulate the Risk Effect of CVB1**

The cord-blood samples and samples taken at the age of 18 months were analyzed to explore whether protective maternal CVB1 antibodies in cord blood can modulate the risk association of CVB1 infections in young infants. The risk association was strongest in the group who experienced CVB1 without maternal CVB1 antibodies (OR 2.6 [95% CI 1.1–5.9]; P = 0.02) (Table 4).

## DISCUSSION

This case-control study nested in the DIPP birth cohort is the first systematic study aimed at identifying enterovirus subtypes possibly associated with the induction of β-cell autoantibodies. The study has several unique strengths. First, it is based on the analysis of neutralizing antibodies, which is the most reliable way to diagnose prior infection caused by a given enterovirus serotype. Second, it covers a large number of different serotypes (n = 41), most of which represent wild-type strains circulating in the background population. Third, it was performed in a prospective birth-cohort study including a longitudinal sample series starting from cord blood, which allowed the timing of the infections to be determined in relation to the time when autoantibodies first appeared. Fourth, the case and control subjects were matched for the most relevant potential confounders such as HLA-defined diabetes risk, sex, time of birth, age at sampling, and the area of residence. Finally, the results provided by the cross-sectional and longitudinal analyses using different infection criteria were coherent.

We believe that the finding that the three serotypes identified are closely related phylogenetically (Fig. 2) is very significant. Indeed, if the signals detected in this study were due to arbitrary random noise in the methods, it would be unlikely that they would cluster together phylogenetically. Close clustering, on the other hand, is precisely what would be expected for serotypes that could be causative or protective, based on the highly plausible hypothesis of some degree of immunological cross-protection, as discussed subsequently.

The outcome reported here is consistent with the diabetogenic role of enteroviruses postulated in the literature and with predictions that can be made in searching for diabetogenic viruses. Prospective studies have shown

**Table 3—The risk for β-cell autoimmunity associated with CVB1 infections according to the time when these infections were diagnosed in 183 cases and 366 matched control children**

| Timing of CVB1 infection* | Sensitive diagnostic criteria | | Specific diagnostic criteria | |
|---|---|---|---|---|
| | OR (95% CI) | P value | OR (95% CI) | P value |
| Whole nested case-control series | | | | |
| No infection | 1 (Reference) | | 1 (Reference) | |
| 12 months or longer before autoantibodies | 1.3 (0.8–2.3) | 0.33 | 1.0 (0.5–2.2) | 0.93 |
| 6 months before autoantibodies | **2.0 (1.1–3.6)** | **0.03** | 1.9 (0.7–5.2) | 0.23 |
| Simultaneously with autoantibodies | 1.5 (0.9–2.4) | 0.11 | **2.1 (1.0–4.4)** | **0.04** |
| Case children who progressed to type 1 diabetes and their control children | | | | |
| No infection | 1 (Reference) | | 1 (Reference) | |
| 12 months or longer before autoantibodies | 1.0 (0.4–2.2) | 0.91 | 0.7 (0.2–2.0) | 0.48 |
| 6 months before autoantibodies | **2.0 (1.0–4.2)** | **0.05** | 1.8 (0.6–5.0) | 0.27 |
| Simultaneously with autoantibodies | 1.6 (0.89–2.9) | 0.11 | **2.5 (1.1–5.6)** | **0.03** |

The diabetes subgroup included 119 case children who progressed to clinical type 1 diabetes and their 239 matched control children. The sensitive and specific diagnostic criteria analyses were performed as defined in the RESEARCH DESIGN AND METHODS. Data in bold type are statistically significant. *Average time in relation to autoantibody seroconversion.

**Table 4—The risk of β-cell autoimmunity in children according to their exposure to CVB1 by the age of 18 months (CVB1 seropositive at that age) and presence of protective CVB1 antibodies in cord blood among 127 case and 254 matched control children**

| CVB1 seropositivity | | Observed risk of β-cell autoimmunity | | |
|---|---|---|---|---|
| Cord blood | 18 months | OR (95% CI) | P value | Expected risk of β-cell autoimmunity*** |
| Pos* | Neg** | 1 (Reference) | | Lowest |
| Neg | Neg** | 1.6 (0.7–3.9) | 0.28 | Low |
| Pos* | Pos | 2.1 (0.8–5.6) | 0.12 | High |
| Neg | Pos | **2.6 (1.1–5.9)** | **0.02** | Highest |

Data in bold type are statistically significant. *Only antibody titers 16 or higher were considered positive in cord blood because low antibody levels disappear rapidly from the child's circulation. **Negative antibody result does not exclude early CVB1 infection due to possible transient antibody responses in these very young infants. ***Expected risk refers to theoretical risk predicted on the basis of CVB1 seropositivity in cord blood (maternal antibodies) and at the age of 18 months.

that the autoimmune process usually begins at an early age (<3 years) (20,21) and that autoantibodies appear annually in "epidemic" peaks (20). Consequently, the causative agent is probably frequent in the background population circulating continuously in very young children. The epidemiology of CVB1 fits with these predictions. CVB1 has been one of the most frequent enteroviruses isolated in recent years in the U.S. (22,23) as well as in Korea (24), India (25), Tunisia (26), Western Germany (27), and Finland (28). It can cause severe systemic infections in young infants (29,30) and infects human pancreatic islets in vitro, being one of the most cytolytic enterovirus serotype in this model (7). In fact, insulitis and islet cell damage have been described in infants who have died of CVB1 infection (31). Certain CVB1 strains induce also persistent infections in mice that lead to chronic inflammatory myopathy (32). One can estimate from the generated data that less than 5% of CVB1-infected children go on to develop type 1 diabetes. This fits with the low attack rate typical for enterovirus diseases; for example, in the beginning of the 20th century, almost the entire population became infected by polioviruses but less than 1% developed motor neuron damage and paralysis. This implies also that the ORs obtained from serological screening studies remain relatively modest, even though CVB1 infection may explain most of the cases.

Surprisingly, the current study revealed that infections by two other CVBs, CVB3 and CVB6, were associated with a decreased risk of β-cell autoimmunity. A possible protective effect of CVB3 has actually been reported in a smaller study where patients with newly

diagnosed type 1 diabetes were found to be less frequently positive for neutralizing antibodies against this serotype than control subjects (26). This phenomenon could be explained by immunological cross-protection induced by CVB3 and CVB6 against the diabetogenic effect of CVB1. Such cross-protection, most likely due to cell-mediated immunity, has been reported in other virus diseases, such as among different rotavirus, papillomavirus, and poliovirus types (33–37). Cross-protection is also supported by the increased CVB1-related risk in children who were infected by CVB1 but none of the protective serotypes. Prevention of lethal CVB1 infection by a prior CVB3 infection has also been observed in a mouse model, fitting nicely with the findings in the current study (38). In addition to cross-protection, other mechanisms related to the induction of β-cell tolerance may mediate the protective effect of viruses against type 1 diabetes as described in NOD mice (39,40). In both cases, the close relationship between the protective and the diabetogenic serotypes suggests a particular impact of the CVB group enteroviruses on the risk of diabetes. Because CVBs are the only enteroviruses to use CAR, it can be hypothesized that they share some specific characteristics in terms of antigenicity and/or tropism.

Despite its virtues, the current study also has limitations. The first relates to the population studied being exclusively from Finland and covering a relatively limited 10-year period. Consequently, we cannot exclude a timing effect of CVB1 infections or a strain-specific effect of this serotype. A timing effect could also explain the low prevalence of the CVB4 serotype, which has been linked to type 1 diabetes in previous studies. Accordingly, confirming these findings in other populations will be important. The statistical power of the current study allowed the identification of viruses with major risk effects, whereas viruses with weaker effects might have been missed. Adding new datasets would also help to assess further the combined effect of the three identified CVB serotypes. The virus strains used in the neutralization assay represent the most common enterovirus serotypes (22,23) but do not include all serotypes known today (many of them are also difficult to cultivate and to produce cytopathic effect in vitro). Therefore, we cannot exclude the possibility that other risk or protective serotypes might have been missed.

The current findings have aspects that fit with causality: First, the CVB1-related risk effect showed logical time relationship: it preceded the initiation of the autoimmune process. In addition, CVB1 infections peaked a few months before autoantibodies first appeared, which overlaps with the previously observed peak in the frequency of enterovirus RNA in serum (41), fitting with the rapid induction of islet autoantibodies in enterovirus-infected mice (42). Second, the accumulation of risk and protective viruses to a small subgroup of phylogenically close enteroviruses supports the

biological relevance of our findings. Third, the discovery of protective viruses fits with immunological cross-protection attenuating infections caused by closely related viruses. Fourth, the observation that maternal CVB1 antibodies modulated the risk effect of CVB1 supports biological plausibility because maternal antibodies protect the child against enterovirus infections (43,44). Finally, we observed a similar risk effect of CVB1 in another study where neutralizing antibodies were analyzed in patients with newly diagnosed type 1 diabetes and control subjects in five European countries (41).

In summary, the results are in line with the previous literature suggesting a link between enterovirus infections and type 1 diabetes. The identification of CVB1 as a potentially diabetogenic virus type is a new discovery that offers possibilities to explore the mechanisms of enterovirus-induced diabetes and may also open the door for the development of an enterovirus vaccine against the disease. Further studies are needed to confirm these findings in other populations. The identification of serotypes with opposite effects on type 1 diabetes implies that serotype-specific methods should be used in such studies.

**Author Contributions.** V.L., J.W.A., M.K., and H.Hy. were part of the steering group that designed the study. O.S., J.I., R.V., M.K., and H.Hy. participated in the recruitment of children to the DIPP study. O.H.L., H.Ho., O.P., S.O., M.M.H., T.R., R.H., M.K., and H.Hy. participated in the virus analyses. S.M.V. was responsible for the dietary data (breast-feeding). H.Hu., P.A., and J.L. analyzed data. O.H.L. and H.Ho. wrote the initial draft of the manuscript. All authors contributed to the data interpretation, to the preparation of the manuscript, and to the final version of the manuscript. H.Hy. and H.Ho. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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